Development of A Multi-stages Membrane Filtration Process for Separation and Purification of Protease from the Wastes of Fishery Industry

Zhenyu Li

A Thesis Submitted in Fulfillment of the Requirements for the Degree of

Doctor of Philosophy in Biotechnology

Prince of Songkla University

2008

Thesis Title	Development of A Multi-stages Membrane Filtration Process for Separation and Purification of Protease from the Wastes of Fishery Industry
Author	Mr. Zhenyu Li
Major Program	Biotechnology

Major Advisor

.....

(Assoc. Prof. Dr. Aran H-Kittikun)

Co-advisor

.....

(Asst. Prof. Dr. Wirote Youravong)

Examining Committee :

.....Chairperson

(Assoc. Prof. Dr. Charun Bunyakan)

.....Committee

(Assoc. Prof. Dr. Aran H-Kittikun)

.....Committee

(Asst. Prof. Dr. Wirote Youravong)

.....Committee

(Prof. Dr. Soottawat Benjakul)

.....Committee

(Asst. Prof. Dr. Anukul Watanasuk)

The Graduate School, Prince of Songkla University, has approved this thesis as fulfillment of the requirements for the Doctor of Philosophy Degree in Biotechnology

.....

(Assoc. Prof. Dr. Krerkchai Thongnoo)

Dean of Graduate School

Thesis title	Development of A Multi-stages Membrane Filtration Process for Separation and Purification of Protease from the Wastes of Fishery Industry
Author	Mr. Zhenyu Li
Major program	Biotechnology
Academic Year	2008

ABSTRACT

In order to reuse the waste of fishery industry, a multi-stage membrane filtration process was developed to separate and partially purify protease from yellowfin tuna spleen which is the waste from tuna canning industry.

For selection of membrane size of microfiltration, the dead-end microfiltrations with membrane pore size 0.10, 0.22 and 0.45 μm were tested to remove suspended solids from yellowfin tuna spleen extract. It was found that 0.10 μm membrane provided the best performance. Almost all trypsin and chymotrypsin were recovered from the permeate of microfiltration while most large particles and half amount of other soluble proteins were rejected by a 0.10 μm membrane at transmembrane pressure 4 psi. The transmissions were about 1 and 0.5 for enzymes and soluble protein, respectively. The pore blocking resistance-limited was the major fouling mechanism for permeate flux decline while cake resistance-limited dominated the most duration of microfiltration process. Increasing both membrane pore size and transmembrane pressure caused partially loss of enzyme activity. Centrifugation and simple pre-filtration changed the particle size distribution in the extract and resulted in the change of fouling mechanism during microfiltration.

Crossflow microfiltration was applied to remove suspended particles from tuna spleen extract. For the condition of sustainable operation with low fouling in batch concentration crossflow microfiltration, the relation between critical ratio of flux (J) to wall shear stress (τ_w) and volume concentration factor (VCF) was found as $J/\tau_w = 3.29\ (\text{VCF})^{-0.74}$ at a given transmembrane pressure of 0.15 bar. The present study revealed a simple method to predict low fouling condition in batch concentration operation during membrane separation process.

Based on total recycle and single-batch concentration crossflow microfiltration, a continuous-batch concentration crossflow microfiltration (CBC-CFMF) with 0.10 μm hollow fiber membrane, crossflow velocity of 0.2 m/s, transmembrane pressure of 0.15 bar and gas injection factor of 0.38 was designed and applied successfully to remove suspended solids from tuna spleen extract. Transmissions of about 1 for both trypsin and chymotrypsin were attained in this study. The optimal gas injection factor (r) of 0.38 resulted in a 300% improvement in flux comparing to the process without gas injection. A clear permeate with slight yellow colour was obtained after CBC-CFMF.

Ultrafiltration was applied for recovery of protease from microfiltration pretreated yellowfin tuna spleen extract. Effect of hydrodynamics and gas sparging on flux enhancement and selectivity was studied by a total recycle mode using a hollow fiber membrane with the molecular weight cutoff 30 kDa. The critical flux varied from 28.8 to 44.2 l/m^2.h and limiting flux varied from 34.3 to 52.4 l/m^2.h while crossflow rate increased from 17.55 to 69.98 l/h without gas sparging. A low gas injection factor of 0.15 could improve critical and limiting flux significantly.. Higher gas injection factors varied from 0.30 to 0.61 did not give remarkable improvement of both critical and limiting flux. The benefit of increasing crossflow rate to enhance critical and limiting flux was great when gas sparging did not applied. Selectivity was increased with increasing permeate flux at sub-critical condition and critical flux condition. It became insensitive to the flux and crossflow rate at limiting flux condition when gas sparging was not applied. Gas sparging gave negative effect on soluble protein and peptide transmission and resulted in the decay of selectivity at sub-critical condition and critical flux condition. The selectivity at limiting flux condition was not sensitive to gas injection factor.

Protease from tuna spleen extract was finally purified by an ultrafiltration with diafiltration mode. Severe fouling was avoid while12-fold purification of protease was achieved in mode 1 (pre-diafiltration followed by post-concnetration) with a critical flux condition operation. A conventional operation, i.e. mode 2 (pre-concentration followed by post-diafiltration) provided 2-fold purification of protease. Fouling was much severer in mode 2 than mode 1. Consequently low flux caused by fouling led to a long operational time in mode 2. The difference of resistance between mode 1 and mode 2 was mainly due to concentration polarization and external membrane fouling.

The purified protease from tuna spleen was compared with two commercial proteases based on degree of hydrolysis of protein. Degree of hydrolysis of 43% were reached after hydrolysis of casein by Alcalase and protease from tuna spleen, respectively while Delvo-Pro showed lower degree of hydrolysis at the same condition. In the case of soybean protein isolate as substrate, protease from tuna spleen provided lowest degree of hydrolysis which was due to the residual activity of soybean protein inhibitor in soybean protein isolate.

The presented work showed that a trypsin-like serine protease with low-cost and qualified hydrolysis efficiency could be obtained from tuna canning waste by membrane filtration.

ACKNOWLEDGEMENTS

First and foremost I would like to thank my supervisor Assoc. Prof. Dr Aran H-Kittikun, and my co- supervisor Asst. Prof. Dr Wirote Youravong, not only for their supervision and guidance but also for their patience and composure, and for giving me the opportunity to complete a PhD.

Very special thanks to Assoc. Prof. Dr. Charun Bunyakan from the Department of Chemical Engineering, Facutly of Engineering, Prince of Songkla University, Prof. Dr. Soottawat Benjakul from the Department of Food Technology, Faculty of Agro-Industry, Prince of Songkla University, and Asst. Prof. Dr. Anukul Watanasuk from the Department of Food Science and Technology, Kasetsart University, for their valuable time and important comments on my dissertation.

My gratitude goes to the Faculty of Agro-Industry and Graduate School, Prince of Songkla University for providing the scholarship and funding to support my study.

Gratitude also goes to Membrane Science and Technology Research Center (MSTRC), Prince of Songkla University. The academic communication in MSTRC has enlarged my view of membrane technology and science.

Many thanks to all lecturers and staffs in Faculty of Agro-Industry, Prince of Songkla University for their smile and kindness.

A big thank you also goes to all my friends in Faculty of Agro-Industry, Prince of Songkla University. They have provided me with great companionship.

Sincere appreciation should go to Prof. Tang Bin and his wife Prof. Zhang Qingqing for their encouragement over the last five years.

I would like to express my sincere gratitude to those who contributed, directly and indirectly, to accomplishing all the laborious work presented in this thesis.

On a more personal level, I'd like to express my heartfelt appreciation to my family for their love and always being there for me.

Very much appreciated.

Zhenyu Li

CONTENTS

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

CONTENTS (continued)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (continued)

LIST OF FIGURES (continued)

LIST OF ABBREVIATIONS AND SYMBOLS

CBC-CFMF = Continuous-batch concentration crossflow microfiltration

CFMF =Crossflow microfiltration

CSE = Centrifugated tuna spleen extrct

CDU = Casein digesting unit

D = Dialysis

DH = Degree of hydrolysis

DV = Diafiltration volume

ED = Electrodialysis

FSE = Pre-filtered tuna spleen extract

FVF = Feeding volume factor

GP = Gas permeation

MF = Microfiltration

NF = Nanofiltration

PV = Pervaporation

RO = Reverse osmosis

RSE = Raw tuna spleen without pre-treatment

SBC-CFMF = Single-batch concentration crossflow microfiltrration

Sp.Act. = Specific activity

TMP = Transmembrane pressure

UF = Ultrafiltration

VCF = Volume concentration factor

J = Flux

J_{crit} = Critical flux

J_{lim} = Limiting flux

ΔP = Transmembrane pressure

Q = Crossflow rate

r = gas injection factor

R = Resistance

LIST OF ABBREVIATIONS AND SYMBOLS (continued)

R_{obs} = Observed rejection

S_a = Apparent sieving coefficient

T_r = Transmission

U_g = Superficial gas flow velocity

U_l = Superficial liquid flow velocity

Ψ = Selectivity

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

As the largest exporter of canned tuna in the world, Thailand costs a large amount of tuna annually (Prasertsan *et al.*, 1988). There is the great economic benefit from tuna canning industry, but the pollution problem for environment. The wastes from production of canned tuna, including both liquid and solid, make the big impact on the environmental protection. In recent years, the reuse of by-products (e.g. wash water, viscera and condensate) of fish products has been an attractive subject (Afonso and Borquez, 2002). The previous researches proved that these by-products contain many valuable materials, especially bioactive compounds. The utilization of these compounds from waste of canned tuna can not only improve economic value but also reduce the pollution load to the environment.

There are several techniques used to separate bioactive compounds from waste of seafood industry successfully, such as chromatography, gel-electrophoresis, etc. These methods provide the possibility for separation of valuable compounds from wastes of seafood industry. Unfortunately, they are only suitable for lab-scale, thus do not help the large-scale utilization for the industry.

Pressure driven membrane processes, e.g. microfiltration, ultrafiltration, nanofiltration and reverse osmosis, have successfully been implemented in many industrial applications. The applications of membrane separation for the recovery of valuable compounds from the industrial by-products have been investigated by many researchers. The results from their works have indicated that the membrane separation is a suitable method for the recovery of valuable compounds (e.g. protein, cells, enzymes and flavor) from industrial wastes with some unique advantages, such as low energy cost, large scale, simple instrument and operation. The applications of membrane processes for utilization of waste from seafood industry also have atttracted more attention.

Literature Review

1. The waste generated from fishery industry and their utilizations

Aquatic foods have become increasingly important due to their high nutrition. Because of the continuous increasing demand of aquatic foods in the global markets, a great amount of raw materials from natural fishery resources has been exploited annually. Apart from increasing yield to gain greater benefit, the environment friendly processes have been widely implemented to utilize raw materials effectively and reduce pollution to keep the industry sustainable. As the consequence, the utilization of processing wastes has been focused to minimize the waste disposal and management.

1.1 Tuna and tuna canning industry

Tuna and related species comprise a single family: the family *Scombridae*. This family, composed of 15 genera and 49 species, is subdivided in two sub-families: the *Gasterochismatinae* and the *Scombrinae*. In the sub-family *Scombrinae*, there are four tribes separated according to characteristic internal bones. These four tribes can be divided in two groups: that of the *Scombrini* and *Scomberomorini*, and that of the *Sardini* and *Thunnini*. The tribe *Thunnini* is more evolved, they are among the bony fishes and have a temperature regulating circulatory system that permits them to conserve a part of their metabolic heat; this characteristic explains why they are widespread in all oceans (Camera Hawaii, Inc., 1995).

All fish composing the family *Scombridae* are marine and epipelagic; they live in midwater in the upper layer (from 0 to 300 meters) and make occasionally important trophic or reproductive migrations. The most important commercial species are yellowfin (*Thunnus albacores*), bigeye (*T. obesus*), skipjack (*Katsuwonus pelamis*), albacore (*T. alalunga*) and bluefin (*T. thynnus*) (Diouf, 1993).

Yellowfin (*Thunnus albacares*) is one kind of large tuna. The major morphological characteristics include that the rays of the second dorsal and anal fins of the yellowfin are longer than those of other species; the flanks and ventral surface carry about 20

almost vertical more or less dotted lines. In the eastern Atlantic, the yellowfin is known from the Azores to South Africa; the common sizes lie between 35 and 180 cm fork length, that is between 0.8 and 111kg (Camera Hawaii, Inc., 1995).

The global production of the principal tuna market has tended to increase continuously, from below 0.5 million tons in the early 1950's to 3.1 million tons in 1994 (Guerard *et al.*, 2001). The demand of canned tuna is still increasing in recent years. Thailand has been the largest exporter of canned tuna since 1980's. Yellowfin is commercially the second most important species of tuna (Guerard *et al.*, 2001) and major raw material for canned tuna in Thailand. In 2002, total Thai canned tuna exports stood at 272,441 tons, up 1 percent from 269,696 tons in 2001 (Food market exchange, 2003).

1.2 The fishery processing by-products

The major part of aquatic animals for fishery processing is only meat. In other words, fishery processing is characterized by high level water consumption. Therefore, a big part of raw materials as well as the effluents from processing are wasted. The wastes generated from fishery processing can be separated to liquid and solid generally. The solid includes bone, skin, dark meat, viscera and pieces from flaking, etc. The liquid includes the water pumped together with the fish during its unloading, wash water, cooking water, oil, blood, mucus, condensate and so on. For example, the production of surimi, a product obtained from minced fish flesh that is washed with water from Pacific whiting (*Merluccius productus*) results in about 80% of the original raw materials is considered waste (Dewitt and Morrissey, 2002b). The fresh water for washing purposes during surimi processing is about 27 m^3/ton of surimi. The organic load of these wastewater is also high (Afonso and Borquez, 2002). In the case of canned tuna processing, there are 25-23% solid waste (e.g. head, skin, viscera) and about 35% liquid waste (e.g. blood, tuna condensate, oil) (Prasertsan *et al.*, 1988). Figure 1 showed the flow-chart for the tuna canning and the wastes from canning process. The tuna wastes constitute a biomass of particular interest to upgrade because of the global economic importance of tuna and their international trade for canning.

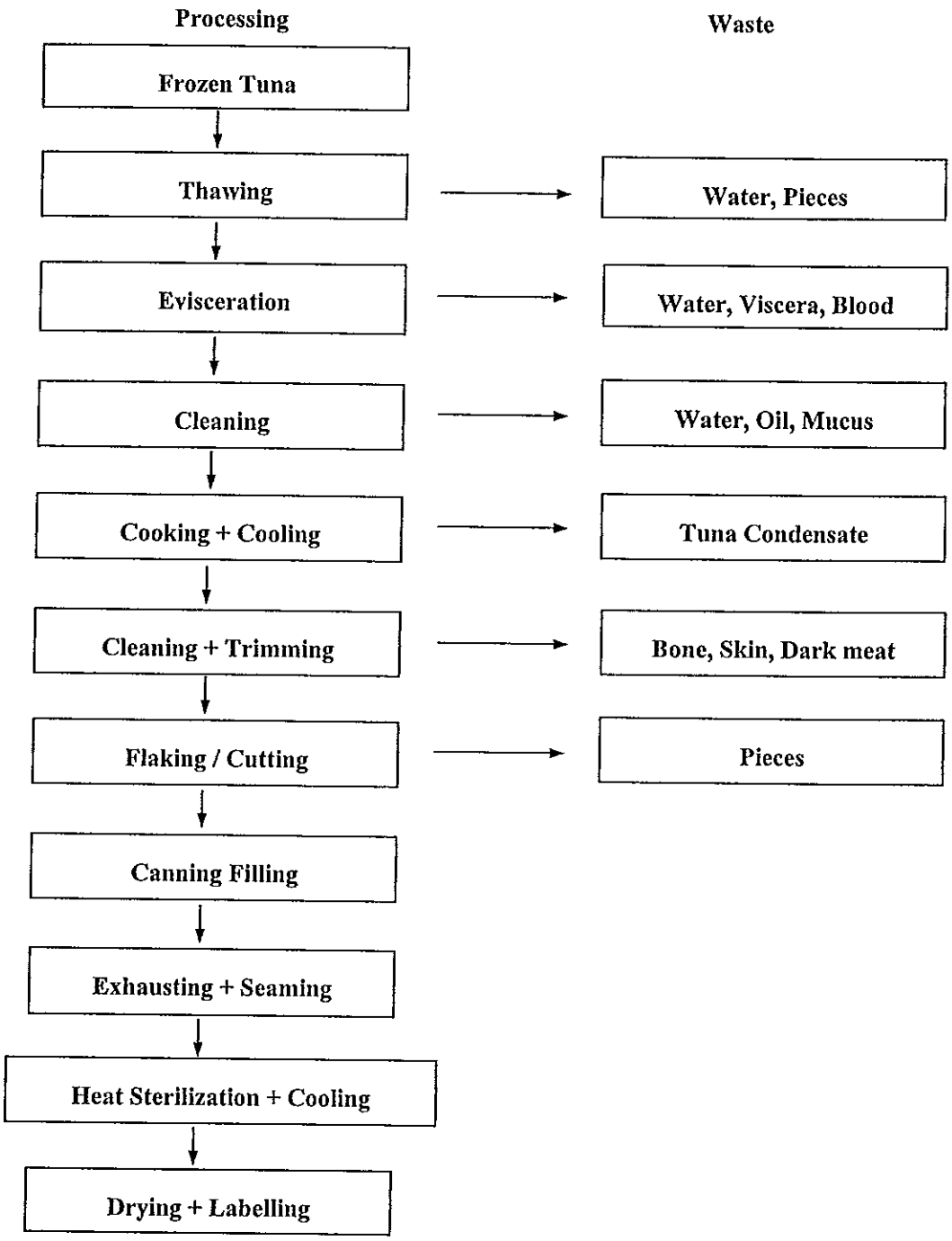

Figure 1. Typical flow-chart for the canning of tuna.

Source: H-Kittikun (2003)

1.3 Utilization of wastes from fishery processing

Both liquid and solid wastes contain high organic content and bioactive compounds which make these wastes to be the potential resources for further reuse. The use of fish waste has been of increasing interest over the past 10 years as biomass from marine origin is considered as a safe material and provides proteins with high nutritional properties and good pattern of essential amino acids (Guerard *et al.*, 2001).

1.3.1 Animal feed

Traditionally, wastes from fishery processing have been used as fish-meal for animal feeding. For instance, most discus fish farmers rely on freshly prepared wet feed based on several ingredients such as shrimp and cockles as protein sources (Chong *et al.*, 2002). Fresh shrimp and crab wastes (shell and viscera) have been used as silage for cattle (Evers and Carroll, 1996). Fresh crab waste has been successfully ensiled with molasses and straw and fed to sheep (Samuels *et al.*, 1991; Abazinge *et al.*, 1994). The silage made from the mackerel and abalone viscera has been used as an ingredient in abalone feed. Silage prepared from fish and abalone viscera are effective dietary protein sources for the juvenile abalone, *Haliotis fulgens*. Significantly higher growth rates occurred when abalone were fed artificial diets containing heated fish silage and unheated fish silage as a protein source compared with the kelp (Viana *et al.*, 1996). Several fishery by-products were investigated by Li *et al.* (2004). It was found that shrimp by-product meal may be a desirable protein feedstuff and more than 50% substitution of menhaden fish meal is possible for both juvenile and sub-adult red drum. Pacific whiting meal was reasonable well digested in the diet of sub-adult red drum. Red salmon head meal may be of special importance in diet formulations intended to limit phosphorus loading and thus deserve heightened attention.

1.3.2 Substrate of microorganism cultivation

Except for feed, the fishery wastes have been used as a substrate for microorganisms. Studies have been conducted by Martin (1999) in the composting of fisheries processing wastes with peat as a means of recovering the valuable biomass present in the fish waste. Subsequently, extracts from this compost were utilized as a substrate source in the growth of microorganisms in submerged fermentation. The production of fish wastes-peat compost was found to be an easy and inexpensive process which required a low energy input. The concentrations of nutrients such as nitrogen were higher in the compost than in the original peat. The utilization of shrimp waste as a substrate for solid-state cultivation of a filamentous fungus, *Aspergillus* sp. S1-13, was investigated by Rattanakit *et al.* (2002). The findings were notable from the viewpoint of chitinase production in that the organism formed almost the same amount of the enzyme or more as a known fungus grown on wheat bran supplemented with colloidal chitin. The crab shell powder prepared by treating shellfish processing waste with boiling and crushing was used as a substrate for isolating chitinolytic microorganisms. Three potential strains (E1, J1, J1-1) were isolated and identified as *Bacillus cereus*, *B. alvei* and *B. sphaericus*, respectively (Wang and Hwang, 2001). In addition, tuna condensate generated by cooking tuna with steam contains a high organic load and could be used as substrate for yeast cultivation. *C. tropicalis* TISTR 5146 was cultivated in the pretreated tuna condensate at room temperature on the shaker with a speed of 200 rpm. Ti was found that *C. tropicalis* TISTR 5146 gave the biomass of 3.72 g/l with a protein content of 58.15% (Chutinut, 1997).

1.3.3 Production of protein and protein hydrolysates

Another important way of upgrading for fishery waste has been the production of protein or protein hydrolysates in controlled conditions (Guerard *et al.*, 2001). Enzymatic hydrolysis of tuna stomach proteins by Alcalase was investigated in a batch reactor (Guerard *et al.*, 2001). The controlled hydrolysis of tuna stomach protein through the action of Alcalase 2.4L provided a high proportion of peptides from 6500 Da to di-peptides and free amino acids. Freeze-dried hydrolysates were tested successfully as nitrogen substrate for microbial cultures. Cano-Lopez *et al.* (1987) applied the trypsin from Atlantic cod for extraction of carotenoprotein from shrimp process wastes. 64% of the astaxanthin and 81% of the protein of shrimp waste was

recovered as carotenoprotein in 24 hours when 25 mg% cod trypsin was added to extraction medium containing 0.5 N EDTA. Gildberg (1992) described a method to produce protein hydrolysates from Atlantic cod stomach and intestine. After autolysis of these viscera, the major part of proteinous material (peptide and amino acid) was recovered from a clear aqueous phase occurred at the bottom of the tanks, and had a palatable taste similar to traditional fish sauce. Gildberg and Stenberg (2001) also developed a new process for utilization of shrimp waste. A high quality chitosan for application in cosmetics was produced from the processing waste of Northern shrimp (*Pandalus borealis*). Another research for acid hydrolysis of shrimp shell waste and the production of single cell protein from the hydrolysate has been done by Ferrer *et al.* (1996). The protein in shrimp-shell wastes was extracted by alkaline solutions at 30°C and a pH of 12 with an extraction time of 2 h and a solid/solvent ratio of 1:20. The results also showed that 90% of the extracted protein was recovered from the extract at a pH of 7-8. The chitin in shrimp-shell wastes was hydrolysed by hydrochloric acid and gives a yield of 80% of its weight of glucosamine. It is, therefore, a simple, fast process with good yields for the production of fermentable sugars which could serve as a substrate for the bioconversion process of the shrimp-shell waste into single cell protein. Morimura *et al.* (2002) developed a process for effective utilization of fish waste (the spine of a yellowtail fish). The process consisted of pretreatment, extraction of high molecular weight protein, and enzymatic hydrolysis of the residue of the extraction process. The extract had useful properties for use as a cosmetic material due to its high water retention capacity, ability to repair rough skin, lack of any odor problem and absence of harmful effects on skin. The hydrolysate also had suitable properties for use as a food additive due to its high anti-radical activity.

1.3.4 Resource of enzymes

The genetic variations with species together with adaptation to different environmental conditions have resulted in that fish enzymes have particular properties. Some these properties include higher catalytic efficiency at low temperature, lower thermal stability and substantial catalytic activity at neutral to alkaline pH (Diaz-Lopez *et al.*, 1998). For example, it was observed that Atlantic cod trypsin is a more effective extraction aid than bovine trypsin for

covering carotenoprotein from shrimp process waste at 4°C (Cano-Lopez *et al.*, 1987). The wastes from aquatic food processing provide ample raw materials for separation and purification of enzymes. The separation or production of valuable products using wastes from aquatic food processing has been done by a number of researchers. Heu *et al* (1995) separated the trypsin and chymotrypsin from viscera of anchovy (*Engraulis japonica*) by a series of steps including ammonium sulfate fractionation, dialysis and chromatography. The purity of trypsin and chymotrypsin were increased 78 and 119 fold, respectively, compared with crude extract. Simpson and Haard (1984) isolated trypsinogen from the pyloric ceca of Greenland cod by ammonium sulfate fractionation followed by acetone precipitation, and the trypsinogen obtained was purified by affinity chromatography on soybean trypsin inhibitor-Sepharose 4B. Bustos *et al.* (1999) purified the trypsin-like enzymes from Antarctic krill processing wastewater. From this wastewater, trypsin-like enzymes were purified 140-fold in one step by affinity chromatography on *p*-aminobenzamidine Sepharose 4B. Tuna viscera were also used for separation of enzymes. Two anionic trypsins (A and B) were purified to homogeneity from yellowfin tuna (*Thunnus albacores*) spleen by a series of column chromatographies including Sephacryl S-200, Sephadex G-50 and DEAE-cellulose. Purity was increased to 70.6- and 91.5-fold with approximately 2.8% and 15.6% yield for trypsin A and B, respectively (Klomklao *et al.*, 2006). Three trypsin isoforms, trypsins A, B and C, from the spleen of skipjack tuna (*Katsuwonus pelamis*) were purified by a series of chromatographies including Sephacryl S-200, Sephadex G-50 and diethylaminoethyl-cellulose (Klomklao *et al.*, 2007).

2. Membrane processes and their applications in fishery industry

Membrane filtration process is an approach to separate different materials by semi-permeable membranes which allow the passage of one or more of the materials much more readily than the others. Pressure plays a role as driven force. These methods have several advantages as following (Cheryan and Rajagopalan, 1998):

(1). This technology is more widely applicable across a wide range of industries.

(2). The membrane is a positive barrier to rejected components. Thus, the quality of the treated water (the permeate) is more uniform regardless of influent variations. These variations may decrease flux, but generally does not affect quality of its output.

(3). No extraneous chemicals are needed, making subsequent recovery easier.

(4). Membranes can be used in-process to allow recycling of selected waste streams within a plant.

(5). Membrane equipment has a smaller foot print.

(6). Energy costs are lower compared to other treatments.

(7). The plant can be highly automated and does not require highly skilled operators.

Over the last 30 years, the membrane processes, including reverse osmosis (RO), ultrafiltration (UF), microfiltration (MF), nanofiltration (NF), dialysis (D), electrodialysis (ED), gas permeation (GP), pervaporation (PV) and liquid membrane, have been widely adopted by different industries. They are used to produce potable water from sea, to clean industrial effluent and recover valuable constituents, to concentrate, purify or fractionate temperature sensitive solutions (food and drug industry, biotechnology), to remove urea and other toxins from the blood stream in an artificial kidney, to release certain drugs at a predetermined rate (controlled release), and so on (Madaeni, 1999).

Large-scale commercial uses of membrane processes have displaced conventional separation processes and more are expected in the future. Membrane processes have always been an integral part of biotechnology processes for fermentation, clarification, purification and concentration (Reis and Zydney, 2001). In particular, microfiltration, ultrafiltration and reverse osmosis are increasingly being applied for treating industrial waste and water according to their capacity of separation. Microfiltration is the separation of particles, whereas ultrafiltration is the separation of macromolecules and reverse osmosis is the separation of ionic components. Figure 2 presents the suitable membrane separation processes for different size ranges and Table 1 shows the pore size and applied pressure for these processes.

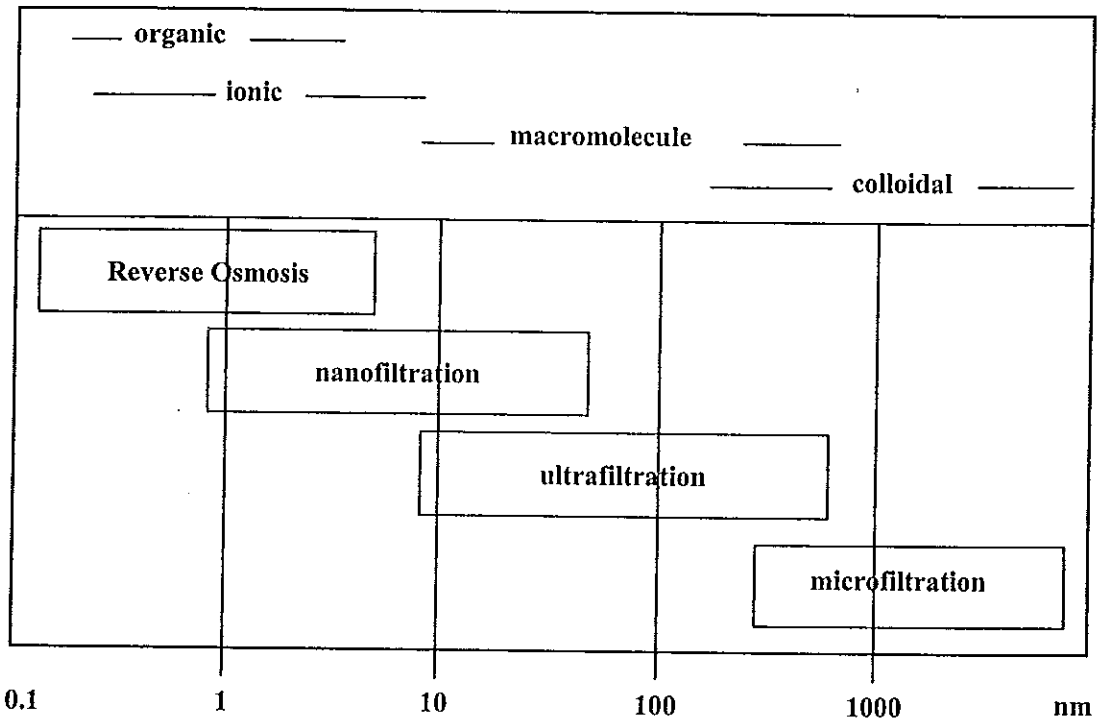

Figure 2. Separation capacity of membrane processes.

Source: Madaeni (1999).

Table 1. Pore size and applied pressure for membrane processes.

	RO	NF	UF	MF
Pore size	No detectable pore size	2-5nm	5-20nm	20nm-1μm
Pressure	30-150atm	5-20atm	2-7atm	1-3atm

Source: Madaeni (1999).

2.1 Ultrafiltration and microfiltration

As mentioned above, the separation capacity of UF and MF covers macromolecules and particles. Their characteristics make them more important for applications in biotechnology, such as purification and concentration of protein, amino acid or enzymes. MF can be used as a pretreatment step for UF. UF membranes typically have pore sizes in the range from 10 to 1000 Angstrom and are capable of retaining species in the molecular weight range of 300-500,000 daltons. Typically rejected species include sugars, biomolecules, polymers and colloidal particles (Kulkarni *et al.*, 1992). When pressure-driven flow through a membrane or other filter medium is used to separate micron-sized particles from fluids, the process is called microfiltration. Although the exact size range is matter of debate, microfiltration is generally defined to be the filtering of a suspension containing colloidal or fine particles with linear dimensions in the approximate range of 0.02 to 10 μm (Davis, 1992). This size range encompasses a wide variety of natural and industrial particles including yeast cell, red blood cell, bacteria, viruses, paint pigment, pulverized coal, colloidal silica, smog, carbon black, silt, clay, and so on. Commercial applications of UF and MF are numerous and found in many different fields. For instance, UF has been used for concentration of blood serum (Pruthi, *et al.*, 1997) and egg-white (Ehsani, *et al.*, 1997), clarification of fruit juices (Girard and Fukunoto, 2000) and gelatin solutions (Dutre and Gragardh, 1995), recovery of protein (Zwijnenberg *et al.*, 2002). MF has been used for concentration and purification of soluble pectin from mandarin peels (Cho *et al.*, 2003), separation of non-living yeast after treatment of waste water (Keskinler *et al.*, 2004), reduction of microbial content in milk (Beolchini *et al.*, 2004), filtration of fermentation broths, upgrading of cleaning solutions in food and metalworking industries (Ripperger and Altmann, 2002).

2.2 Factors affecting the performance of ultrafiltration and microfiltration

The liquid-phase membrane separations, e.g. UF and MF, universally rely on the preferential retention and passage of at least one solute and one solvent. Both these processes often involve selective separation of multiple solutes. The primary objectives of multiple-solute separations are to achieve both a desired yield of the product and an acceptable level of

purification, from one or several impurities (Reis and Saksena, 1997). However, one cannot achieve complete retention of the retained solute with current membrane technology in practice. There is a compromise between yield and purification. Separating solutes with similar retention properties, such as selective protein filtration, is a particularly difficult problem (Nakassuka and Michaels, 1992). The function of UF or MF process is affected by a series of factors. Major factors include pressure, feed velocity, solute concentration and temperature. Interaction between feed solution and membrane, characteristic of solute and cleaning operation also should be considered in many cases.

Normally, the flux (J) of permeate is described as a function of the driving force and the total resistance (Meindersma *et al.*, 1995)

$$J = \frac{\Delta P}{\mu R} \tag{1}$$

where, ΔP is the differential pressure(or transmembrane pressure); μ is the dynamic viscosity of the UF; R is total resistance.

From equation (1), the total resistance R could be written as the sum of R_m which is the clean membrane hydraulic resistance, R_{ir} which is an irreversible fouling and R_r which includes reversible fouling and the concentration polarization (CP) effect.

The transmission of a partially rejected protein through a membrane is expressed by the apparent sieving coefficient conveniently (Ghosh and Cui, 2000).

$$S_a = \frac{C_P}{C_b} \tag{2}$$

where C_b is the protein concentration in the bulk feed; C_p is the protein concentration in permeate.

The diamensionless ratio of sieving coefficients has been termed as selectivity (Reis and Saksena, 1997) and it defined as:

$$\Psi = \frac{S_{a1}}{S_{a2}} \quad (3)$$

where Ψ is the selectivity, and S_{a1} and S_{a2} are the observed sieving coefficients for the lesser- and greater- retained solute, respectively.

From equation (3), a larger ration (Ψ) between the observed sieving coefficients is correlated with a better separation between two solutes.

2.2.1 Concentration polarization and fouling

During UF or MF process, the major problems are concentration polarization and fouling. Both concentration polarization and fouling cause the reduction of the flux to far below the theoretical capacity of the membrane.

Concentration polarization is the accumulation of retained solutes to a high concentration on the membrane surface. As a result of this accumulation, the membrane effectively experiences a higher feed-side concentration resulting in reduced flux as well as reduced apparent rejection. Concentration polarization is considered to be reversible and can be controlled in a membrane module by means of velocity adjustment, pulsation, ultrasound or an electric field (Sablani *et al.*, 2001).

The occurrence of fouling affects the performance of the membrane either by deposition of a layer onto the membrane surface or by blockage or partial blockage of the pores. Fouling changes the effective pore size distribution and reduces both membrane flux and selectivity (Field *et al.*, 1995).

Both concentration polarization and fouling strongly depend on the operation condition and characteristic of membrane.

2.2.2 Transmembrane pressure

Transmembrane pressure is a key operating parameter for pressure driven membrane process (Youravong, 2001). UF or MF process can be separated to two regions

according to the profile of flux versus transmembrane pressure. The first region is a pressure-dependent region. During this region, the permeate flux increases with the increase of transmembrane pressure. On the other hand, higher transmembrane pressure also increases membrane fouling (Taddei *et al.*, 1989). The second one is a pressure-independent region where the permeate flux keeps almost constant even the transmembrane pressure increase. Therefore, a suitable pressure reduces not only cost of energy but also fouling.

2.2.3 Feed velocity

Feed velocity or shear stress at the membrane surface is another key factor influencing membrane flux and fouling, especially, for reduction of concentration or reversible fouling resistance. High shear rates generated at the membrane surface tend to shear off deposited material and thus reduce the hydraulic resistance of the fouling layer. Fouling may be more severe when a solution filtered at low feed flow rate (Torres *et al.*, 2002). But there are some exceptions. Ghost *et al.* (2000) observed that cross flow velocity had a negligible effect on the observed transmission of lysozyme. They thought that lysozyme was very easily transmitted through the membrane used in their works, hence the extent of concentration polarization of lysozyme molecules was negligible.

2.2.4 Temperature

The effect of temperature on separation performance is not too clear. In general, increasing temperature could decrease viscosity of feed which results in higher permeate flux. In addition, the temperature should be set carefully during the separation of thermo-sensitive subjects, such as enzyme.

2.2.5 Feed solution property

Feed solution property also plays an important role during separation process. Generally, as the concentration of solutes in the feed increases, its viscosity and density increase

and its diffusivity decreases. These changes in the physical properties affect the absolute value of the flux and higher feed concentration usually aggravates fouling. On the other hand, different solutes, such as protein, fat, oil, carbohydrates, salts or biofoulant, have different modes of fouling. A brief review about relationship between fouling and feed component has been done by Li *et al.* (2004). In many cases, a pretreatment of feed is reasonable.

2.2.6 Membrane property

Membrane properties also affect the formation of fouling during UF or MF as well as other membrane separation processes. Both materials of membrane and surface topography are the reasons related to differences of fouling. For example, fouling problem is more severe for hydrophobic membrane than hydrophilic membrane. And cellulose membrane can reduce fouling much more than other hydrophobic membranes (Shi *et al.*, 2001). The surface of cellulose acetate membranes is smooth and uniform. In contrast, polyamide thin-film membranes have protuberances on the surface, which could act as hooks for suspended matter in the feed, thus leading to greater fouling. So polyamide based membranes tend to foul more than cellulose acetate membrane (Murthy and Gupta, 1999).

2.3 Methods for improvement of membrane separation process

As mentioned above, concentration polarization and fouling lead to a performance loss of membrane separation processes. The causes of these phenomena are variable. Successful operation of membrane plant requires careful management of concentration polarization and fouling of the membrane. The avoidance of these two problems is probably not possible, but its impact can be limited by a variety of techniques. The choice of membrane, module, process configuration and pre-treatment are all important for varying extents. A high degree of separation should be achieved with acceptable productivity. In addition to improving filtration rates, avoidance of fouling also results in easier cleaning of membranes. This may limit the need for severe cleaning regimes, and hence prolong the lifetime of polymeric membranes (Wakeman and Williams, 2002).

2.3.1 Pretreatment of feed bulk

It is well known that fouling of membrane process can be reduced by a suitable pretreatment of feed. Pretreatment of feed is used either to remove particulates that may cause clogging in the module or to prevent particulates or macromolecules from reaching and depositing on the membrane surface or to reduce the total contaminant load in downstream membrane modules (Wakeman and Williams, 2002). Both physical methods and chemical methods are available for pretreatment. Physical methods, such as pre-filtration and centrifugation are usually used to remove large particles from the feed. Chemical methods without any harmful effect on the target of separation can be used for pretreatment. For instance, pH adjustment is often used to make molecular or colloidal foulants far from their isoelectric point, thereby reducing their tendency to form a gel layer. In many cases, chemicals methods and physical methods are combined to achieve a desirable result. Precipitation, coagulation or flocculation can be achieved by chemical treatments, then pre-filtration or centrifugation can be applied to remove solids generated from chemical pretreatments. In addition, heat is also a common method for pretreatment.

2.3.2 Membrane materials

Membrane materials also influence the fouling. Appropriate choice or modification of membrane materials can lead to looser binding of the solutes to the membrane surfaces, which can have the effect of lessening any membrane-solute interaction that leads to a reduction of membrane permeability (Wakeman and Williams, 2002). The work from Ma *et al.* (2001) indicated that a significantly higher permeate volume was obtained for membrane modified with acrylic acid (hydrophilic, negatively charged), and a lower permeate volume was obtained with membranes modified with dimethyl aminoethyl methacrylate (hydrophilic, positively charged), compared to that for the unmodified polypropylene membrane (hydrophobic, neutral)

2.3.3 Flow manipulation

Concentration polarization and cake formation at membrane surface are strongly controlled by wall shear stress at membrane surface or turbulent flow. Therefore, hydrodynamic methods play important roles to improve performance of membrane process.

2.3.3.1 Increasing feed velocity

It has been proved that increasing feed cross flow rate (or cross flow velocity) can increase permeate flux. Torres *et al.* (2002) observed that fouling (i.e. decreasing permeate flux) was more severe when the solution was ultrafiltered at both low feed flow rate and membrane molecular weight cut-off and the highest flux was obtained at the maximum recirculation flow during their study of operating conditions in concentration of chicken blood plasma proteins by ultrafiltration. Ghosh *et al.* (2000) worked for separation of lysozyme by hollow fiber ultrafiltration. In their study, the permeate flux was found to increase with increase in the value of cross flow velocity. The increase in cross flow velocity caused reduction in concentration polarization and this led to an increase in the permeate flux. They also found that effective selectivity was generally insensitive to the cross flow velocity in their research. Increased cross flow rate also enhanced the permeate flux during separation of proteases from fish viscera by ultrafiltration (Li *et al.*, 2006).

2.3.3.2 Turbulent/vortex promotion

On the other hand, increased flow velocity causes the increase of energy cost. It should be considered in industrial applications. Thus turbulent or vortex promoter attracts more and more attention from researchers. Turbulence is encouraged by appropriately designed feed spaces or static mixers (Wakeman and Williams, 2002).

The vortex flow filtration (VFF) system containing cylindrical annulus with an rotating inner cylinder can be characterized by the taylor number, T_a (Roth *et al.*, 2001):

$$T_a = \frac{\omega R_1 (R_2 - R_1)}{\nu} \frac{[2(R_2 - R_1)]^{0.5}}{(R_2 + R_1)^{0.5}} \quad (4)$$

where ω is the rotational velocity of the inner cylinder; R_1 is the inner radius of the annular region; R_2 is the outer radius of the annular region; ν is the kinematic viscosity of the fluid.

For $41 < T_a < 800$, the system has laminar flow with vortices. For $800 < Ta < 2000$, transitional vortex flow occurs, and for $2000 < T_a < 10{,}000\sim15{,}000$ turbulent flow with vortices occurs. The flow in the axial direction can be characterized by an axial Reynolds number:

$$R_{e_a} = 2V_z (R_2 - R_1)/\nu \quad (5)$$

where ν is the kinematic viscosity of the fluid; R_1 and R_2 are the inner and outer radii, respectively. V_z is the velocity in the axial direction.

In most cases where $R_1\omega >> V_z$, the flow field within the annular region is dominated by the rotation of the cylinder. Consequently, the shear force at the wall is mainly dependent on the angular rotation and hence the Taylor number.

There are a lot of types of turbulent or vortex promoter. The nut-shaped external helix design was successful in filtering whole blood (Millward *et al.*, 1995). Filtration performances of between three and five times that were obtained in existing commercial devices were claimed. The vortex wave produced an enhancement factor of 3.5 relative to a flat unobstructed channel in this study. Ding *et al.* (2002) investigated the application of dynamic filtration using a disk equipped with vanes rotating at high speed near a membrane to total protein concentration of UHT skimmed milk. The addition of vanes to the disk increases the permeate flux by about 56% due to higher fluid core velocity resulting in higher shear rate at the membrane. The performance of helical inserts in existing tubular filters has been investigated by Bellhouse *et al.* (2001). The insert and filter combination was found to deliver high filtration fluxed at low cross flow rates over a wide range of applications covering reverse osmosis to microfiltration. In addition, a flat-plate ultrafilter equipped with a ladder-like flow deflector was used to perform partial separation of globulins from albumin in bovine plasma successfully (Najarian and

Bellhouse, 1996).

2.3.3.3 Backpulsing

High feed or retentate velocity can reduce cake formation and concentration polarization, but it also causes a high pressure drop on the feed side. Thus the permeate channel is often at or near to atmospheric pressure. The transmembrane pressure at the feed end of the filter is greater than at the retentate discharge end, causing more cake compaction at the feed end. Backpulsing (also known as backshocking or backwashing) has been proved to be effective to overcome this problem. Backpulsing is a short duration (about 0.1s or shorter) and may be operated continuously or periodically. Backpulsing makes a reversed flux of permeate flow back through the membrane, lifts off the cake and flushes it out of the module (Serra *et al.*, 1999). The average permeate flux per cycle of backpulsation is given by the amount of permeate collected per membrane area during forward filtration between backpulsing, minus that lost during the short backfiltration period, divided by the cycle duration (Ramirez and Davis, 1998).

$$\langle J \rangle = \frac{\left(\int_0^{t_f} J_f(t)\mathrm{d}t - \int_{t_f}^{t_f+t_b} J_b(t)\mathrm{d}t \right)}{t_f + t_b} \tag{6}$$

where J_f is flux magnitude(volume permeate per time per membrane area) during forward filtration of duration t_f and J_b is the flux magnitude during a backpulse of duration t_b.

During the backpulse part of the cycle, it is assumed that a fraction of the membrane area is instantly and completely cleaned, so that the reverse flux during the backpulse is simply a weighted average of the clean membrane flux, $J_o$and the fouled membrane flux, J_s.

$$J_b = \beta J_o + (1 - \beta) J_s \tag{7}$$

where β is treated as a fitted parameter which may determined from measurement of the net permeate collected at different backpulsing frequencies.

In comparison to forward filtration, long-term flux enhancements of up to 4-fold

were obtained by using water backpulsing for filtration of 1.0m diameter carboxylate modified latex particles using unmodified polypropylene membranes (Ma *et al.*, 2001). For optimization of the membrane purification of a vaccine using backpulsing, Meacle *et al.* (1999) observed the implementation of membrane backpulsing significantly improved the performance of this membrane based purification step for removal of unreacted polysaccharide from the conjugate vaccine product. Buffer requirement and cycle time were appreciably reduced without affecting product quality or product yield and backpulsing was successfully scaled-up from the lab scale. The authors also observed that backpulsing was most effective at low shear rates, when the protein gel-layer was expected to be thickest and sieving was significantly better at low shear rates when backpulsing was used.

2.3.3.4 Crossflushing

Except backpulsing, crossflushing is also occasionally used in membrane process. Crossflushing involves periodic stoppage of the permeate flow while maintaining cross flow across the membrane. If sufficiently non-adhesive, the particles in the external cake may erode from the cake and be swept away due to the cross flow. Kuruzovich and Piergiovanni (1996) studied crossflushing of yeast suspensions using corssflushing intervals of 5-90 s every 30-360 s. The authors observed more than 100% flux increase under optimal conditions. Redkar and Davis (1995) also studied crossflushing with washed yeast suspensions. The authors observed nearly 10-fold flux increase due to crossflushing of 2 s every 9 s. But this flux increase was much smaller than the nearly 30-fold flux increase observed when backpulsing for 2 s was done every 9 s. A series of studies has proved that crossflushing is a much weaker cleaning technique than backpulsing. Net flux is nearly steady with bachpulsing, but it continuously declined with crossflushing. Crossflushing is also expected not to be effective for the filtration of the adhesive foulants such as unwashed or even washed bacterial cells (Kuberkar and Davis, 2001).

2.3.3.5 Operation according to critical flux

The concept of critical flux (J_{crit}) introduced by Field *et al.* (1995) suggests that

there is a flux below which species have negligible interaction with the membrane. If permeate flux is above the critical flux, an irreversible deposit appears. The critic flux results in a force balance between drag forces and surface interaction in a mass boundary layer (Espinasse *et al.*, 2002). The critical flux may be a desirable operational target for a clean membrane plant. If non-fouling operation can be sustained and low energy operation promoted, then costs of cleaning are reduced and energy is saved. There are two types of critical flux. In the first definition a 'strong form' of critical flux exists if the flux of a suspension is identical to the flux of clean water at the same transmembrane pressure. In the second definition a 'weak form' of the critical flux exists if the relationship between transmembrane pressure and flux is linear, but the slope of the line differs from that for clean water (Wu *et al.*, 1999). Chang and Judd *et al.* (2002) carried out a cross flow ultrafiltration of binary protein solutions using flux-stepping and constant flux experiments to identify the apparent critical flux. The results indicated that the formation of a dynamic layer of the retained species was the main contributor to the rapid increase in transmembrane pressure. Metsamuuronen *et al.* (2002) determined the critical flux by constant flux experiments under laminar flow conditions in ultrafiltration of myoglobin and baker's yeast. In their work, critical flux increased with increasing flow velocity and decreasing solute concentration. In the case of baker's yeast below the critical flux, the flux was about the same as the pure buffer solution flux showing a strong form of the critical flux. Howell (1995) showed an example for sub-critical flux operation of microfiltration in an activated sludge plant in which clarified effluent was removed from the aeration tank through membrane immersed in the tank. A constant flux operation at a value lower than critical flux was maintained ant the system had been operated for over 12 months without chemical cleaning or backflushing. The only cleaning activity was the constant flushing of the membrane surface by air bubbles generated to aerate the bioreactor and by the periodic suspension of permeate withdrawal. Youravong (2001) investigated the critical flux during ultrafiltration of milk components. The author observed that the critical fluxes of milk components including sodium caseinate, whey protein concentrate suspensions, reconstituted skimmed milk and skimmed milk were weak form.

2.3.3.6 Gas sparging

As mentioned above, various methods have been used to reduce the negative effect of concentration polarization and fouling during membrane processes. Compared to other methods, a novel gas sparging (or gas bubbling) technique has been proved to be effective, simple and economical. Gas sparging is a hydrodynamic method to enhance membrane process by introducing gas (e.g. compressed air or nitrogen gas) into module to generating secondary flow to promote local mixing near the membrane surface (Li *et al.*, 1998).

The two-phase flow pattern depends on the air injection factor (r) which defined as (Chang and Judd, 2002; Psoch and Schiewer, 2005):

$$r = \frac{U_g}{(U_g + U_l)} \tag{8}$$

where U_g and U_l are the superficial gas velocity and superficial liquid flow velocity, respectively.

According to the value of ε, the flow pattern can be characterised as bubble flow ($r < 0.2$) in which air bubble are dispersed in the liquid phase; slug flow ($0.2 < r < 0.9$) which comprises alternate slugs of gas and liquid; and annular flow ($r > 0.9$) in which continuous gaseous phase occupies the center of the pipe (Cabassaud *et al.*, 2001). It is well known that slug flow is the most efficient regime for significant enhancement of flux (Marcier *et al.*, 1997). The vertically posited tubular and hollow fiber membranes are most common membrane units for gas sparging application.

In the case of a gas sparging UF in the condition of upward slug flow using tubular membrane unit, one unit of gas slug followed by a liquid slug can be separated to three distinct zones, i.e. (1) the falling film flow zone corresponding to the length of the gas slug; (2) the wake zone; and (3) the remaining liquid slug zone. the average permeate flux ($J_{v,ave}$) of gas sparged ultrafiltration can be calculated by:

$$J_{v,ave} = [L_1 J_{v1} + L_2 J_{v2} + L_3 J_{v3}] / L_t \tag{9}$$

where L_1, L_2, L_3 are the lengths of tubular membrane in the falling film flow zone, the wake zone and the remaining liquid slug zone, respectively. L_t is the length of tubular membrane. J_v is the permeate flux in each zone.

A gas-sparged hollow fiber ultrafiltration with aqueous 144 kDa dextran solution was performed by Smith and Cui (2004). It was found that flux enhancement was higher under continuous rather than intermittent gas sparging. Enhancement of up to 102% was found with a 10 g/L feed concentration. Results gave good insight into how design and operation parameters affect permeate flux in gas-sparged hollow fiber ultrafiltration. The gas injection technique was also introduced to achieve flux improvement in cross flow ultrafiltration of oil emulsion. (Um *et al.*, 2001). By the nitrogen gas injection, homogeneous liquid phase oil/water emulsion was changed to heterogeneous gas-liquid phase. The injected gas caused positive effect of promoting turbulence. With gas injection, as long as a certain level of gas fraction was maintained, much higher flux was achieved than as expected from the film theory. However gas injection also had negative effect of decreasing the effective membrane area due to the partial occupation of membrane pores by bubbles. The efficiency of the gas injection was found out to be dependent on bubble fractions in the mixture. The potential use of cross flow ultrafiltration with gas sparging to filter and break a foam solution of an aqueous polymer was investigated by Abdel-Ghani (2000). A solution made of 300 kDa polyvinylpynolidone dissolved in water was introduced into a tubular ultrafiltration membrane module having a 100 kDa MWCO. The polymer has the property of being able to form metastable foam when air is injected into solution. The foam was injected into the membrane module at different pressure and concentration. It was found that, under same transmembrane pressure, the permeate flux was higher with the foam present than without. Depending on the flow conditions, up to 125% increase in flux was observed. Li *et al.* (1997a) investigated the effect of bubble size and frequency on the permeate flux of gas sparged ultrafiltration with tubular membranes. Solutions of dextran and human serum albumin were used as test media. It was found that permeate flux increased with increasing bubbling frequency in the examined range. The effect of bubble size on flux was divided into two regions, an increasing region for small bubbles and a plateau region for larger slugs. On the other hand, some negative effects of gas sparging should be considered (Cui *et al.*, 2003). There is the risk of foaming and

possible bubble damage to proteins or microorganisms. The injected gas may dissolve into the liquid at elevated pressure, the dissolved gas could be released into the permeate side which may result in the build up of back pressure and reduce the efficiency of the process.

In addition, there are also some other methods to improve membrane processes. A high shear stress can be developed at the membrane surface by rotating the surface at high speed rather than pumping feed across the surface at a high cross flow velocity. Wronski *et al.* (1989) reviewed several designs of rotating membranes. Ghosh (2006) provided a novel cascade ultrafiltration configuration. The configuration including three stages was specifically designed for continuous, high-resolution protein-protein fractionation. By suitably adjusting the flow streams within this novel configuration, high recovery as well as high purity of each target protein was achieved.

2.4 Applications of membrane processes for treatment of wastes from fishery industry

Waste generated from fishery industry can be reused for many applications. Nowadays, according to their unique advantages, membrane processes have served more and more benefits for treatments of wastes from fishery industry.

Dewitt and Morrissey (2002a) studied the parameters for the recovery of proteases from surimi wash water. In their study, laboratory scale ultrafiltration experiments were performed to establish conditions for recovery of a heat stable, acid protease from Pacific white (*Merluccius productus*) surimi process water. 10-fold concentration of protease activity was achieved after ultrafiltration under experimental conditions. Same authors (Dewitt and Morrissey, 2002b) also set a pilot plant recovery of catheptic proteases from surimi wash water by ultrafiltration. After pretreatment, surimi wash water was pumped from the feed tank to the filtration unit at 3.75-4.00 gal/min under an average transmembrane pressure of 20-21.5 psi. The filtration system with molecular weight cut-off 30 or 50 kDa polyethersulfone membrane. After ultrafiltration, enzyme purity was increased about 100-fold, and yield was approximately 80%. Gildberg (1992) developed simple methods for diversified utilization of stomach and intestine of Atlantic cod (*Gadus morhua*). A high concentration of pepsin (9 g/l) was obtained by ultrafiltration of the aqueous phase from the cod stomach silage preserved with formic acid. A

concentration of trypsin-like enzymes could be obtained by ultrafiltration of fish sauce produced by salt fermentation of cod intestines. The permeate from the ultrafiltration contained the major part of proteinous material which had a palatable taste similar to traditional fish sauce. Tryptic enzymes were also obtained by ultrafiltration of fish sauce made from cod viscera (Gildberg and Shi, 1994). Recovery of a enzyme mixture containing trypsin and chymotrypsin activity from yellowfin tuna spleen was achieved by a using plate membranes (regenerated cellulose) with molecular weight cut-off of 30 kDa (Li *et al.*, 2006). The purification of about 18-folds for proteases was obtained at the number of diafiltration volume of 6.2 during crossflow rate of 360 l/h and TMP of 1.5 bar. Vandanjon *et al.* (2002) developed an application of membrane process to utilize wastes from fishery industry. Authors recovered marine flavors from seafood cooking waters of shrimps, buckies or tuna. A good retention of marine flavors was achieved by using ultrafiltration following by reverse osmosis, and the polluting load was considerably decreased after reverse osmosis (COD reduced by 95% for shrimps and buckies and 85% for tuna). Afonso and Borquez (2002) provided a review of the treatment of seafood processing wastewaters and recovery of proteins therein by membrane separation processes. The review surveyed many researches in this field and these results showed that membrane process is a strong tool for the treatment of almost all types of seafood wastewaters from different sources such as surimi, fish unloading, fish meal, fish fillets, shellfish and crustaceans, miscellaneous, and so on.

3. Production and applications of protein hydrolysates

The aim of every hydrolysis method is the quantitative liberation of all amino acids of the substrate and the quantitative recovery of them in the hydrolysate. Protein hydrolysis can be accomplished by either chemical or enzymatic method. Chemical hydrolysis can be performed under acidic or alkaline condition.

Protein hydrolysates can be classified into three major groups depending on the degree of hydrolysis, which determines their applications: hydrolysates with a low degree of hydrolysis with improved functional properties, hydrolysates with a variable degree of hydrolysis that are used mostly as flavourings, and extensive hydrolysates that are mostly used as nutritional supplements and in special medical diets (Pedroche *et al.*, 2004).

3.1 Acidic hydrolysis

Acidic hydrolysis can be performed in a liquid- or gas-phase mode. Hydrolysis is usually performed by heating a sample in a presence of high concentration acids using thermal or microwave energy (Weiss *et al.*, 1998). The hydrochloric acid (HCl) is the most common reagent for acidic hydrolyisis. This is due to the convenience of application of this reagent, as it can be used in both the liquid- or gas-phase modes and can be evaporated afterwards, so that the hydrolysate is recovered in a small volume of the reconstitution buffer (Fountoulakis and Lahm, 1998). Other acidic reagents include sulfonic acid, methanesulfonic acid (MSA), toluenesulfonic acid, Mercaptoethanesulfonic acid or the mixture of several acids. Both liquid- and gas-phase hydrolysis of BSA and recombinant human interferon α_2 (IFN α_2) was described by Weiss *et al.* (1998). Conventional hydrolysis of protein with HCl and MSA yielded very accurate composition data. Hydrolysis using microwave energy was responsible for the higher racemization. Chiou and Wang (1988) applied hydrolysis with 4 M methanesulfonic acid at 160°C for 45 min. Oxygen was removed from the hydrolysis mixture by flushing with nitrogen instead of removal by evaporation. They obtained amino acid recoveries similar to those achieved following hydrolysis at 110°C for 24 h. Hayashi and Suzuki (1985) reported the hydrolysis with 3 M p-toluenesulfonic acid at 110°C for 22 h. Methionine and methionine sulfone were quantitatively recovered, whereas methionine sulfoxide was partially converted to methionine during hydrolysis (about 94% recovery).

3.2 Alkaline hydrolysis

Alkaline hydrolysis is almost exclusively used for determination of tryptophan which is stable under basic conditions. Alkaline hydrolysis is also applied if the protein sample contains a large percentage of carbohydrates, as it is the case with foods (Fountoulakis and Lahm, 1998) and in the formulation solutions of pharmaceutical proteins, which usually include high percentages of monosaccharides (Gupta *et al.*, 1997). Alkaline hydrolysis is usually performed with NaOH, KOH or more seldom with barium hydroxide (Fountoulakis and Lahm, 1998; Huet

and Pernollet, 1986). Delgalo-Zamarreno *et al.* (1995) applied an alkaline hydrolysis to hydrolyse Vitamin A, D_3 and E in milk using alcoholic sodium hydroxide solution consisting of 50 ml ethanol and 15ml of 60% aqueous NaOH solution and 5 ml 10% of ascorbic acid. Kintz and Cirimele (1997) applied alkaline hydrolysis of protein in human hair for testing human hair for drug of abuse. The hydrolysis process was performed by incubating a 30 mg quantity of powdered hair in 1 ml of 1 M sodium hydroxide for 10 minutes at 95°C in the presence of 100 ng of amphetamine-d, methamphetamine-d, methylenedioxyamphetamine-d and methylenedioxymethamphetamine-d_5. In the study of tryptophan determination in proteins and feed stuffs (Ravindran and Bryden, 2005), the chicken egg white was hydrolysed with NaOH in Teflon containers under an atmosphere of nitrogen at 120°C for 15 hours.

3.3 Enzymatic hydrolysis

There is an increasing interest in the development of fast and gentle enzymatic production methods as an alternative to mechanical and chemical treatments which often damage the products and reduce product recovery (Gildberg, 1993). Therefore, enzymatic methods are widely used for protein hydrolysis. Enzymatic hydrolysis has some advantages over the chemical methods. First, enzymatic methods can hydrolyse both collagenouos and non-collagenous proteins. Second, enzymatic methods have the potential of reducing the volume of waste. Furthermore, the enzymatic hydrolysis process is simple, efficient and involves the mild conditions (Fonkwe and Singh, 1996). According to these advantages of enzymatic hydrolysis, numerous enzymes have been employed for protein hydrolysis in many fields, especially in food industry, biotechnology and pharmaceutical. For instance, Alcalase was used for the hydrolysis of tuna stomach proteins at pH 8 and 50°C. The action of this enzyme provided a high proportion of peptides from 6500 Da to di-peptides and free amino acids (Guerard *et al.,* 2001). Fonkwe and Singh (1996) applied papain for protein recovery from mechanically deboned turkey residue (MDTR) at 60°C. The hydrolysis reduced the weight of the original MDTR by 51% on a dry weight basis and recoverd 46% the MDTR proteins. The hydrolysate contained 78% protein, 4.6% ash and only 5.7% fat. The proteins consisted mainly of amino acids and small peptides with molecular masses lower than 6.5 kDa and were very soluble in water over a wide pH range.

The efficiency of the Alcalase and Flavourzyme protease combination for hydrolysis of the unheated and heat-treated soybean meals at pH 7.6 and 40°C for 16 hours was examined by Fischer *et al.* (2001). For all soybean meals, enzymatic treatment extracted most of the original protein (89-94%). In addition, alkaline protease Optimase™ APL-440 was used for the enzymatic hydrolysis of crayfish processing by-products (Bake and Cadwallader, 1995). Atlantic cod trypsin and bovine trypsin were used to aid the extraction of carotenoprotein from shrimp wastes at 4°C (Cano-Lopez *et al.*, 1987). The limiting of enzymatic hydrolysis is the capital cost of enzyme purchase and the fact that a bitter hydrolysis may be formed. However, the animal protein hydrolysates generally do not have a bitter taste (Fonkwe and Singh, 1996). The cost of enzyme also can be reduced by utilization of industrial waste.

3.4 Applications of protein hydrolysates

Hydrolysis of proteins is performed for various purposes, varying from reduction of allergenic properties to changing functional properties like solubility, gelation, emulsifying and foaming properties (Ven *et al.*, 2001).

In recent years, the protein hydrolysates have received a great deal of attention, and are main constituents of geriatric products, high-energy supplements, enteral and parenteral solutions, and hypoallergenic foods. The use of protein hydrolysates as a source of proteins for hospitalized patients has been steadily increasing for the last two decades (Morbahan and Trumbore, 1991).

The fact that protein is absorbed in the form of both peptides and amino acids, and that the transport mechanism of peptides in the intestinal mucous membrane is different from that of free amino acids, suggests that protein hydrolysates may offer physiological advantages of speed and efficiency over amino acids (Matthews, 1975; Silk *et al.*, 1980). Since the 1970s, pancreatic hydrolysate from casein and egg albumin has been used in commercialized diets (Young *et al.*, 1975) for patients presenting with problems of digestion or poor absorption (Silk, 1987), and for those with defects in amino acid metabolism (Netto and Gaieazzi, 1998), as well as in cases of transitory intestinal diseases due to food allergies, the short bowel syndrome, Crohn's disease and pancreatic insufficiency (Milla, 1991). Protein hydrolysates are also used as a food

supplement for people who have special (nonmedical) protein needs such as the elderly, athletes and people on weight-control diets (Frokjear, 1994;)

Fish protein hydrolysates are potential feed ingredients that are used in aquaculture feeds mainly as protein supplements, attractants and palatability enhancers (Oliva-Teles *et al.*, 1999). The use of dried fish protein hydrolysate produced by pre-processing the raw material with hydrolytic enzymes in diets has been shown to improve growth and feed utilization of salmonids (Berge and Storebakken, 1996) and carp larvae (Carvalho *et al.,* 1997). This positive effect may be attributed to increased digestibility of the meal due to the enzymatic treatment; free amino acid released in the process might also act as attractants for some species, increasing feed intake and growth (Berge and Storebakken, 1996), although free amino acids seem to have only a minor effect as attractants for turbot (Mackie and Adron, 1978).

The first investigation into fish protein hydrolysis for human consumption was described in 1970s (Abdul-Hamid *et al.*, 2002). The hydrolysates were reconstituted to milk-like products and they had excellent nutritional properties (Yanez *et al.,* 1976). In recent years, interest in the use of fish protein hydrolysates for human consumption has been increasing. Yu and Tan (1990) found that a fish cracker, containing protein hydrolysate from *Oreochromis mossambicus* (Black Tilapia), was acceptable in terms of appearance, crispiness and color.

In addition, protein hydrolysates have been employed in food systems as additives for beverage and infant formulae, as food texture enhancers (Chiang *et al.*, 1999). Protein hydrolysates such as hydrolyzed vegetable protein and autolyzed yeast extract have been used as flavor enhancers (Nagodawithana, 1992). Protein hydrolysates can be used to produce Maillard reaction flavor (processing flavor, e.g. meat and savory flavor) by heat treatment (Dziezak, 1986).

Although lots of work has been done to utilize the wastes from fishery industry by membrane process. But there are still some unsatisfactory factors. For instance, the production of protein hydrolysates from wastes often involves adding commercial enzymes which increases the capital cost. Another way to produce protein hydrolysates is autolysis. The reaction is difficult to control accurately during the autolysis process. On the other hand, most previous researches are successful at lab-scale. It is necessary to scale-up for industrial applications.

The present work will aim the development of an improved membrane process

for industrial treatment of fishery waste. The proteases in tuna processing waste will be recovered by membrane process and applied back to tuna canning waste to produce valuable products.

Objectives

1. To develop a multi-stages membrane filtration process for separation and purification of protease from yellowfin tuna spleen.
2. To study fouling mechanism during membrane filtration process.
3. To study effect of hydrodynamic factors on performance of membrane filtration process.
4. To develop optimal parameters of process for minimizing fouling and promoting recovery of products.
5. To apply the protease separated and purified by membrane filtration for protein hydrolysis.

CHAPTER 2

SUSPENDED SOLIDS REMOVAL AND FOULING MECHANISM DURING MICROFILTRATION OF TUNA SPLEEN EXTRACT

2.1 Abstract

The dead-end microfiltrations with membrane pore size 0.10, 0.22 and 0.45 μm, were applied to remove suspended solids from yellowfin tuna spleen extract before recovery of proteases by ultrafiltration. All suspended particles were removed by microfiltration process. However 0.1 μm membrane provide the best performance. Almost all trypsin and chymotrypsin were recovered from the permeate of microfiltration while most large particles and half amount of other soluble proteins were rejected by a 0.1 μm membrane at transmembrane pressure 4 psi. The transmissions of enzymes were about 1 and the transmission of soluble protein was about 0.5. The membrane fouling was separated to membrane resistance-limited, pore blocking resistance-limited and cake resistance-limited. The pore blocking resistance-limited was the major fouling mechanism for permeate flux decline while cake resistance-limited dominated the most duration of microfiltration process. The change of membrane pore size and transmembrane pressure resulted in the change of fouling mechanism transition and the change of duration for each fouling mechanism. A critical pressure for the highest resistance to the flow was hypothesized when membrane with large pore size (0.45 μm) was used. Increasing both membrane pore size and transmembrane pressure caused partially loss of enzyme activity. Centrifugation and pre-filtration removed a part of suspended solids in tuna spleen extract before microfiltration, thus changed the particle size distribution in the extract and changed fouling mechanism during microfiltration. It was observed that particle size distribution was more important than other factors for membrane fouling mechanism.

2.2 Introduction

The demand of fishery product has increased significantly in recent years. As one of the largest exporters of canned tuna in the world, Thailand produced about 311,070 tons canned tuna in the year 2004 (National Food Institute, 2005). Since the major part of aquatic animal for fishery processing is only meat, thus a large amount of wastes has been generated from the fishery industry annually. In tuna canning process, there are 23-25% solid waste (e.g. head, skin, viscera) and about 35% liquid waste (e.g. blood, tuna condensate, oil) (Prasertsan *et al.*, 1988). The utilization of fishery waste has caught more attention. The pressure driven membrane processes, e.g. microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) have successfully been implemented in many fishery industrial cases (Gildberg and Shi, 1994; Afonso and Borquez, 2002; Vandanjon *et al.*, 2002; Dewitt and Morrissey, 2002a). In previous studies, the valuable compound, especially protein or enzyme can be recovered from fishery waste by ultrafiltration. However the wastes from fishery processing contain many impurities, such as fat, meat debris and blood which may limit the function of ultrafiltration process. According to its separation range in size from approximately 0.02 μm to 10 μm, microfiltration may serve to remove most impurities, especially suspended solids before recovery of valuable compound from fishery waste by ultrafiltration process.

2.2.1 General theories for membrane process

The permeate flux of a membrane process is described by Darcy's law as:

$$J = \frac{\Delta P}{\mu R} \tag{10}$$

where J is the volumetric flux of permeate across the membrane (m/s), ΔP is the transmembrane pressure (Pa), μ is the feed fluid viscosity(Pa.s) and R is the hydraulic resistance (m^{-1}).

The transmission of the solute through a membrane is expressed as the apparent sieving coefficient (S_a) (Ghosh and Cui, 2000):

$$S_a = \frac{C_p}{C_b} \tag{11}$$

where C_p is the concentration of the solute in the permeate and C_b is the concentration of the solute in the bulk feed.

The permeate flux is normally used to indicate the performance of the microfiltration process. Fouling is the major problem causing the functional decay of microfiltration process. The feed property and operation condition (e.g. pressure, shear rate) can affect the fouling formation. The resistance to flow is the membrane itself when the membrane is clean. But the fouling forms immediately and permeate flux decreases gradually as soon as the flow starts to contact membrane surface and permeate passes through the membrane (Tansel *et al.*, 2000). External fouling occurs on the membrane surface due to the accumulation of large particles that do not enter the membrane pores, whereas internal fouling occurs within the internal pore structure of the membrane due to deposition and adsorption of small particles and other macromolecules which are able to pass into the pores (Tracey and Davis, 1994). Therefore, the analysis of fouling mechanism can help to choose optimal conditions for operation of membrane separation process.

2.2.2 Models for membrane fouling

Some theoretical models have been used to describe the fouling mechanism. For example, Hermia (1982) derived four different filtration laws based on Darcy's law. Complete blocking describes that every single particle blocks a single pore without superimposition. Intermediate blocking describes that every single particle blocks a single pore, or deposits on the membrane surface (superimposition is possible). Standard blocking describes that particles deposit on the inside of the membrane pores. Cake filtration describes that particles form a cake layer on the membrane surface. The characteristic form of the filtration laws is shown in equation (12):

$$\frac{d^2t}{dV^2} = \alpha\left(\frac{dt}{dV}\right)^{\beta} \tag{12}$$

where t is operation time (s), V is cumulative permeate volume (m^3), α is the multiplicative constant in the characteristic laws ($s^{1-\beta}/m^{6-3\beta}$) and β is the exponent in the characteristic laws (dimensionless).

β equals to 2, 1.5, 1, 0 for complete blocking, standard blocking, intermediate blocking and cake filtration, respectively.

For the microfiltration of suspensions, Lim and Bai (2003) modified equation (10) as:

$$J = \frac{\Delta P}{\mu(R_m + R_p + R_c)} \tag{13}$$

where R_m is the resistance of clean membrane, R_p is the resistance due to pore blocking and R_c is the resistance arising from cake formation.

From equation (13), the total hydraulic resistance was separated to three types. Then, Lim and Bai (2003) rewrote the equations from Wiesner *et al.* (1992) to develop new models for each resistance as following:

Membrane resistane-limited: $$\frac{1}{J} = K_m t + \frac{1}{J_0} \tag{14}$$

Pore blocking resistance-limited: $$\ln J = -K_p t + \ln J_0 \tag{15}$$

Cake resistance-limited: $$\frac{1}{J^2} = K_c t + \frac{1}{J_0^2} \tag{16}$$

where J_0 is the initial permeate flux. K_m, K_p and K_c are system parameters relating to membrane resistance, pore blocking resistance and cake formation resistance, respectively.

The fouling mechanism can be analyzed by fitting experimental data to equation (14), (15) and (16). The linear stages in the figures based on equation (14), (15) and (16) indicate the type and duration of the fouling.

In previous report, it was proofed that ultrafiltration could be used to separate proteases from yellowfin tuna spleen extract (Li *et al.*, 2006). However, the wastes from fishery processing contain a number of impurities. Therefore, a suitable method to remove these impurities was important. Microfiltration may serve for this objective. On the hand, some researchers have analyzed fouling mechanism in membrane processes using model feed fluid, such as BSA solution (Pradanos *et al.*, 1996) However, the investigation of fouling mechanism using industrial waste instead of model solution is more valuable for industrial applications of the membrane processes. The aim of present study was to investigate the feasibility of using microfiltration to remove suspended solids from yellowfin tuna spleen extract before the recovery of proteases by ultrafiltration and the fouling mechanism during microfiltration.

2.3. Materials and methods

2.3.1 Preparation of tuna spleen extract

The spleen of yellowfin tuna (*Thunnus albacares*) was kindly provided by Chotiwat Canning Co., Ltd., Hat Yai, Thailand. The spleen was chopped into about 1 cm^3 pieces and kept at –20°C. The spleen was thawed at 4°C overnight before extraction. The tuna spleen extract was prepared by mixing 100 g of spleen and 300 ml of Tris-HCl buffer (pH 8.0, 0.02 M containing 5 mM $CaCl_2$ and 0.02% NaN_3). Then the mixture was homogenized for 3 minutes by a kitchen blender (National MX-T2GN, Japan) at high speed scale.

In order to study the effect of pre-treatment methods before microfiltration on membrane fouling mechanism, the tuna spleen extract was pre-treated by centrifugation or pre-filtration after homogenization. The centrifugation was performed at 13,000 *g* and 4°C using a refrigerated centrifuge (Model Himac SCR-20B, Japan). The pre-filtration process was performed by filtering tuna spleen extract through nylon net, cotton pad and cloth in series.

2.3.2 Microfiltration system

Dead-end microfiltration was carried out with a 44.5-mm-diameter stirred cell (Model 8050, Millipore, USA) connected to a stainless steel reservoir (maximum capacity 3 liters) which was pressured from 4 psi to 20 psi by compressed nitrogen gas. The membranes used were 0.10 μm and 0.22 μm polyvinylidene fluoride (PVDF) membrane (Durapore® membrane filter, Millipore, USA), and 0.45 μm mixed cellulose esters membrane (MF™-membrane filter, Millipore, USA). All membranes are hydrophilic. The reservoir was initially filled with deionized water and the water flux was measured as a function of time at a constant transmembrane pressure (TMP) until a steady state flux was obtained. Then, the reservoir and stirred cell were emptied and refilled with the tuna spleen extract. The system was re-pressurized quickly. The stirring speed was set at 100 rpm. The permeate flux was measured by timed collection using a digital balance (GF-3000 Precision balance, A&D Co. Ltd, Japan) connected to a PC computer. All filtration processes were performed at room temperature (28 ± 2°C).

2.3.3 Analytical methods

The compositions and total solid content in tuna spleen extract was measured by the method of AOAC (1999). Soluble protein concentrations in both permeate and feed bulk were measured by the Lowry method (Lowry *et al.*, 1951). The particle size distribution in tuna spleen extract was analyzed by laser particle size analyzer (Beckman Coulter LS230)

The activity of trypsin was determined by the method of Cano-lopez *et al.* (1987) using 10 mM N-toluenesulfonyl-L-arginine methyl ester (TAME) as substrate. The activity of chymotrypsin was determined by the method of Ramakrishna *et al.*(1987) using 1.07 mM benzoyl-L-tyrosine ethyl ester (BTEE) in 50% methanol (v/v) as substrate.

2.4 Results and discussion

The compositions of yellowfin tuna spleen extract included total protein (0.06 g/ml), salt (0.001 g/ml), crude fat (0.01 g/ml), ash (0.005 g/ml). The typical permeate flux of MF process was observed corresponding to all conditions of microfiltration in this study. At the beginning of the filtration process the flux decreased sharply, and later slowly, then the flux was

almost constant for a long period. A clear permeate with slightly yellow colour was obtained after microfiltration. Nearly all visible solids were rejected to the retentate.

2.4.1 Effect of membrane pore size and transmembrane pressure on flux behavior and filtration resistance

The tuna spleen extract without pre-treatment was used as feed in this step. It was observed that the steady state flux of 0.1 μm membrane was always higher than those of 0.22 μm membrane and 0.45 μm membrane (Figure 3a, 4a, 5a) at all TMPs. The flux decline in microporous membrane filtration could be related to the ratio of feed molecular or particle size to membrane mean pore size. The larger ratio resulted in a higher steady state flux (Pradanos *et al.*, 1996). The membrane with smaller pore size provided a higher ratio of feed molecular size to membrane mean pore size when the same feed was used. So the highest steady state flux at same filtration condition (i.e. TMP) was observed from 0.1μm membrane.

On the other hand, the permeate flux related to the total resistance of filtration for these membranes (Fiure 6). At each TMP, the 0.1μm membrane showed the lowest total resistance which resulting in the highest steady state permeate flux. It might be due to continued internal fouling of the larger pore size membranes and a more compact cake layer on the larger pore size membranes. Tracey and Davis (1994) observed that the increase in resistance of the larger pore size membrane was due to internal fouling followed by the development of a cake layer on the top of the membrane. This combined (external and internal) resistance to flow led to higher total resistance for the larger pore size membrane than for the smaller pore size membrane.

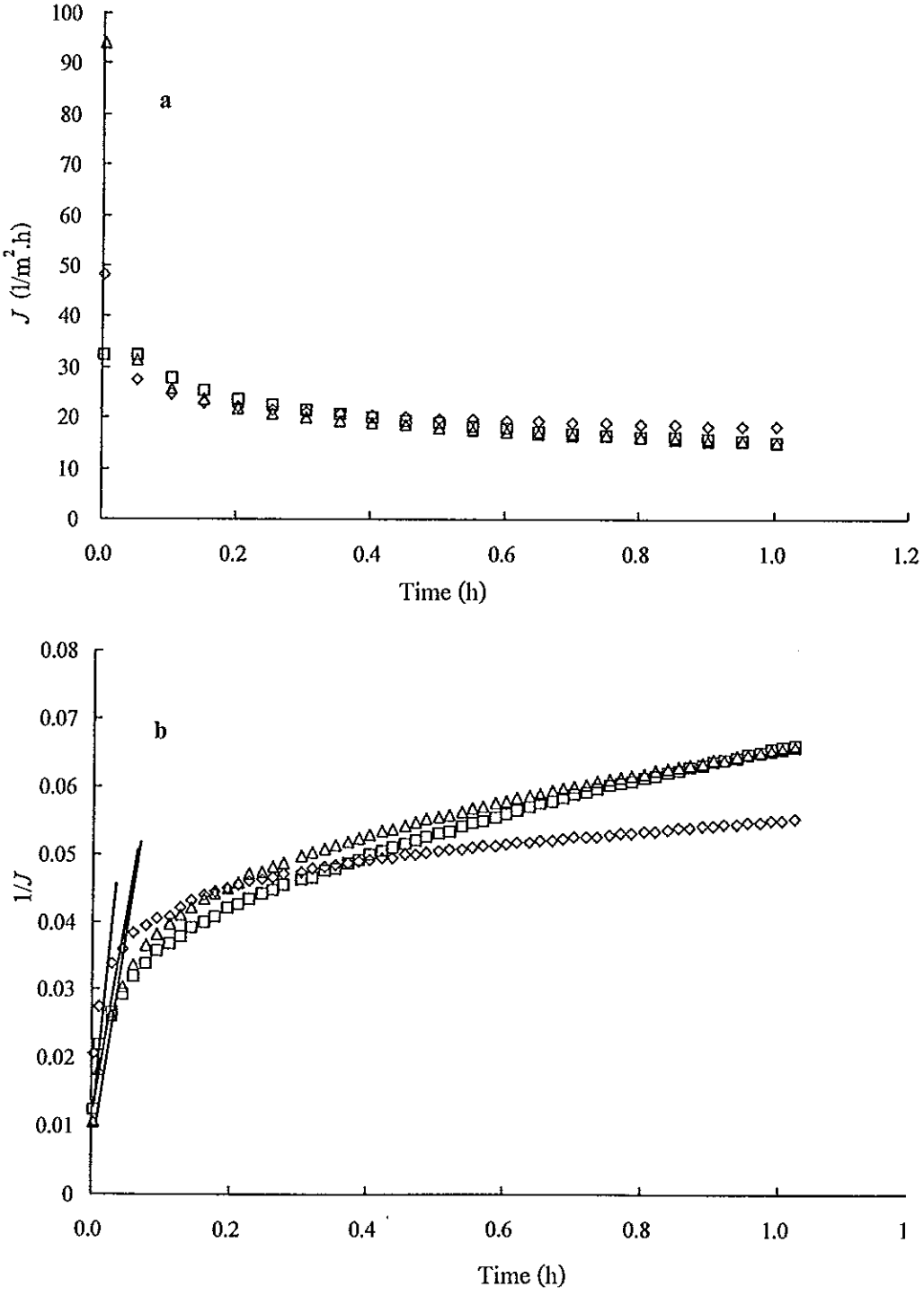

Figure 3. Permeate flux (*J*) profile and determination of fouling by fitting models in microfiltration of tuna spleen extract without pre-treatment at 4 psi (a, permeate flux profile, b, membrane resistance-limited model; c, pore blocking resistance-limited model; d, cake resistance-limited model; ◇ 0.10 μm membrane; □ 0.22 μm membrane; △ 0.45 μm membrane).

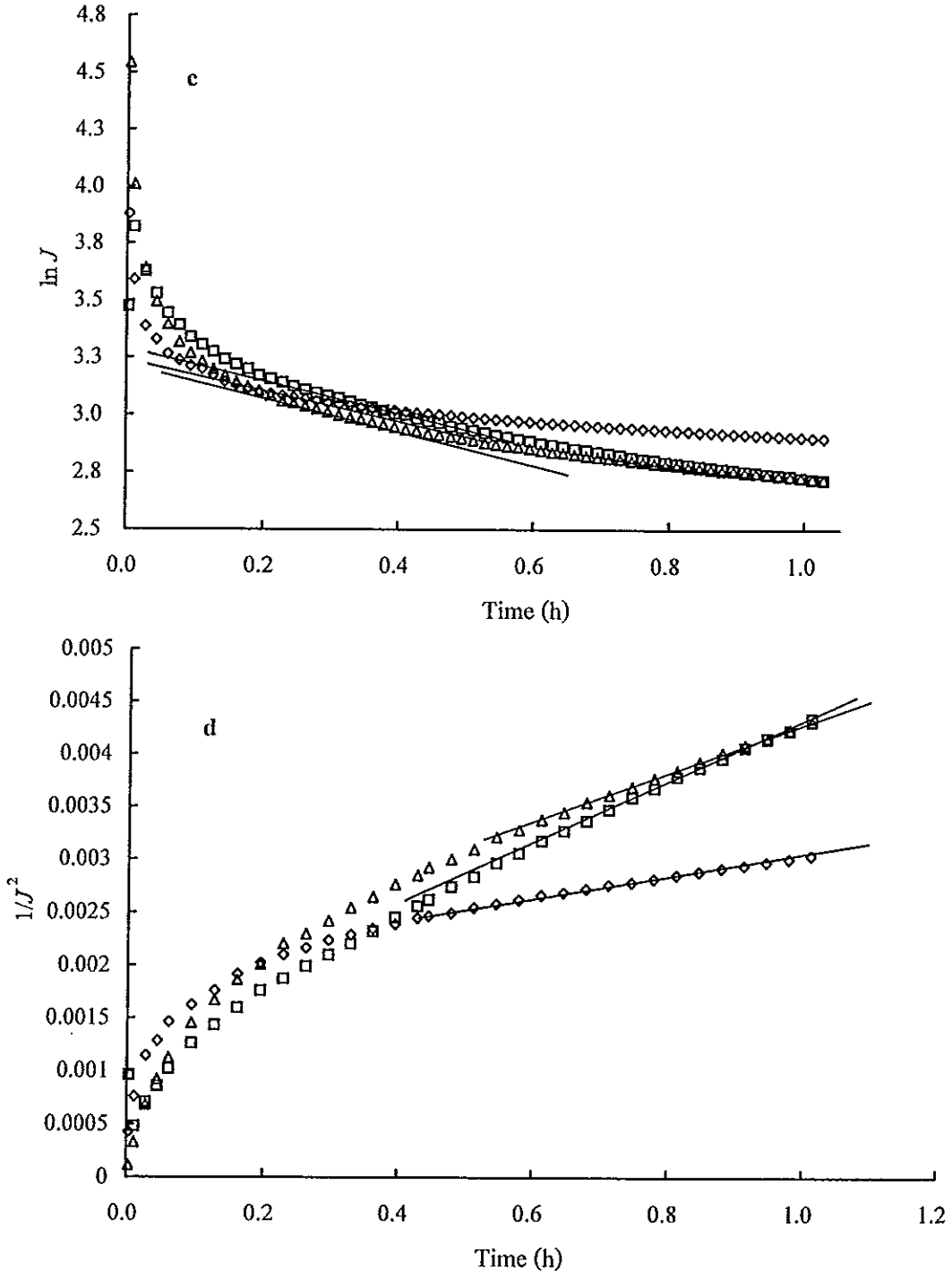

Figure 3. (Continued)

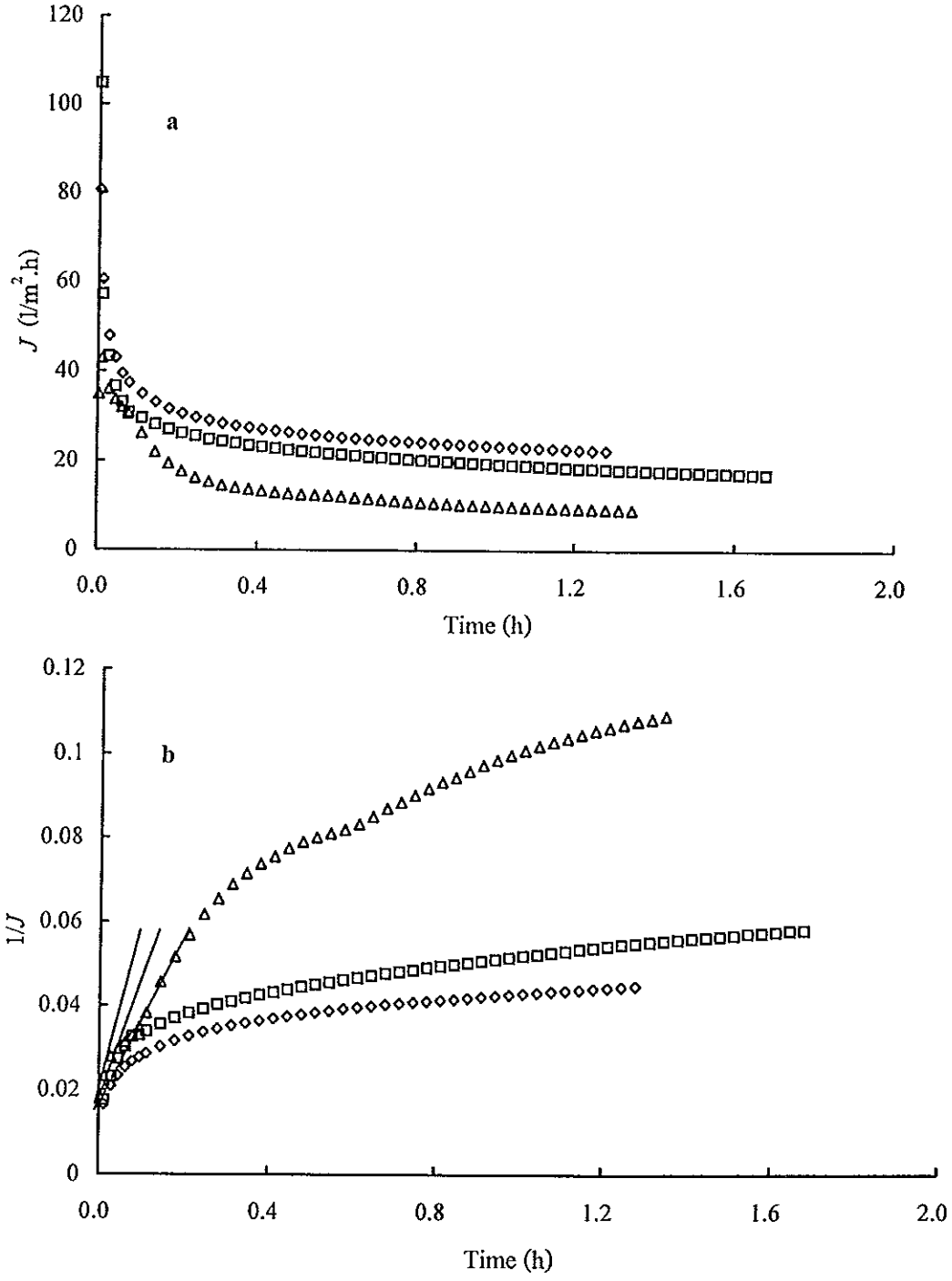

Figure 4. Permeate flux (J) profile and determination of fouling by fitting models in microfiltration of tuna spleen extract without pre-treatment at 10 psi (a, permeate flux profile, b, membrane resistance-limited model; c, pore blocking resistance-limited model; d, cake resistance-limited model; ◇ 0.10 μm membrane; □ 0.22 μm membrane; △ 0.45 μm membrane).

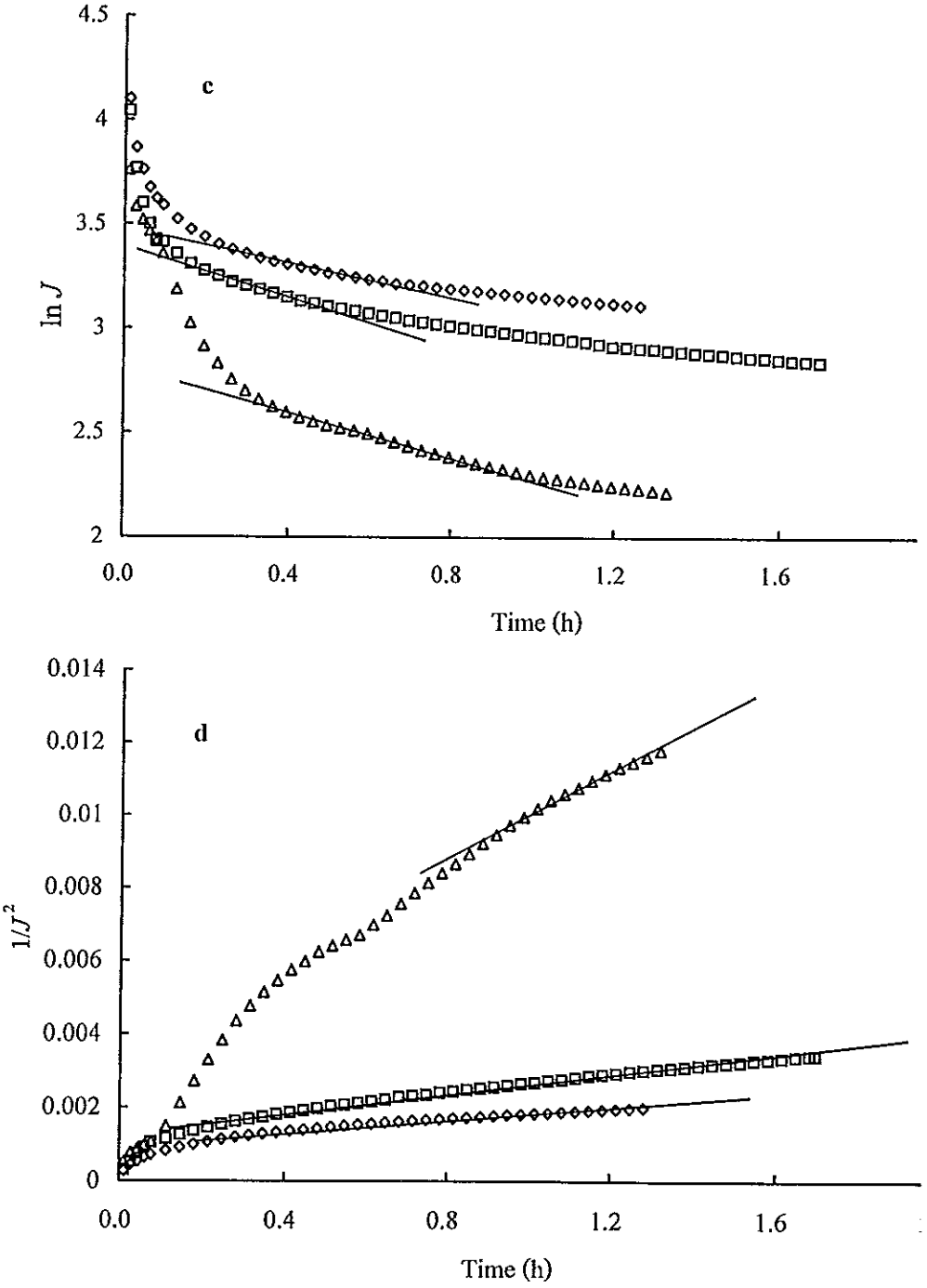

Figure 4. (continued)

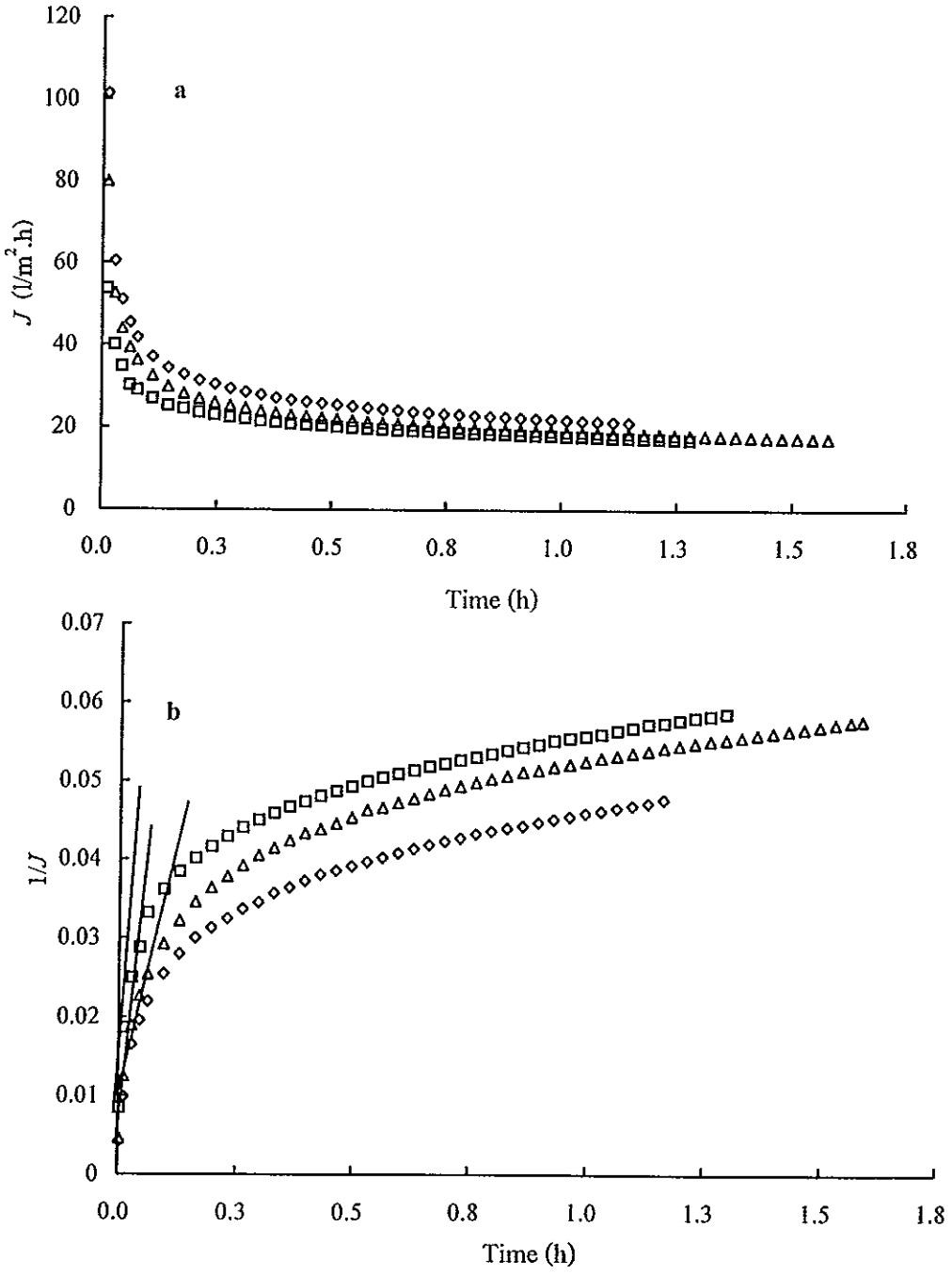

Figure 5. Permeate flux (*J*) profile and determination of fouling by fitting models in microfiltration of tuna spleen extract without pre-treatment at 20 psi (a, permeate flux profile, b, membrane resistance-limited model; c, pore blocking resistance-limited model; d, cake resistance-limited model; ◇ 0.10 μm membrane; □ 0.22 μm membrane; △ 0.45 μm membrane)

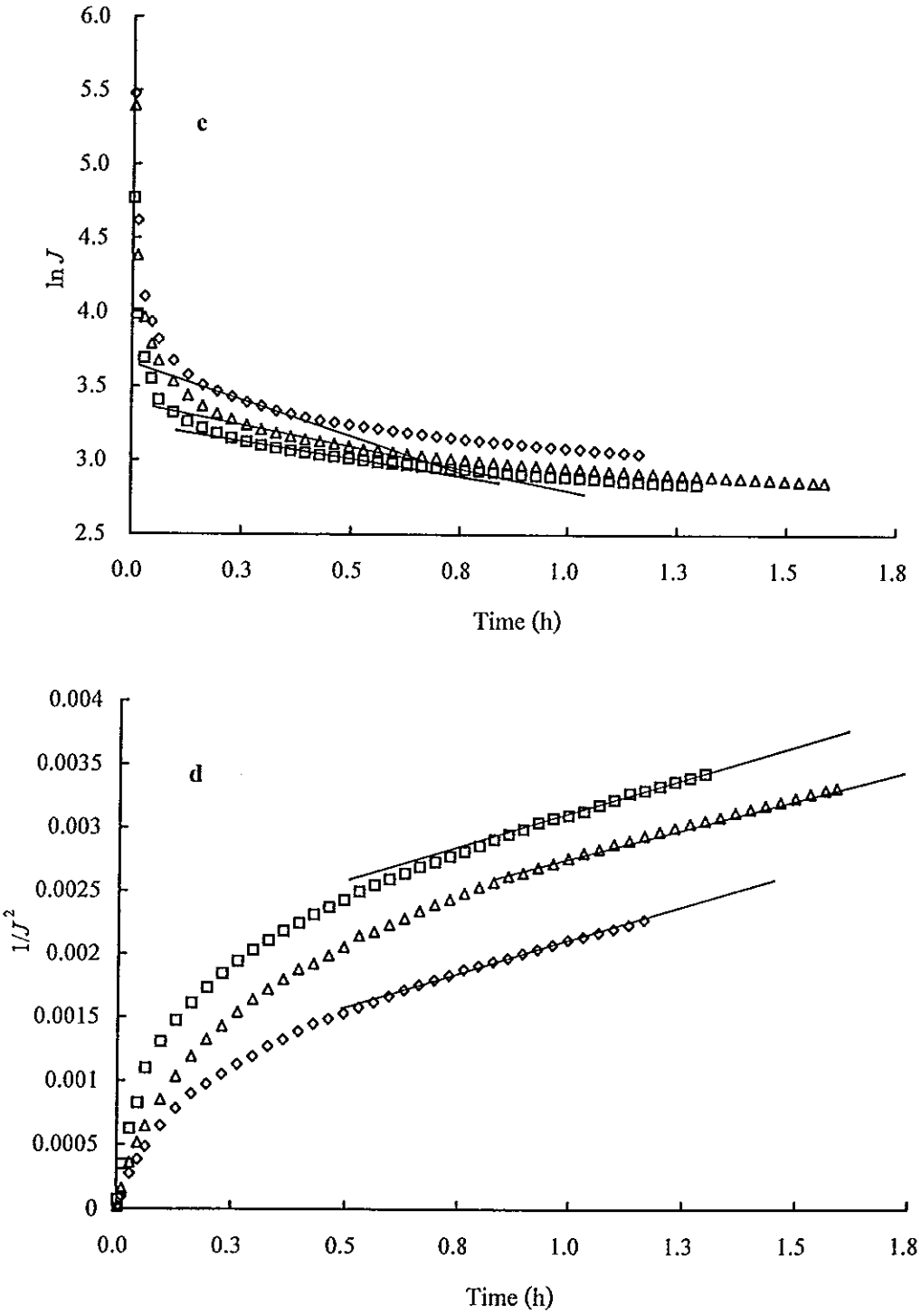

Figure 5. (continued)

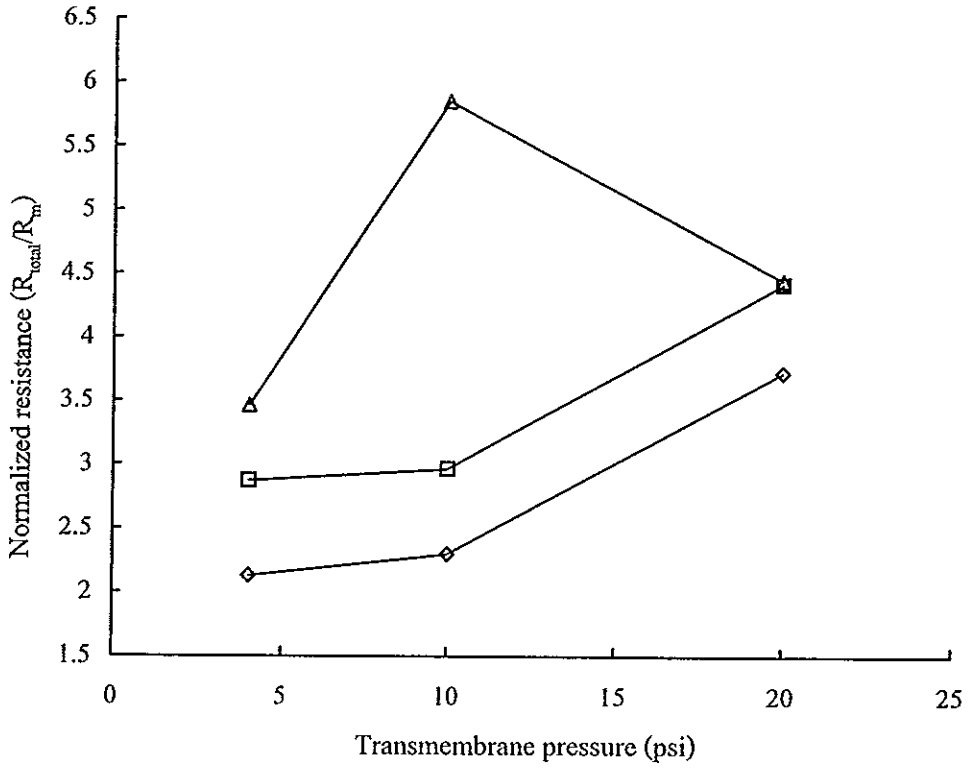

Figure 6. Total filtration resistance (R_{total}) of different membranes at different transmembrane pressures using tuna spleen extract without pre-treatment as feed (◇ 0.10 μm membrane; □ 0.22 μm membrane; △ 0.45 μm membrane)

For 0.1 μm and 0.22 μm membranes, the total resistance was increased with increasing TMP (Figure 6). It was due to that the loss of protein was promoted by shear in the pores with increasing applied pressure (Bowen and Gan, 1991). At the same time, the higher transmembrane pressure resulted in a higher initial permeate flux, thus, more solutes (e.g. protein) could penetrate into the internal space of membrane, so more solutes might be absorbed onto the membrane pore wall by the solute-membrane interaction. In addition, thicker and compacter cake layer formed at high pressure. Thus the fouling in the pores and on the top of membrane became more severe at higher pressure in this case. However, the highest total resistance of 0.45 μm membrane was found at 10 psi (Figure 6). This phenomenon was probably due to the transition of

fouling mechanism according to the applied pressure. It was found that fouling was essentially internal (pore blocking) for low applied pressures. A gradual change toward the appearance of a cake on the membrane occurred when the applied pressure was increased (Velasco *et al.*, 2003). According to the structure geometry, 0.45 μm membrane had larger internal pore space than 0.1 μm and 0.22 μm membrane. During filtration, the internal space of the smaller pore size membrane could be 'saturated' in a short period by internal fouling, thus the change of applied pressure affected the thickness and compactness of external fouling layer on the membrane surface rather than the internal fouling inside the membrane. So the resistance increased gradually with increasing applied pressure. In the case of the membrane with larger pore size, when a high transmembrane pressure was applied, the thick and compact fouling layer could form and acted as a secondary membrane in a short period in which the internal space of membrane had not been 'saturated' by internal fouling. If the low pressured was applied, the internal fouling could achieve the saturation, but less resistance arose from the external fouling layer comparing to that at the high pressure. Therefore, there was possibly a critical pressure at which both internal fouling and external fouling could achieve the maximum rate to yield the highest filtration resistance.

2.4.2. Effect of transmembrane pressure and membrane pore size on fouling mechanism

The tuna spleen extract without pretreatment was used as feed in this step. The fouling modeling equation (14), (15) and (16) were verified with data collected from a series of test runs. It was observed that all three models could be fitted with the experimental data. In the initial stage of filtration, the experimental data were fitted with membrane resistance-limited model since the resistances from pore blocking and cake layer could be neglected in the beginning stage of microfiltration. However, this period was very short (Figure 3b, 4b, 5b). As mentioned above, the fouling occurred as soon as the operation started and the feed solution passed through the membrane (Tansel *et al.*, 2000). After this short period dominated by membrane resistance-limited, the pore blocking resistance-limited model seemed to fit the second stages of all microfiltration processes (Figure 3c, 4c, 5c). The third stage was cake resistance-limited model (Figure 3d, 4d, 5d).

At different transmembrane pressures and membrane pore sizes, there were different behaviors of these linear phases fitting to fouling modeling equations. At all applied pressures, 0.1 μm membrane had shortest pore blocking duration which was due to its smaller pore size comparing with other two membranes (Figure 3c, 4c and 5c). For the smaller pore size membrane, the permeate concentration dropped off immediately, the fouling appeared to be due to immediate blocking of the pores and a growing protein layer on the membrane surface. The formation of cake layer occurred sooner on the surface of the membrane with smaller pores than the membrane with larger pore size (Tracey and Davis, 1994). Therefore, the pore blocking duration of 0.1 μm membrane was shorter than 0.22 μm and 0.45 μm membranes. In the case of 0.45 μm membrane, the duration of pore blocking was about 0.4 hour at 4 psi, 0.5 hour at 10 psi, and 0.2 hour at 20 psi. It confirmed the assumption mentioned above, i.e. the pore blocking could achieve the highest rate at 10 psi rather than 20 psi in the case of 0.45 μm membrane. After pore blocking stage, the experimental data could be fitted to the cake resistance-limited model for a long period (Figure 3d, 4d and 5d). At 4 psi and 10 psi, the cake layer resistance-limited rate (the slope of the linear phase) of 0.45 μm membrane was higher than those of 0.1 μm and 0.22 μm membrane (Figure 3d and 4d). The more compact or compressed cake layer on the larger pore size membranes was presumably due to the higher initial flux and greater pressure drop across the cake layer on larger pore size membranes (Tracey and Davis, 1994). However, the slope of linear phases in cake resistance-limited model for all three membranes became approximate at high pressure (20 psi) (Figure 5d). The reason was probably that the high pressure minimized the difference of the higher initial flux and greater pressure drop across the cake layer among these membranes. In this study, the stage of pore blocking appeared to be the major part of microfiltration process for the permeate decline. A similar phenomenon was determined during the microfiltration of the activated sludge wastewater by an asymmetric PVDF hollow fiber MF membrane (Lim and Bai, 2003).

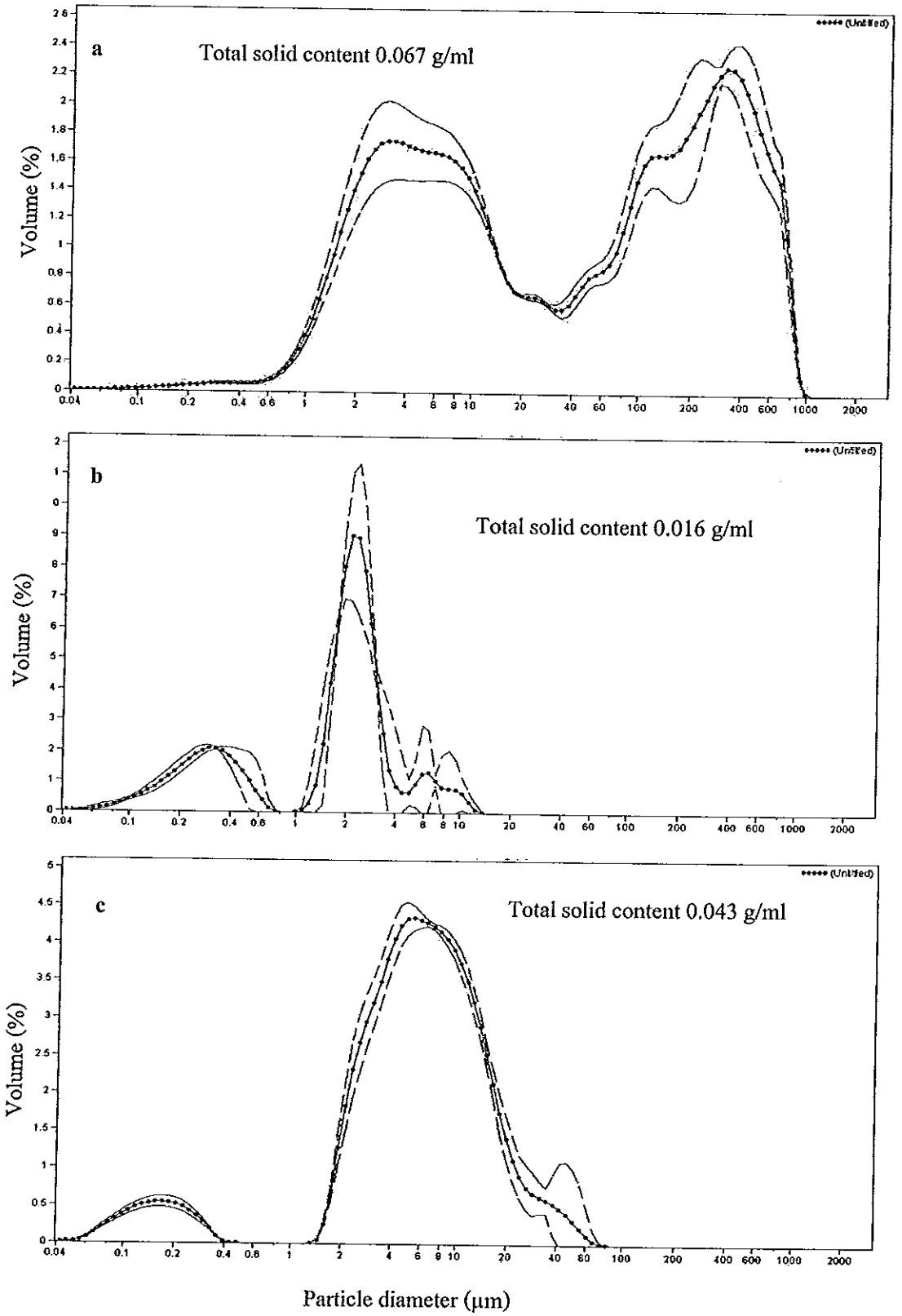

Figure 7. Particle size distribution in feed (a, extract without pre-treatment; b, pre-filtered extract; c, centrifugated extract).

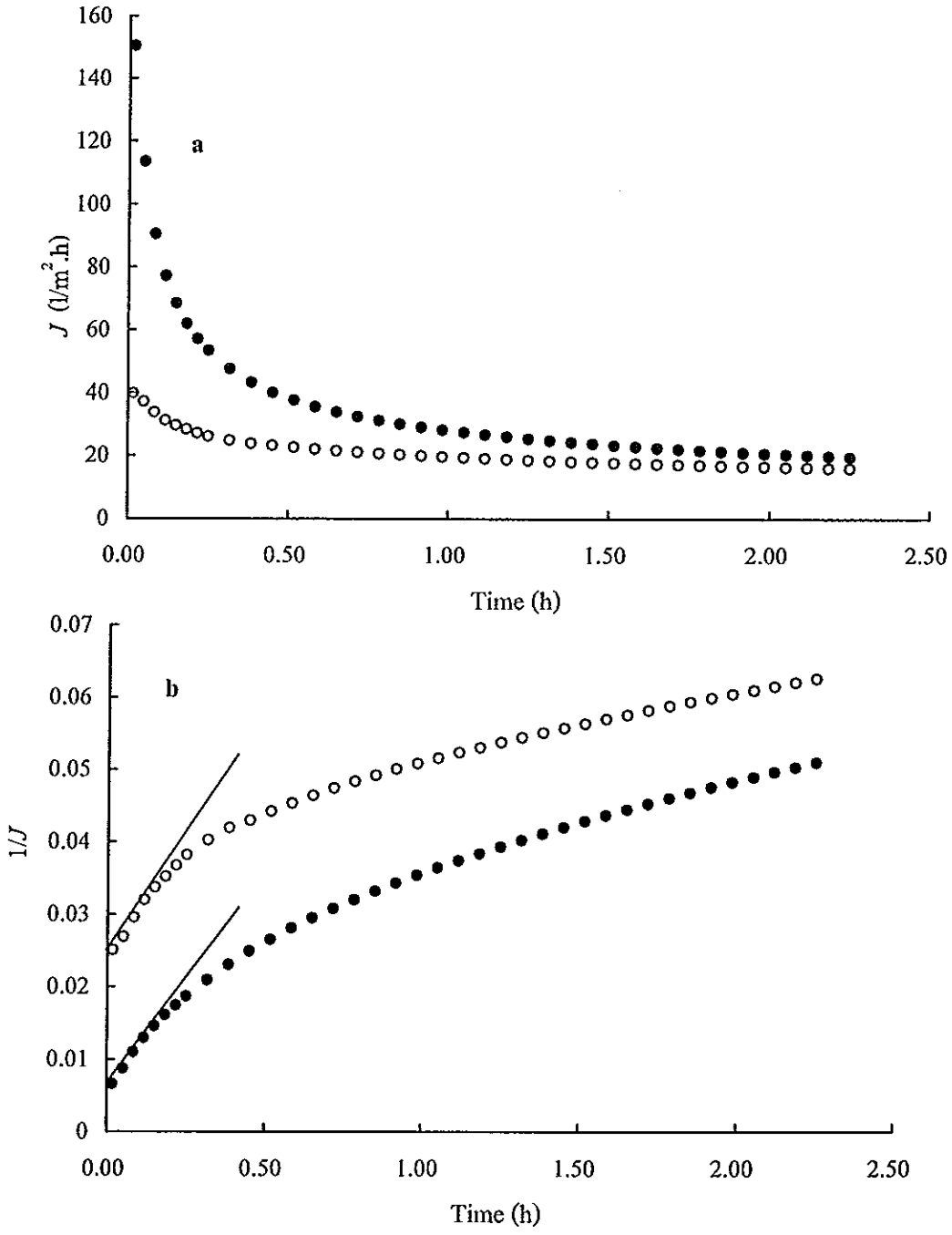

Figure 8. Permeate flux (*J*) profile and determination of fouling by fitting models in microfiltration of pre-treated tuna spleen extract at 4 psi using 0.10 μm membrane (a, permeate flux profile, b, membrane resistance-limited model; c, pore blocking resistance-limited model; d, cake resistance-limited model; ● centrifugated extract; ○ pre-filtered extract)

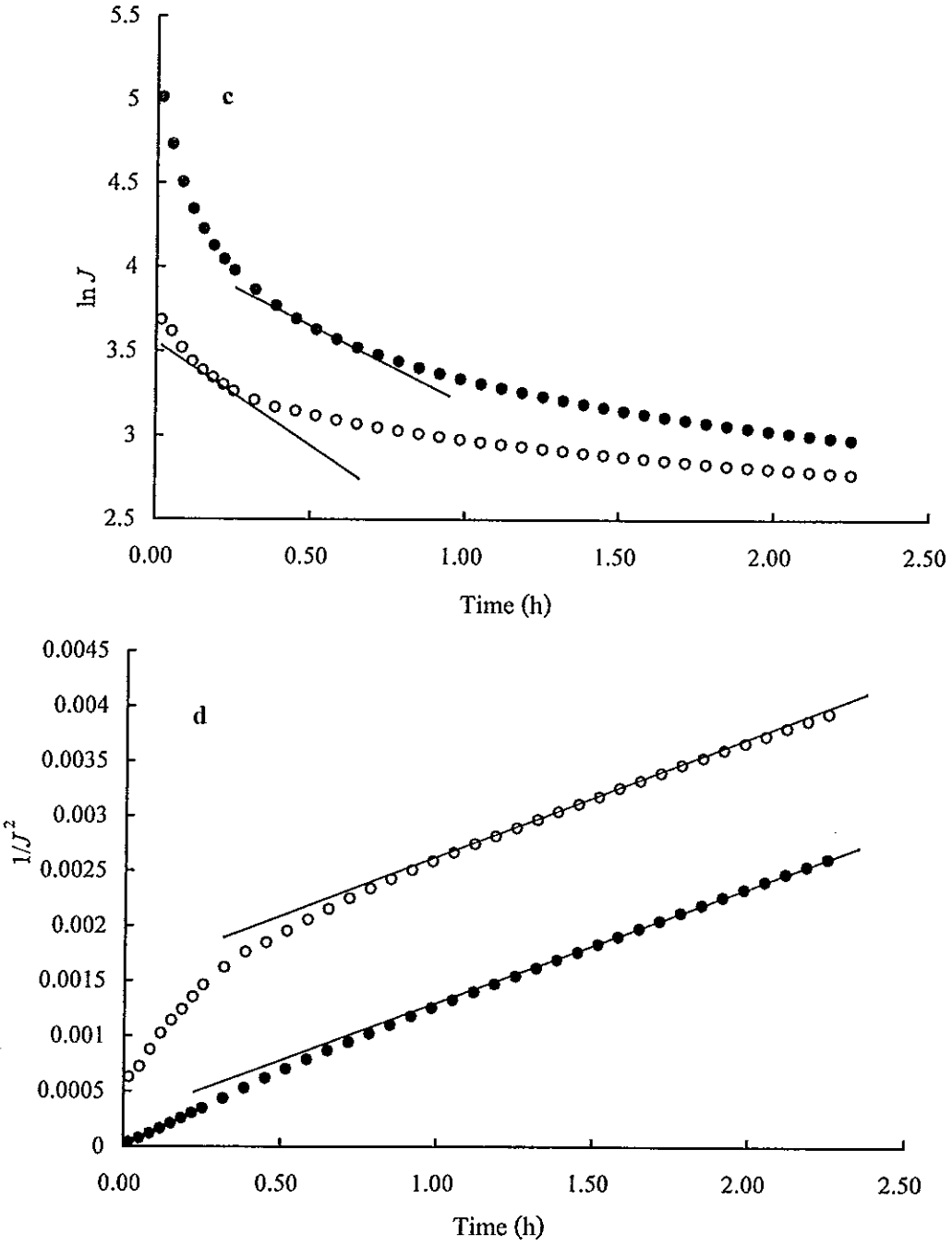

Figure 8. (continued)

2.4.3 Effect of pre-treatment on flux behavior

In some membrane filtration processes, especially hollow fiber membrane, the large particles would block fiber channel. Therefore, the pre-treatment to remove large particles before microfiltration process became important. In this study, the effect of pre-treatment was studied by using 0.1 μm membrane at 4 psi. The centrifugated tuna spleen extract and pre-filtered tuna spleen extract were applied as feed. Figure 7 showed the particle size distribution and total solid content in the tuna spleen extracts before and after pre-treatment. It was observed that both centrifugation and pre-filtration removed a part of large particles from tuna spleen extract. The range of particle size distribution and total solid content in centrifugated tuna spleen extract were larger than those in pre-filtered tuna spleen extract (Figure 7b, 7c). The reason was probably that the floating fat cake wrapped particles at low temperature. These fat cakes could be removed by filration rather than centrifugation. The lower steady state flux was observed when the pre-filtered tuna spleen extract was used as feed (Figure 8a). The reason might be same as that mentioned above. The higher ratio of feed molecular size to membrane pore size resulted in the higher steady state flux of permeate. The particle sizes in tuna spleen extract without pre-treatment and centrifugated tuna spleen extract were larger than the particle size in pre-filtered tuna spleen extract. The lowest ratio of particle size in the feed to membrane pore size was achieved when the pre-filtered tuna spleen extract was used as feed during microfiltration by 0.1 μm membrane.

2.4.4 Effect of pre-treatment on fouling mechanism

Centrifugated and pre-filtered tuna spleen extracts were used as feed. Microfiltration was also applied using 0.1 μm membrane at 4 psi in this step. The pattern of fouling mechanism was similar to that of the tuna spleen extract without pre-treatment, i.e. the fouling process could be separated to three types according to the determination of linear stage based on the equation (14), (15) and (16) (Figure 8b, 8c, and 8d). Comparing with the microfiltration of tuna spleen extract without pre-treatment, the first linear stage, i.e. membrane resistance-limited was more significant when pre-treated tuna spleen extract was used (Figure 8b). It was due to the change of feed property after pre-treatment. The feed solution became much

clearer after centrifugation or pre-filtration (Figure 7). It could be assumed that the property of centrifugated or pre-filtered tuna spleen extract was closer to the property of water than tuna spleen extract without pre-treatment. In theory, only membrane resistance-limited could occur during microfiltration of pure water. Therefore, more significant membrane resistance-limited stage was observed when centrifugated or pre-filtered tuna spleen extract was used. On the other hand, there was not significant difference in membrane resistance-limited stage between centrifugated and pre-filtered tuna spleen extract (Figure 8b). The reason was probably that the particles of sizes close to or smaller than the membrane pore size caused internal and external pore blocking (Bai and Leow, 2001). The amounts of particles with the size close to or smaller than membrane pore size (i.e. 0.1 μm) were approximate (about 0.4%) in both centrifugated and pre-filtered tuna spleen extract (Figure 7b, 7c). Therefore, these two microfiltration processes underwent similar membrane resistance-limited extension until pore blocking became the dominant fouling.

After the membrane resistance-limited stage, the pore blocking-resistance limited stage was observed (Figure 8c). The centrifugated tuna spleen extract caused a longer duration of pore blocking resistance-limited than the pre-filtered tuna spleen extract. It was due to that more large particles in this feed obstructed the small particles in contacting membrane surface and entering the pore, thus prolonged the duration of pore blocking. Due to these larger particles as well, the cake resistance-limited became dominant fouling earlier when centrifugated tuna spleen extract was used as feed. The dominant fouling changed to cake resistance-limited after microfiltration of centrifugated tuna spleen extract for about 0.5 hour and microfiltration of pre-filtered tuna spleen extract for about 1.25 hours (Figure 8d). It was found that this change occurred after microfiltration of tuna spleen extract without pre-treatment for about 0.5 hour as well at same conditions of filtration (Figure 3d) although there were higher amount and larger size of particles in this extract. The similar transition time from pore blocking resistance-limited to cake resistance-limited for centrifugated tuna spleen extract and the tuna spleen extract without pre-treatment implied that the particles gave little effect on the formation of cake layer once the sizes of particles were much larger than membrane pore size.

In addition, most figures of cake resistance-limited showed two distinct stages, i.e. a non-linear curve at initial stage, a linear curve (cake resistance-limited) at final stage (Figure

3d, 4d and 5d). However, this curve was almost linear from initial point when centrifugated tuna spleen extract was used as feed (Figure 8d). The differences among centrifugated tuna spleen extract, pre-filtered tuna spleen extract and tuna spleen extract without pre-treatment were particle size distribution, viscosity, solute composition, total solid content and so on. All of these factors might affect the fouling mechanism. But one or several of them might be the major reason causing the difference of cake resistance-limited curve between centrifugated tuna spleen extract and others. A clue was found from the study of Lim and Bai (2003). These researchers studied the fouling in microfiltration of activated sludge wastewater by an asymmetric PVDF hollow fiber MF membrane (pore size 0.1 μm). The curves of fouling mechanism in their study were extremely similar to those curves of centrifugated tuna spleen extract in our study. The property between centrifugated tuna spleen extract and activated sludge wastewater should be different. The coincidence was that both of them showed a similar particle size distribution. It could be hypothesized that particle size distribution played more important role than other properties of feed for membrane fouling.

2.4.5 Effect of microfiltration process on transmissions of soluble protein and enzymes

Table 2 showed the average transmissions of soluble protein and target enzymes under different filtration conditions. The transmissions of soluble protein were about 0.50 at all conditions. Even pore size of microfiltration membrane was much larger than protein molecular size, about half amount of protein was still rejected by these membranes. The rejection of protein was thought to be due to protein aggregation occurring in solution or at the mouths of the pores under dynamic or convective conditions (Tracey and Davis, 1994). Jim *et al.* (1992) observed a heavy cake of aggregates and sheets on the membrane surface after 40 minutes of the microfiltration of BSA, even a substantial amount of protein could pass the membrane. Stirring also caused aggregation over time. The aggregates became large enough to be rejected by the membrane and served as sites for further deposition of other foulants (Meireles *et al.*, 1991). In addition, increasing transmembrane pressure also resulted in forming larger protein aggregates which deposited on the membrane surface since more denaturation and aggregation could also be induced by high compression from high pressure (Velasco *et al.*, 2003). The change of

aggregation and denaturation of protein might be the reason that the protein transmission did not increased significantly when the transmembrane pressure and membrane pore size were increased.

The aggregated protein and other large particles in the spleen extract deposited on the membrane surface might form a loose layer. At the same time, the expected enzymes (trypsin and chymotrypsin) kept stable structure in the optimal buffer used in the study. Therefore, the average transmissions of small molecules, such as trypsin (about 24 kDa) and chymotrypsin (about 27 kDa) still kept at the high rate. Bowen and Gan (1992) also observed that no significant loss in activity of permeating enzyme occurred under solution conditions where the enzyme was stable. At 4 psi, the average transmissions of all expected enzymes were about 1 by using 0.1 μm membrane. It indicated that 100% expected enzyme could be recovered from the permeate after microfiltration at 4 psi using 0.1 μm membrane. However, both increasing membrane pore size and increasing transmembrane pressure caused the loss of enzyme activity. For example, the average transmission of chymotrypsin was 0.56 at 4 psi using 0.45 μm membrane. And the average transmission of trypsin decreased to 0.49 at 20 psi using 0.1 μm membrane. It indicated that both membrane geometry (pore size) and transmembrane pressure affected the activity of enzyme recovered in the permeate. The mechanism for the loss of enzyme activity was probably a membrane-enzyme interaction resulting from a shear induced deformation of the enzyme structure (Bowen and Gan, 1992). The high pressure induced a faster flow passing through the membrane. Therefore, a higher shear force could be generated at high pressure and distorted enzyme native structure on passage through the micropores. In addition, the repeated adsorption-desorption of enzyme molecules onto the membrane pore wall also leaded to the loss of enzyme activity (Bowen and Gan, 1992). For a larger pore size membrane, more enzyme molecules were hold in the large pore size membrane and underwent the adsorption-desorption process. This repeated contact promoted structural damage of enzyme molecules. Therefore, more enzyme activities were lost using the larger pore size membrane as well as the higher transmembrane pressure.

Table 2. Effect of microfiltration process on transmissions of soluble protein and enzymes using different extracts as feed.

TMP (psi)	Membrane pore size (μm)	Protein transmission			Trypsin transmission			Chymotrypsin transmission		
		RSE*	FSE*	CSE*	RSE	FSE	CSE	RSE	FSE	CSE
4	0.10	0.59	0.45	0.53	1.03	0.98	1.01	1.01	0.95	0.99
	0.22	0.56	0.47	0.54	1.06	1.02	1.04	0.59	0.44	0.52
	0.45	0.49	0.42	0.51	1.02	0.97	0.99	0.56	0.45	0.49
10	0.10	0.54	0.49	0.54	0.88	0.73	0.78	0.22	0.19	0.23
	0.22	0.55	0.51	0.50	0.60	0.52	0.59	0.18	0.13	0.15
	0.45	0.46	0.43	0.47	0.44	0.37	0.47	0.19	0.11	0.15
20	0.10	0.54	0.50	0.56	0.56	0.44	0.46	0.19	0.12	0.16
	0.22	0.45	0.51	0.49	0.49	0.38	0.40	0.17	0.07	0.16
	0.45	0.41	0.44	0.47	0.33	0.31	0.38	0.14	0.09	0.13

* RSE, raw tuna spleen without pre-treatment; FSE, pre-filtered tuna spleen extract; CSE, centrifugated tuna spleen extract

On the other hand, the high relative velocity between dissolved protein molecules and adsorbed protein molecules within the larger pore size membrane could resulted in collisions which overcame the repulsive forces between protein molecules that stabilize a static dispersion (Bowen and Gan, 1991). The enzyme molecules aggregated without the repulsive forces and blocked the pore. Furthermore, both shear force and adsorption-desorption process might change the secondary structure of proteins during passage through microfiltration membranes (Truskey *et al.*, 1987). However, the transmissions of enzymes from pre-treated tuna spleen extract were slightly lower than those from tuna spleen without pre-treatment. It was due to the change of particle size distribution. The pre-treated tuna spleen extract contained more small particles than tuna spleen extract without pre-treatment. These small particles caused severer pore blocking and formed compacter layer on the membrane surface than larger size particles, thus, blocked more pathways for the transmission of enzymes. As mentioned above, the large size particles in tuna spleen without pre-treatment played a role of shelf in the structure of fouling layer on the membrane surface. Therefore, a looser layer formed on the membrane surface when tuna spleen without pre-treatment was used as feed. The small particles (e.g. trypsin and chymotrypsin) passed this layer easily and a higher transmission of solutes could be maintained. According to this phenomenon, it could be expected that gas spray method could be used to reduce fouling when pre-treated tuna spleen extract was used as feed. The bubble generated from gas spray in feed line might play a similar role as large particles to change the structure of fouling layer on the membrane surface (Cui *et al.*, 2003), then enhance the permeate flux and transmissions of solutes. According to the total solid content in the extracts, it should be mentioned that pre-filtration could remove more suspended solids in the tuna spleen extract than centrifugation before microfiltration. Therefore, pre-filtration could be chosen as pre-treatment method rather than centrifugation.

2.5 Conclusion

The microfiltration was successfully used to remove suspended solids from the yellowfin tuna spleen extract before recovery of proteases. All visible suspended solids were removed and a clear permeate fluid with slightly yellow colour was obtained by one-step microfiltration process. About half amount of soluble proteins was rejected by three membranes

with pore size 0.10, 0.22 and 0.45 μm, respectively. Both transmembrane pressure and membrane structural geometry could affect the recovery of enzyme activity. Larger membrane pore size and higher transmembrane pressure could cause the loss of enzyme activity. All proteases could be recovered in permeate from a 0.10 μm membrane at transmembrane pressure 4 psi. The fouling during microfiltration was separated to three models, i.e. membrane resistance-limited, pore blocking resistance-limited and cake resistance-limited. The change of membrane pore size and transmembrane pressure resulted in changing the extension of each fouling mechanism. Although high pressure gave high initial filtration rates, the overall volume filtered at a given time could become greater for lower pressure operation. Pore blocking was thought as a major fouling mechanism for the permeate flux decline whereas cake formation on the membrane surface dominated the most process of a microfiltration in the later and long period. It should be emphasized that all fouling phenomena, e.g. adsorption, pore blocking and cake layer, might be involved simultaneously because of the complexity of the feed composition, operation condition and membrane property. However, the dominant fouling mechanisms changed with time and caused the transition of dominant fouling in the different period of filtration process. The centrifugation or pre-filtration of tuna spleen extract removed a part of suspended solids and changed particle size distribution in the extract. Particle size distribution in tuna spleen extract played an important role for membrane fouling. The pre-filtration of feed could be employed before microfiltration process to remove a part of suspended solids and improve microfiltration performance since it was a simple method and removed more suspended solids. It also would reduce the cleaning load of membrane after microfiltration process.

CHAPTER 3

REMOVAL OF SUSPENDED PARTICLES FROM EXTRACT OF TUNA SPLEEN BY MICROFILTRATION: EXPERIMENTAL EVALUATION OF A LOW FOULING CONDITION

3.1 Abstract

A batch concentration microfiltration with a hollow fiber membrane (pore size of 0.10 μm) was successfully employed as a pretreatment process to remove suspended particles from extract of yellowfin tuna spleen before recovery of proteases by ultrafiltration. It was found that all visible suspended particles and more than half amount of soluble proteins were rejected. The transmission was about 0.8 and 0.6 for trypsin and chymotrypsin, respectively. A clear permeate with slightly yellow colour was obtained. Based on critical ratio of permeate flux (J) to wall shear stress (τ_w), the condition for sustainable operation with low fouling was experimentally evaluated. An empirical model of relation between critical J/τ_w and volume concentration factor (VCF) was found as $J/\tau_w = 3.29\ (\mathrm{VCF})^{-0.74}$ at a given transmembrane pressure of 0.15 bar. The equation indicated a condition, below which there is no marked fouling and above which sharp increase of fouling could occur. The present study revealed a simple method to predict low fouling condition in batch concentration operation during membrane separation process.

3.2 Introduction

Tuna is a main source of canning product in Thailand with production exceeding 311, 070 tons annually (National Food Institute, 2005). There are 23-25% solid waste (e.g. head, skin and viscera) and about 35% liquid waste (e.g. blood, condensate and oil) generated from tuna canning plant (Prasertsan *et al.*, 1988). These wastes contain a high organic load, and therefore a number of progresses have been made toward better utilization of these wastes. In previous study, the proteases could be recovered from yellowfin tuna spleen by ultrafiltration (Li *et al.*, 2006).

Since wastes from fishery processing contain many impurities, such as fat, meat debris and blood which could limit the function of ultrafiltration (UF) process, microfiltration (MF) may serve as a simple and economical method to remove most impurities, especially suspended particles and provide a clear feed bulk for further recovery of valuable compound, such as proteases by UF.

Concentration polarization and fouling are the major problems causing the functional decay of membrane separation process. In order to control concentration polarization and fouling, the critical flux was introduced and investigated by a number of researchers (Howell, 1995; Howell *et al.*, 2004; Espinasse *et al.*, 2002). Furthermore, in crossflow membrane filtration, it has been observed that the permeate flux (J) governs convective mass transport to the membrane and wall shear stress (τ_w) rules the transport by erosion to diffuse molecules and particles back from the membrane towards the fluid bulk (Lu *et al.*, 1993; Geasan-Guiziou *et al.*, 1999). Thus, a critical value of J/τ_w has been applied to indicate a sustainable membrane filtration process with negligible fouling (Le Berre and Daufin, 1999; Jeantet *et al.*, 2000). However, most researches of critical value of J/τ_w focus on the total recycle mode which holds a constant feed property. Few results have been reported for a commonly used process, batch concentration mode in which the properties of feed are changed continuously with time during the process.

The present study was therefore based on critical value of J/τ_w to evaluate a low fouling condition in a batch concentration crossflow microfiltration (CFMF) which was applied as a pretreatment process to remove suspended particles from extract of yellowfin tuna spleen before recovery of proteases.

3.3 Materials and methods

3.3.1 Analytical methods

Soluble protein concentrations (mg/ml) in both permeate and feed bulk and activities of trypsin and chymotrypsin (U/ml) were determined by the methods described in the previous work (Li *et al.*, 2006). The particle size distribution in extract of tuna spleen was measured by a Laser Particle Size Analyzer (LS230, Beckman Coulter, USA). The viscosity was measured by a viscometer (LVDV-I, Brookfield, USA). The permeate from microfiltration

process was collected and weighed continuously using a digital balance (GF-3000, A&D, Japan) connected to a PC to measure its flow rate. All filtration processes were performed at ambient temperature (28 ± 2°C).

3.3.2 Preparation of extract as feed bulk

The yellowfin tuna spleen was provided by Chotiwat Manufacturing Co. Ltd (Thailand), Hat Yai. The extract was prepared by homogenizing spleen of yellowfin tuna with Tris-HCl buffer (Li *et al.*, 2006). Before microfiltration, the tuna spleen extract was pretreated by simply filtrating extract through nylon net, cotton pad and cloth in series to remove largest particles which may block the port of hollow fiber membrane. The pretreated extract was used as feed bulk for microfiltration process.

3.3.3 Crossflow microfiltration process

The membrane used for CFMF was a hollow fiber membrane (CFP-1-E-5A, Amersham Biosciences, UK) with pore size of 0.10 μm, fiber diameter of 1 mm, flow length of 30 cm and effective area of 0.12 m^2. Pressure transducers (MBS3000, Danfoss, Denmark) were used to detect the pressure at the inlet and outlet of the membrane, and of the permeate. The crossflow velocity was controlled by a feed pump (Procon 814V230, Millipore, USA) combined with a retentate valve and measured by a digital flowmeter (Magflo5000, Danfoss, Denmark). Two CFMF modes i.e. total recycle and batch concentration were operated. The transmission of solute (T_r) was calculated by Equation (17) (Li *et al.*, 1997b):

$$T_r = \frac{C_p}{C_b} \tag{17}$$

where C_p is the solute concentration in permeate and C_b is the solute concentration in feed bulk.

In CFMF, the wall shear stress τ_w (Pa) was calculated as following (Geasan-Guiziou *et al.*, 1999):

$$\tau_w = \frac{F_a}{2}\rho_r \upsilon^2 \quad (18)$$

where F_a is Fanning number, ρ_r (kg/m^3) is the density of retentate and υ is the crossflow velocity (m/s).

For laminar flow in a hollow fiber membrane, F_a is 16/Re with a Reynolds number (Re) defined as

$$\mathrm{Re} = \frac{\rho_r \upsilon d}{\mu_r} \quad (19)$$

where d (m) is fiber diameter and μ_r (Pa.s)is dynamic viscosity of the retentate.

From Equation (18), (19) and F_a, wall shear stress could be expressed as a function of μ_r and υ as below:

$$\tau_w = \frac{8\upsilon}{d}\mu_r \quad (20)$$

Critical ratio of J/τ_w was investigated in both total recycle and batch concentration CFMF.

During total recycle CFMF, both retentate and permeate were recycled to feed tank to keep a constant property of feed bulk. The extracts were pre-concentrated 1.5, 2, 3, 5, 7 and 9-fold, respectively by the same type of membrane used in this study. The original extract and the pre-concentrated extracts were used as feed, respectively to investigate critical ratio of J/τ_w in total recycle CFMF. The critical flux was investigated by the 'step by step technique' (Chiu and James, 2005). The initial TMP was 0.01 bar and TMP was increased at fixed interval of 0.01 bar in time steps of 30 minutes prior to the onset of non-linearity in the increase of permeate flux, which was indicative of critical flux, thereafter 20 minutes time steps were used. The critical

fluxes for different feed concentrations were investigated at same wall shear stress range of 1.38 to 5.50 Pa by varying crossflow velocity.

The batch concentration CFMF was performed from an initial feed volume of 10 liters until 1 liter feed bulk was left, i.e. 9 liters permeate was collected. Retentate was recycled to feed bulk while permeate was removed from the system. The crossflow velocity of 0.2 to 0.8 m/s was applied. The original extract was used as feed for batch concentration CFMF. Viscosities of retentate and permeate were detected from samples taken at different volume concentration factor (VCF). VCF was defined as the ratio of initial volume to retentate volume at any time:

$$VCF = \frac{V_0}{V_r} = \frac{V_0}{V_0 - V_p} \tag{21}$$

where V_0 is initial volume of feed, V_r is volume of retentate and V_p is volume of collected permeate.

3.3.4 Resistance analysis

The resistance to the fluid flow during CFMF was defined by Darcy's law:

$$J = \frac{TMP}{\mu R_t} \tag{22}$$

where J is the permeate flux across the membrane (m/s), TMP is the transmembrane pressure (Pa), μ is the permeate viscosity (Pa.s) and R_t is the total hydraulic resistance (m^{-1}).

The total resistance (R_t) to fluid flow during CFMF was divided into membrane resistance (R_m), resistance caused by reversible fouling (R_{rf}) and resistance caused by irreversible fouling (R_{if}). R_t was the sum of R_m, R_{rf} and R_{if}. R_m was measured by filtering deionized water through the new membrane at TMP of 0.10 bar for 10 minutes. After CFMF of tuna spleen extract, fouled membrane was rinsed and flushed by deionized water at crossflow velocity of 0.8 m/s and TMP of 0.10 bar for 20 minutes to remove R_{rf}. Total wash volume of water was 30 liters. R_{if} was calculated from water flux of flushed membrane at TMP of 0.10 bar for 10 minutes.

3.4 Results and discussion

The particle size distribution in extract of tuna spleen simply filtered by nylon net, cotton pad and cloth in series was shown in Figure 9. The major particle size was larger than 0.1 μm which resulted in a high turbidity. After CFMF, a clear permeate with slightly yellow colour was obtained. The size of particles possibly presenting in this permeate was too small to respond to the detection by the Laser Particle Size Analyzer. Considering the minimum measurement capacity of 0.04 μm of this analyzer, it could be concluded that all suspended particles has been removed.

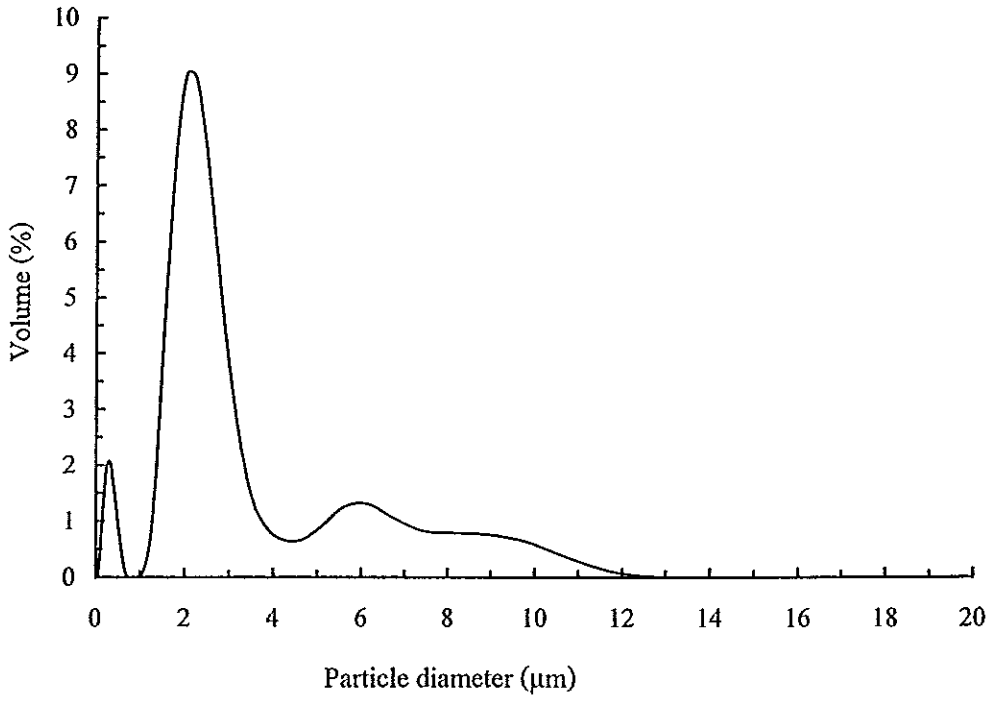

Figure 9. Particle size distribution in extract of tuna spleen simply filtered through nylon net, cotton pad and cloth in series before microfiltration.

3.4.1 Critical value of J/τ_w in total recycle CFMF with original extract as feed

The original extract without pre-concentrating process was initially used as feed in total recycle CFMF. It was observed that the critical flux in this study was weak form, i.e. the relation between flux and TMP is linear, but the slope of the line differs from that for clean water (Metsamuuronen *et al.*, 2002). The critical flux was increased from 7.6 to 20.9 $l/m^2.h$ while τ_w was increased from 1.38 to 5.50 Pa (Figure 10). The critical TMP corresponding to each J_c was ranged from 0.05 to 0.10 bar. A critical J/τ_w of 3.16 was obtained from the slope of J_c versus τ_w (Table 3).

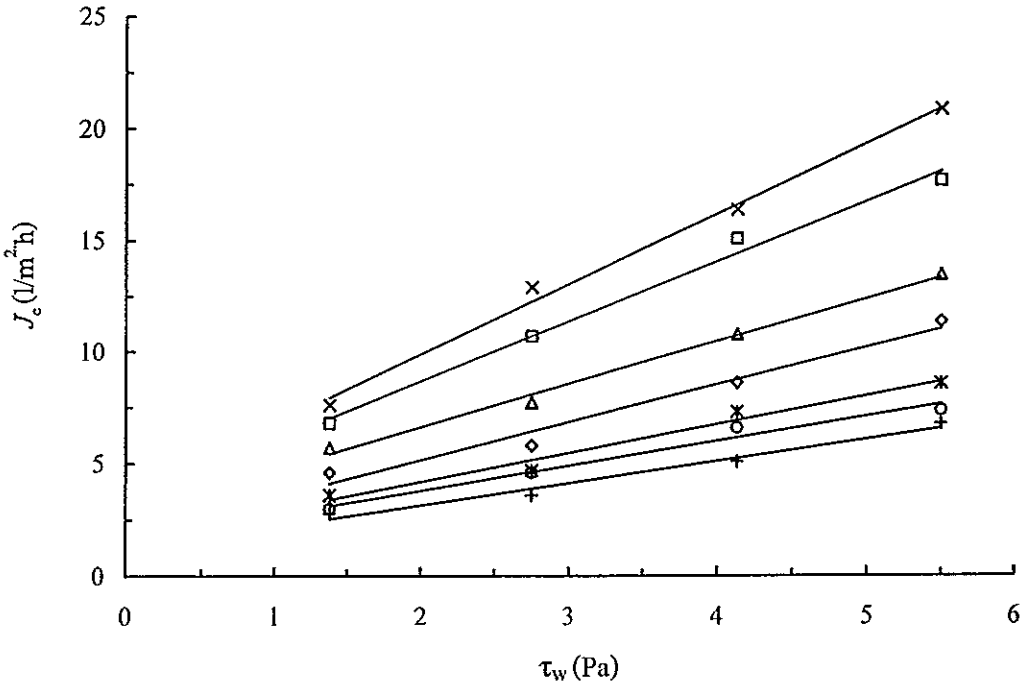

Figure 10. Critical permeate flux (J_c) versus wall shear stress (τ_w) in total recycle CFMF with different feed concentration (×, original extract; □, 1.5-fold; △, 2-fold; ◇, 3-fold; *, 5-fold; ○, 7-fold; +, 9-fold).

However, even a critical J/τ_w was obtained from total recycle CFMF, it was still insufficient for operation once mode was switched to batch concentration. During batch concentration process, the properties of feed bulk (e.g. viscosity, solute concentration) were changed continually with increasing VCF, thus critical value J/τ_w could not be a constant. A new empirical model should be evaluated for batch concentration process.

Table 3. Relation between critical permeate flux (J_c) and wall shear stress (τ_w) and evaluation of critical J/τ_w during total recycle CFMF with different feed concentration.

Feed	J_c vs. τ_w	R^2 value	critical J/τ_w
Original	$J_c=3.16\tau_w+3.59$	0.99	3.16
1.5-fold	$J_c=2.70\tau_w+3.29$	0.99	2.70
2-fold	$J_c=1.93\tau_w+2.79$	0.99	1.93
3-fold	$J_c=1.69\tau_w+1.72$	0.97	1.69
5-fold	$J_c=1.28\tau_w+1.64$	0.98	1.28
7-fold	$J_c=1.11\tau_w+1.59$	0.98	1.11
9-fold	$J_c=0.98\tau_w+1.19$	0.97	0.98

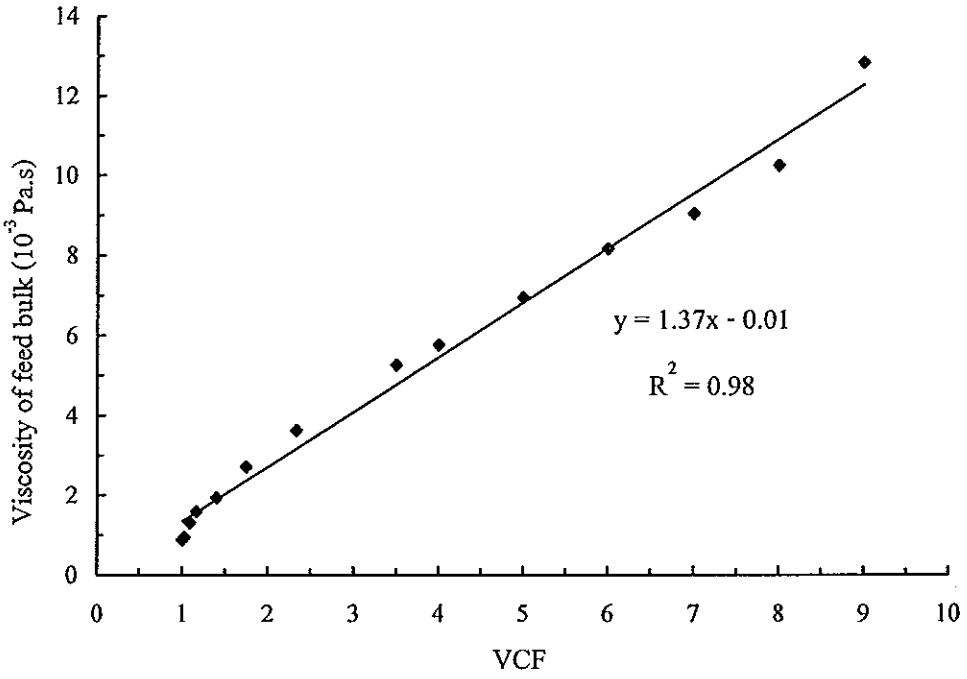

Figure 11. The relation between viscosity of feed bulk and VCF during batch concentration CFMF with TMP of 0.15 bar.

3.4.2 Flux profile in batch concentration CFMF

In order to obtain a relatively high permeate flux for more efficient operation and observe the linkage between operation condition and fouling formation, a TMP of 0.15 bar was applied in batch concentration operation. Since TMP of 0.15 bar was higher than critical TMP range in total recycle CFMF and the viscosity of feed bulk was increased significantly with increasing VCF (Figure 11), the concentration polarization and the fouling could occur rapidly in batch concentration CFMF. Regardless of changing concentration and viscosity of feed bulk with increasing VCF, a typical permeate flux of MF process was still observed in batch concentration CFMF (Figure 12). The permeate flux also could be divided into three periods, i.e. an initial period of rapid decrease of flux, a second period of less severe decrease of flux and a third period corresponding to a small decrease of flux until a steady-state was reached (Constenla and Lozano, 1997).

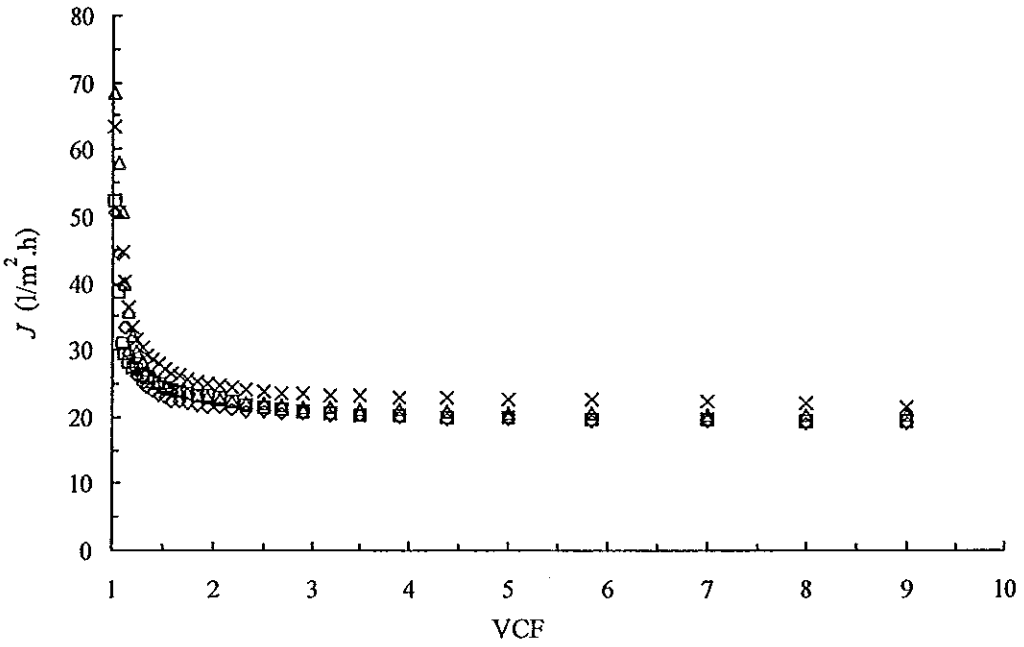

Figure 12. Effect of crossflow velocity (υ) on permeate flux in batch concentration CFMF with TMP of 0.15 bar (◇, υ of 0.2 m/s; □, υ of 0.4 m/s; △, υ of 0.6 m/s; ×, υ of 0.8 m/s).

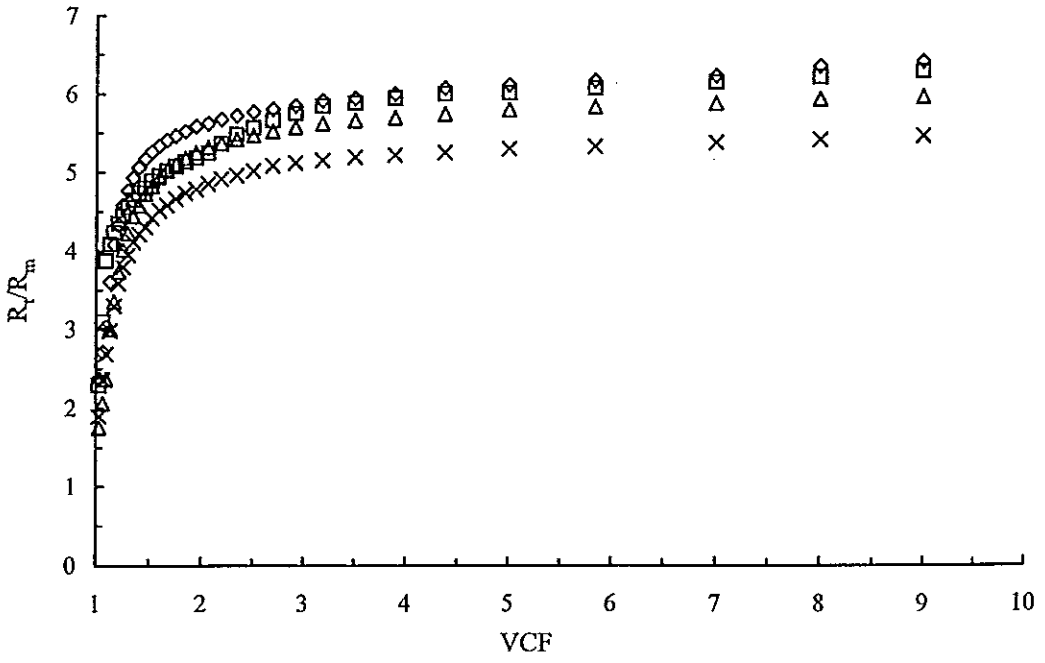

Figure 13. The profile of normalized total resistance (R_t/R_m) in batch concentration CFMF with TMP of 0.15 bar (◇, υ of 0.2 m/s; □, υ of 0.4 m/s; △, υ of 0.6 m/s; ×, υ of 0.8 m/s).

3.4.3 Resistance analysis in batch concentration CFMF

The membrane resistance (R_m) of 6.0×10^{11} m^{-1} was measured from the clean membrane before microfiltration process. Based on the flux of extract (Figure 12) and viscosity of permeate (it was constant during the process since the permeate with nearly identical properties at different VCF was obtained), R_t was worked out by Equation (22). Hence Figure 12 was replotted to a figure of normalized resistance versus VCF (Figure 13). After each run, the membrane was rinsed and flushed thoroughly, R_{if} was measured from flushed membrane by water flux (Figure 14). Compared with R_t/R_m, it was found that R_{if} was much lower than R_t. Most fouling could be classified to reversible fouling because the low value of R_{if} and negligible change of R_{if} were observed when crossflow velocity increased (Figure 14). It could be assumed that R_{if} was due to protein adsorption on the wall of the membrane and resulted in irreversible fouling of the walls and pores in just a short period after starting microfiltration. This irreversible fouling could not be eliminated by increasing crossflow velocity (Vadi and Rizvi, 2001). An average value of R_{if}/R_m was almost constant as 0.16 (Figure 14).

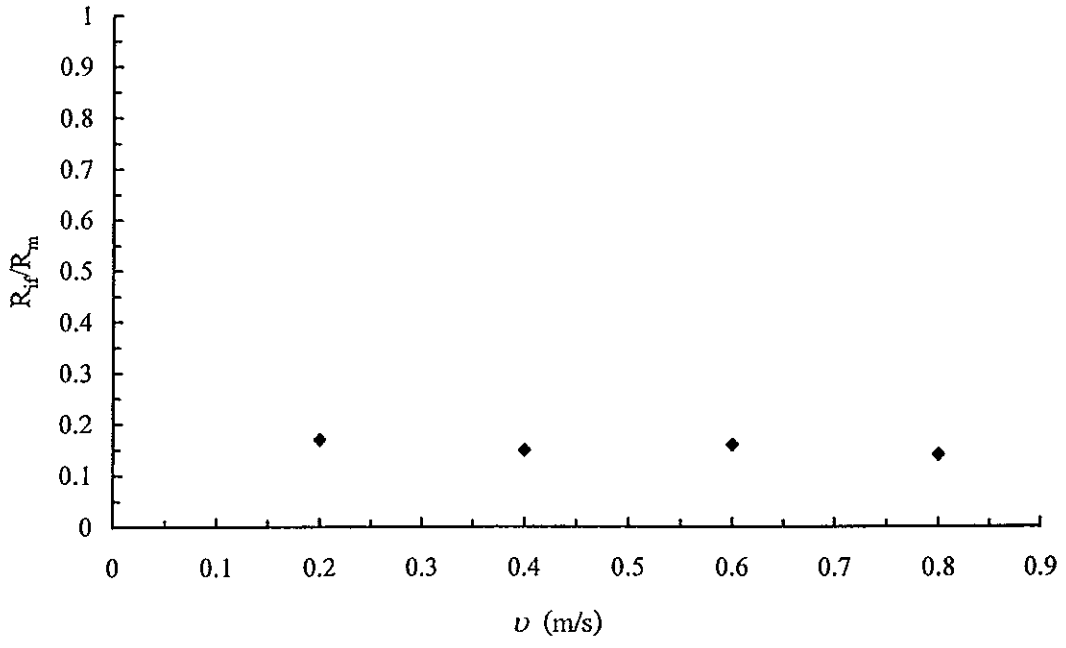

Figure 14. Normalized resistance of irreversible fouling (R_{if}/R_m) versus crossflow velocity (υ) in batch concentration CFMF with TMP of 0.15 bar.

3.4.4 Evaluation of critical J/τ_W in batch concentration CFMF

The critical flux in batch concentration CFMF could be hypothesized as similar as the definition of critical flux in other modes of operation, i.e. a flux at which membrane fouling was negligible. Since R_{if} occurred immediately once filtration began and could not be avoided, the critical flux in this study was considered as the weak form at which R_{rf} did not occur. The critical flux of 94.6 $l/m^2.h^1$ could be calculated by Equation (22) from TMP, viscosity of permeate and intrinsic resistance of system (i.e. $R_{if}+R_m$).

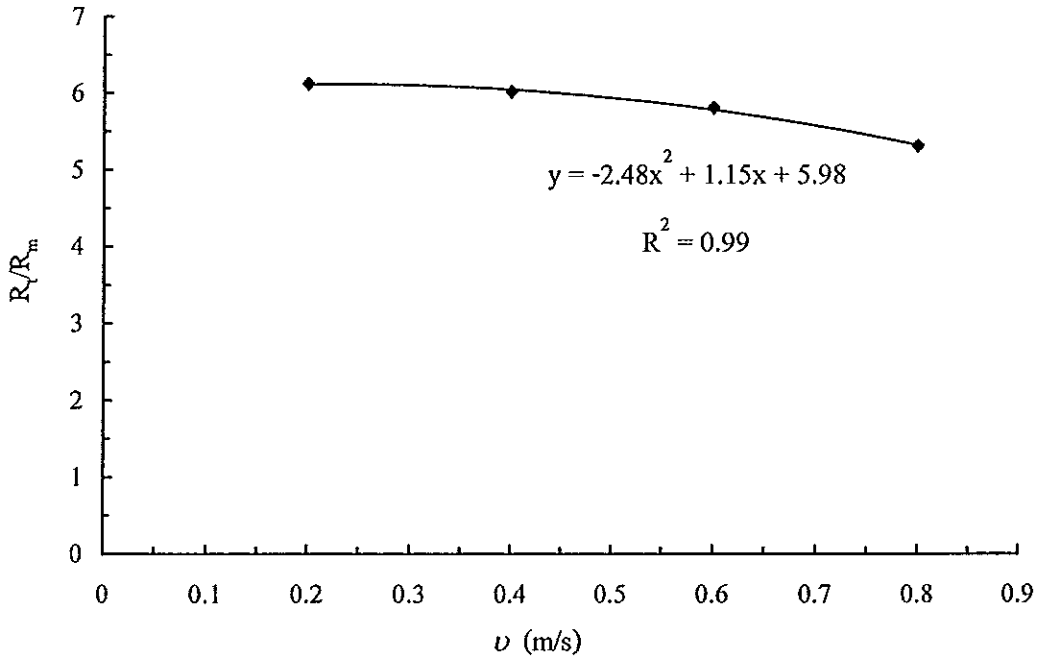

Figure 15. Normalized total resistance (R_t/R_m) versus crossflow velocity (υ) at VCF of 5 in batch concentration CFMF with TMP of 0.15 bar.

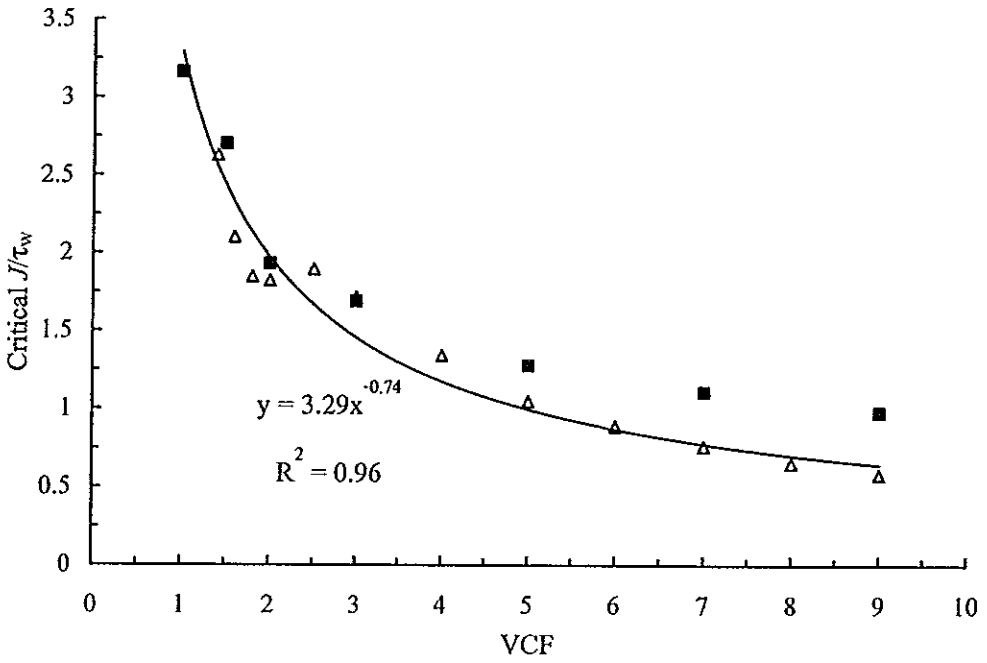

Figure 16. Profile of critical J/τ_w versus VCF in batch concentration CFMF with TMP of 0.15 bar (△, experimental data from batch concentration CFMF; ——— empirical model based on batch concentration CFMF; ■, experimental data from total recycle CFMF).

As mentioned by Equation (20), τ_w was related to viscosity of retentate and crossflow velocity. It was observed that increasing crossflow velocity decreased R_t/R_m (Figure 13) at each VCF. In order to determine a crossflow velocity which could eliminate reversible fouling at each VCF, R_t/R_m versus crossflow velocity at different VCF was plotted. Polynomial equations were applied to mathematically represent the relation of R_t/R_m versus crossflow velocity. Since R_t was divided into R_m, R_{rf} and R_{if}, R_t/R_m could be calculated as $(R_m+R_{if})/R_m$ if R_{rf} was eliminated by crossflow. Hence the critical crossflow velocity (υ_c) to eliminate R_{rf} could be found when R_t/R_m equaled to $(R_m+R_{if})/R_m$ (i.e. 1.16). Figure 15 exemplified the evaluation of υ_c at VCF of 5. υ_c of 1.65 m/s at VCF of 5 was worked out by a polynomial equation when R_t/R_m equaled to 1.16. Other υ_c at different VCF were obtained by the same method. Based on υ_c and viscosity of feed bulk (Figure 11) at different VCF, critical τ_w at each VCF was calculated by Equation (20).

Finally, a critical value of J/τ_w was obtained from critical flux of 94.6 l/m^2.h and critical τ_w at different VCF. Different mathematic trendlines were used to fit data of critical J/τ_w versus VCF. A trendline with power relation was found to yield the best fitting (Figure 16). The result from Figure 16 modeled the relation between critical J/τ_w and VCF as Equation (23) at given condition in this study.

$$\frac{J}{\tau_w} = 3.29(\mathrm{VCF})^{-0.74} \tag{23}$$

Under the curve, it was the sustainable operation area with negligible fouling. Once operation came to the area above the curve, the severe but reversible fouling would occur rapidly. Higher VCF required lower J/τ_w (i.e. low J and high τ_w) to avoid severe fouling for a sustainable operation. Once J and τ_w are decided, the maximum concentration factor (VCF_{max}) could be worked out by Equation (23). Lower than VCF_{max}, the fouling could be negligible and the fouling would form rapidly if VCF is higher than VCF_{max}. On the other hand, once VCF_{max} is attained, the operation could be switched to feed-and-bleed mode (product was permeate) or diafiltration mode (product was retentate). Change of mode would decrease the viscosity of feed bulk, therefore increase actual critical J/τ_w and keep operation under sustainable condition until a desired recovery rate of product is achieved.

In order to prove reliability of empirical model of Equation (23), pre-concentrated extract were used as feed in total recycle CFMF. The critical J/τ_w were investigated and compared to the modeled trendline (Figure 16). During total recyle CFMF, critical J/τ_w decreased with increasing feed concentration (Figure 16). Critical J/τ_w based on different feed concentration were evaluated in Table 3. It was observed that there was a good agreement between experimental data based on total recyle CFMF and the empirical model at low VCF (Figure 16). The difference between experimental data and model was seen at high VCF. It was probably due to fouling mechanism and extent. The difference in fouling mechanism and extent between total recycle and batch concentration CFMF gave minor influence at low VCF. However, this difference should be significant at high VCF. In total recycle CFMF, even the feed was pre-concentrated to different VCF, the feed property was constant during each filtration process and fouling initially formed on a clean membrane surface. During batch concentration CFMF, the

fouling at high VCF actually based on the previously formed fouling at low VCF. The fouling mechanism was much more complex in batch concentration CFMF than that in total recycle CFMF due to continuously change of feed properties, such as concentration and viscosity. Furthermore, higher fouling extent in batch concentration CFMF made the value of empirical model lower than results from total recycle CFMF.

3.5 Conclusion

The microfiltration was successfully used to remove suspended particles from extract of yellowfin tuna spleen before recovery of trypsin and chymotrypsin. All visible suspended particles were removed and a clear permeate fluid with slightly yellow colour was obtained by one-step microfiltration process. Most trypsin and chymotrypsin could pass through the membrane with pore size of 0.10 μm while about half amount of soluble protein was rejected. An empirical model of low fouling condition in batch concentration process at given operation condition indicated a power relation between critical J/τ_w and volume concentration factor (VCF). The profile of critical J/τ_w versus VCF divided operation into a sustainable condition with negligible fouling and an unsustainable condition with severe fouling. Validation of model was tested on the data from total recycle CFMF with different feed concentration. The good agreement between model and experimental data was obtained at low VCF stage. However, the empirical model under-predicted critical J/τ_w at high VCF stage. Considering the difference of fouling mechanism and extent between total recycle and batch concentration CFMF, the proposed model could be acceptable and useful to predict sustainable operation during batch concentration process. On the other hand, due to the high fouling load in feed bulk, the critical J/τ_w was relatively low when VCF reached high levels in present study. Anyway this study provided a simple method to modeling a batch concentration membrane filtration process. A desirable critical J/τ_w could be attained in other applications if the feed property is adjusted to be reasonable by additional methods, such as dilution, pre-filtration. Batch concentration operation in sustainable condition combined with feed-and-bleed (product in permeate) or diafiltration (product in retentate) could get both low fouling operation and desired recovery rate of product.

CHAPTER 4

REMOVAL OF SUSPENDED SOLIDS FROM TUNA SPLEEN EXTRACT BY MICROFILTRATION: A BATCH PROCESS DESIGN AND IMPROVEMENT

4.1 Abstract

In order to reduce the fouling load during separation of trypsin and chymotrypsin from yellowfin tuna spleen extract by ultrafiltration, a batch concentration microfiltration was designed as a pretreatment process to remove suspended solids from this extract. Based on total recycle and single-batch concentration CFMF, a continuous-batch concentration CFMF (CBC-CFMF) with 0.10 μm hollow fiber membrane, crossflow velocity of 0.2 m/s, transmembrane pressure of 0.15 bar and gas injection factor of 0.38 was designed and applied successfully to remove suspended solids from tuna spleen extract while transmissions of about 1 for both trypsin and chymotrypsin were attained in this study. The negative effects of shear stress, high concentration of feed and long holding time in batch operation process were minimized by CBC-CFMF combined with gas bubbling technique. The optimal gas injection factor (r) of 0.38 resulted in a 300% improvement in flux comparing to the process without gas injection. Higher gas injection factor caused damage on both transmission and activity of expected enzymes. A clear permeate with slight yellow colour was obtained after CBC-CFMF.

4.2 Introduction

The demand of fishery product has increased significantly in recent years. As one of the largest exporters of canned tuna in the world, Thailand produces more than 311,070 tons canned tuna annually (National Food Institute, 2005). Since the major part of aquatic animal for fishery processing is only meat, a large amount of wastes has been generated from the fishery industry. In the tuna canning process, there are 23-25% solid waste (e.g. head, skin, viscera) and about 35% liquid waste (e.g. blood, tuna condensate, oil) (Prasertsan *et al.*, 1988). The utilization of fishery waste has caught more attention. The pressure driven membrane processes, e.g.

microfiltration (MF), ultrafiltration (UF), nanofiltration (NF) and reverse osmosis (RO) have successfully been implemented in many fishery industrial cases (Gildberg and Shi, 1994; Afonso and Borquez, 2002; Vandanjon *et al.*, 2002; Dewitt and Morrissey, 2002a). In previous studies, the proteases were recovered from yellowfin tuna spleen by UF (Li *et al.*, 2006). However the wastes from fishery processing contain many impurities, such as fat, meat debris and blood. After extraction, the extract is normally turbid and contains a lot of suspended solids which may limit the function of UF process. A suitable pretreatment method is required. According to its separation range in solute size varying from approximately 0.02 μm to 10 μm and operational advantages, MF may serve as a simple, low cost and continuous operation to substitute for other methods such as centrifugation as pretreatment process to remove suspended solids from tuna spleen extract and provide a clear permeate as feed bulk for further recovery of valuable compound by UF process.

It is well known that concentration polarization and fouling including reversible and irreversible types are major problems causing functional decay of MF process and low permeate flux to far below the theoretical capacity of the membrane. The total avoidance of these two problems is not possible, but their impact could be limited by a variety of techniques, such as pretreatment of feed bulk, modification of membrane material and flow manipulation. Gas bubbling is a novel hydrodynamic method to enhance membrane processes by introducing gas into a module to promote the local mixing near the membrane surface. It was observed that the injection of gas increased the permeate flux by 7% to 50% in HSA and IgG UF process with a flat sheet membrane (Li *et al.*, 1998). A 175% increase in the permeate flux for MF of yeast using a polymeric membrane module with the gas injection was also observed (Cui *et al.*, 2003). Comparing to other methods, gas bubbling is simple and economical. Gas bubbles pose less risk to the membrane and are easily separated from the process stream.

The present study was to develop a MF process as the pretreatment to remove the suspended solids from yellowfin tuna spleen extract before recovery of proteases from this extract by UF. The optimal conditions, i.e. transmembrane pressure, crossflow velocity, gas injection factor and mode of operation were studied.

4.3 Materials and methods

4.3.1 Analytical methods

The major compositions in yellowfin tuna spleen extract were analyzed by the methods of AOAC (1999). The concentrations of soluble protein in both permeate and feed bulk and activities of trypsin and chymotrypsin were determined by the methods described in the previous work (Li *et al.*, 2006). The particle size distribution in this extract was measured by a Laser Particle Size Analyzer (LS230, Beckman Coulter, USA). The permeate from MF process was collected and weighed continuously by a digital balance (GF-3000, A&D, Japan) connected to a PC to measure its flux. All filtration processes were performed at the ambient temperature (28± 2°C).

4.3.2 Preparation of tuna spleen extract

The yellowfin tuna spleen was provided by Chotiwat Manufacturing Co. Ltd (Thailand), Hat Yai. The extract was prepared by homogenizing the yellowfin tuna spleen with Tris-HCl buffer (Li *et al.*, 2006) and simply filtered through nylon net, cotton pad and cloth in series to remove large solid particles which may block the port of the hollow fiber membrane.

4.3.3 Crossflow microfiltration for removal of suspended solids

The membrane used for the crossflow MF (CFMF) was a hollow fiber membrane (CFP-1-E-5A, Amersham Biosciences, UK) with pore size of 0.10 μm, fiber diameter of 1 mm, flow length of 30 cm and effective area of 0.12 m^2. Pressure transducers (MBS3000, Danfoss, Denmark) were used to measure the pressure at the inlet and outlet of the membrane, and of the permeate. The crossflow velocity was controlled by a reciprocal displacement pump (Procon 814V230, Millipore, USA) and a retentate valve. The crossflow velocity was measured by a digital liquid flowmeter (Magflo5000, Danfoss, Denmark). The transmission of solute (T_r) was calculated by Equation (24) (Li *et al.*, 1997b):

$$T_r = \frac{C_p}{C_b} \tag{24}$$

Where C_p is the solute concentration in permeate and C_b is the solute concentration in feed bulk or retentate.

4.3.3.1 Total recycle crossflow microfiltration

A total recycle CFMF with feed volume of 5 liters was used to investigate the suitable operational TMP. Both retentate and permeate were recycled to the feed tank to keep the constant property of feed bulk. Critical flux was defined as a permeate flux below which the formation of fouling on membrane is neglectable (Howell, 1995). The critical flux is investigated based on Dancy's law:

$$J = \frac{TMP}{\mu R_{tol}} = \frac{TMP}{\mu(R_m + R_{rf} + R_{if})} \tag{25}$$

where: J is volumetric flux of permeate across the membrane (m/s), TMP is the transmembrane pressure (Pa), μ is the permeate viscosity (Pa.s), R_{tol} is the total hydraulic resistance (m^{-1}), R_m is the membrane resistance, R_{rf} is the resistance caused by reversible fouling and R_{if} is the resistance caused by irreversible fouling.

Below the critical flux, the relation between flux and TMP is linear since resistances caused by fouling (i.e. R_{rf} and R_{if}) are neglectable. The critical flux was investigated by a "step by step" technique (Chiu and James, 2005). The initial TMP was 0.01 bar and TMP was increased at fixed interval of 0.01 bar in time steps of 30 minutes prior to the onset of non-linearity in the increase of permeate flux, which was the indication of critical flux. Thereafter time steps of 20 minutes were used. The critical flux was investigated at crossflow velocities varying from 0.2 to 0.8 m/s. TMP corresponding to each critical flux was considered as the critical TMP at the given conditions.

4.3.3.2 Batch concentration crossflow microfiltration

Two types of batch concentration CFMF named single-batch concentration CFMF (SBC-CFMF) and continuous-batch concentration (CBC-CFMF) were investigated for removal of suspended solids from yellowfin tuna spleen extract. In both SBC-CFMF and CBC-CFMF, the retentate was recycled to the feed bulk while the permeate was removed from module.

SBC-CFMF with an initial feed volume of 10 liters in the feed tank was performed until 1 liter feed bulk was left, i.e. the permeate of 9 liters was collected. TMP was controlled at 0.03 and 0.15 bar, respectively. The crossflow velocities were controlled as same as those in the total recycle run. The volume concentration factor (VCF) was defined as the ratio of the initial feed volume to the feed volume left in the tank during SBC-CFMF process:

$$VCF = \frac{V_0}{V_f} = \frac{V_0}{V_0 - V_p} \tag{26}$$

where V_0 is the initial feed volume; V_f is the feed bulk left in the tank during concentration process and V_p is collected permeate volume.

CBC-CFMF was performed with an initial feed volume of 1 liter. Crossflow velocity of 0.2 m/s and TMP of 0.15 bar were applied. The initial feed volume of 1 liter in the feed tank was kept as a constant volume by continuously replacing the lost liquid (permeate) with the newly added feed, i.e. tuna spleen extract. The filtration process was maintained until the permeate of 9 liters was collected. The feeding volume factor (FVF) was defined as the ratio of the newly added tuna spleen extract volume to the initial feed volume:

$$FVF = \frac{V_F}{V_0} = \frac{V_p}{V_0} \tag{27}$$

where V_F is the volume of newly added feed extract , V_0 is the initial volume in feed tank and V_p is the collected permeate volume.

4.3.3.3 Continuous-batch concentration crossflow microfiltration aided by the gas bubbling

A gas bubbling technology was applied to enhance the permeate flux during CBC-CFMF in this study. The compressed nitrogen gas was injected into the inlet of feed pipe

through a Y-tubular piece. A fixed liquid crossflow velocity of 0.2 m/s and a fixed TMP of 0.15 bar were applied. The gas flow velocity was controlled and measured by a gas flowmeter (RMB-53D-SSV, Dwyer, USA) combined with a pressure gauge (2419-2C-P, CKD, Japan). The gas-liquid dual flow pattern depends on the gas injection factor (r) which equals to $U_g / (U_g + U_l)$. U_g and U_l are the superficial gas and liquid flow velocity, respectively. The superficial velocity is defined as the velocity if only gas or liquid is in the pipe. The dual flow pattern changes from bubble flow ($0 < r < 0.2$) over slug flow ($0.2 < r < 0.9$) to annular flow ($0.9 < r < 1.0$) (Psoch and Schiewer, 2005).

The superficial gas flow velocity was controlled at 0, 0.04, 0.12, 0.25 and 0.37 m/s, respectively in this work. The corresponding r was 0, 0.16, 0.38, 0.56, and 0.65 which varied from bubble flow to slug flow.

4.3.3.4 Resistance analysis

The resistance to the fluid flow during CFMF was defined by Darcy's law (Equation 18). The total resistance R_{tol} was divided into membrane resistance (R_m), resistance caused by reversible fouling (R_{rf}) and resistance caused by irreversible fouling (R_{if}). R_{tol} was the sum of R_m, R_{rf} and R_{if}. R_m was measured by filtering deionized water through the new membrane at TMP 0.1 bar for 10 minutes. After each CFMF process of the tuna spleen extract, the fouled membrane was flushed by deionized water at the crossflow velocity of 0.8 m/s and TMP of 0.1 bar to eliminate R_{rf}. Total wash volume of water was 30 liters. R_{if} was calculated from the water flux of flushed membrane at TMP of 0.1 bar for 10 minutes.

4.4 Results and discussion

The compositions analyzed in the yellowfin tuna spleen extract were soluble protein (9.97 mg/ml), salt (1.00 mg/ml), crude fat (10 mg/ml) and ash (5.00 mg/ml). The extract contained trypsin (20.56 U/ml) and chymotrypsin (12.14 U/ml). The particle size distribution was shown in Figure 17. The black dots indicated the particles' size and their volume percentage presented in the tuna spleen extract simply filtered by nylon net, cotton pad and cloth in series.

The mean particle size in this feed was 2.2 μm, and 99% particles were smaller than 10 μm. The typical permeate flux of MF process was observed from CFMF in this study, i.e. at the beginning of filtration processes the flux decreased sharply, and later slowly, then the flux was almost constant for a long period.

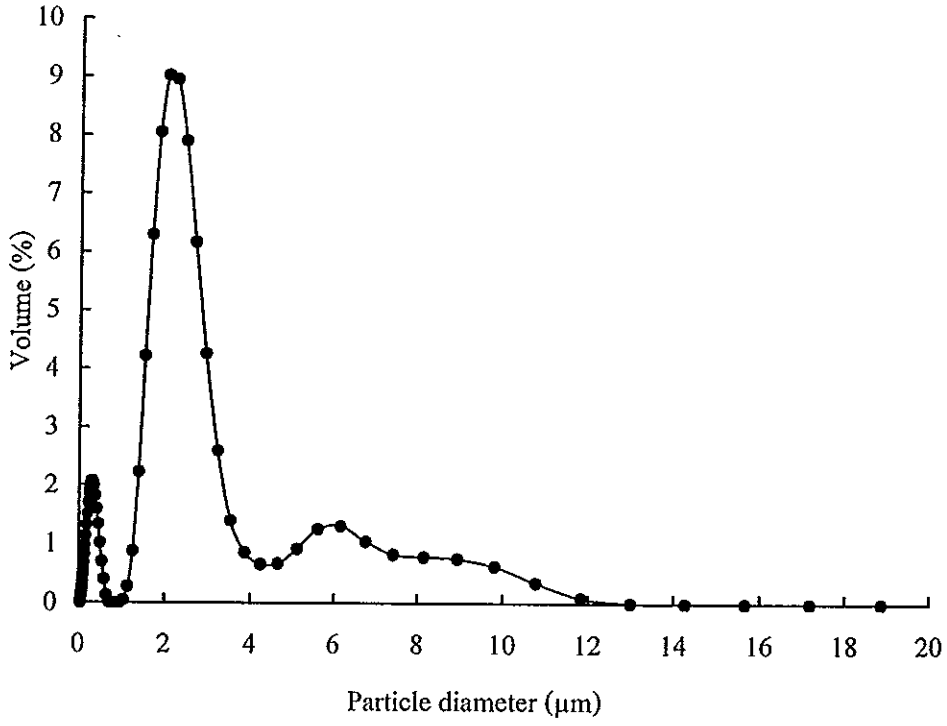

Figure 17. Particle size distribution in yellowfin tuna spleen extract simply filtered through nylon net, cotton pad and cloth in series.

4.4.1 Detection of critical transmembrane pressure by total recycle crossflow microfiltration

A hollow fiber membrane with pore size 0.1 μm was selected for CFMF. Crossflow velocity varying from 0.2 to 0.8 m/s was applied. As mentioned above, critical flux was measured by a 'step by step' technique. An example at crossflow velocity of 0.6 m/s was

illustrated by Figure 18. TMP was increased with a fixed interval of 0.01 bar from an initial value of 0.01 bar. Since fouling was neglectable, the increase of permeate flux with increasing TMP was linear when permeate flux was below critical flux. Once the permeate flux exceeded the critical flux, the fouling occurred and the relation between permeate flux and TMP altered to be non-linear. The critical transition point, i.e. critical flux of 16.4 $l/m^2.h$ was detected at TMP 0.08 bar. Other critical fluxes were detected by the same method at different crossflow velocities. It was observed the line of permeate flux versus TMP did not go through the origin in Figure 18. Theoretically, the permeate flux should come to origin zero if TMP is zero. The deviation of the line from origin in Figure 18 was probably due to the temporary formation of local vacuums in the closed pipe. These vacuums formed by lack of liquid at somewhere, especially at the space of joint in the pipe and broke the transmembrane pressure distribution, thus made the line of permeate versus TMP to cross X- or Y-axis. Similar phenomena were also observed in many other studies (Kaichang *et al.*, 2003; Dengxi *et al.*, 1999). Anyway, the initial linear dependency indicated the negligible fouling and directed to the critical flux. Figure 19 shows critical flux and corresponding TMP versus crossflow velocity. The critical flux was increased from 7.6 to 20.9 $l/m^2.h$ while crossflow velocity was increased from 0.2 to 0.8 m/s. The critical TMP for each critical flux ranged from 0.05 to 0.1 bar at each given crossflow velocity.

Total recycle operation is a closed system. However, the target enzymes were in the permeate which should be removed and collected for further recovery. Therefore a batch concentration CFMF should be applied to collect permeate. In order to compare the effect of pressure, a TMP of 0.03 bar which was lower than critical TMP range and a TMP of 0.15 bar which was above critical TMP range were selected for further SBC-CFMF.

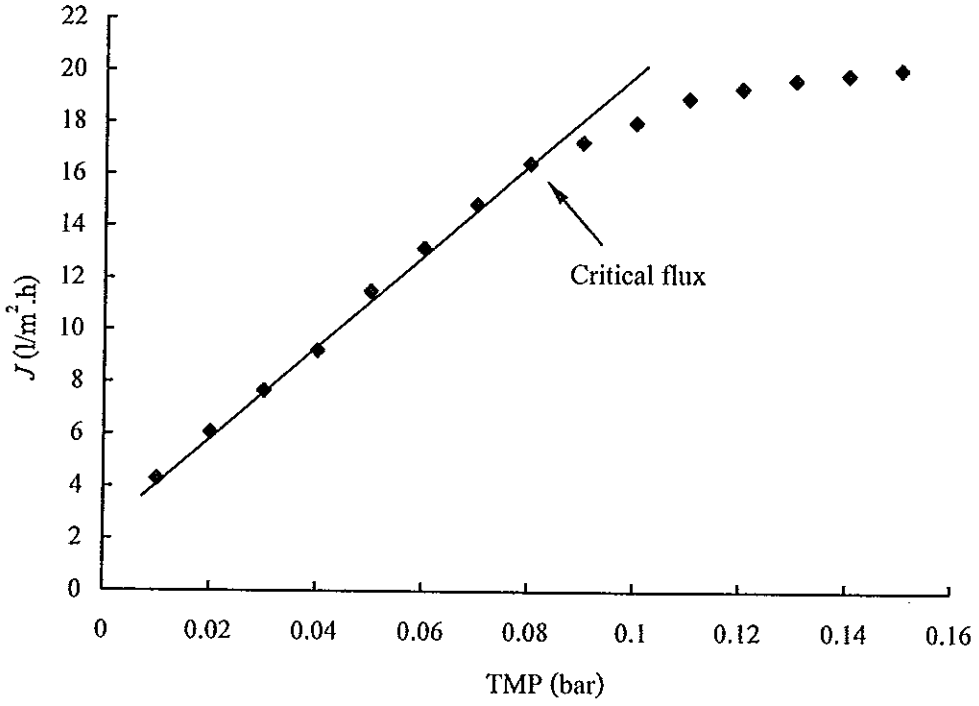

Figure 18. Detection of critical flux by a 'step by step' method in the total recycle CFMF at the crossflow velocity of 0.6 m/s.

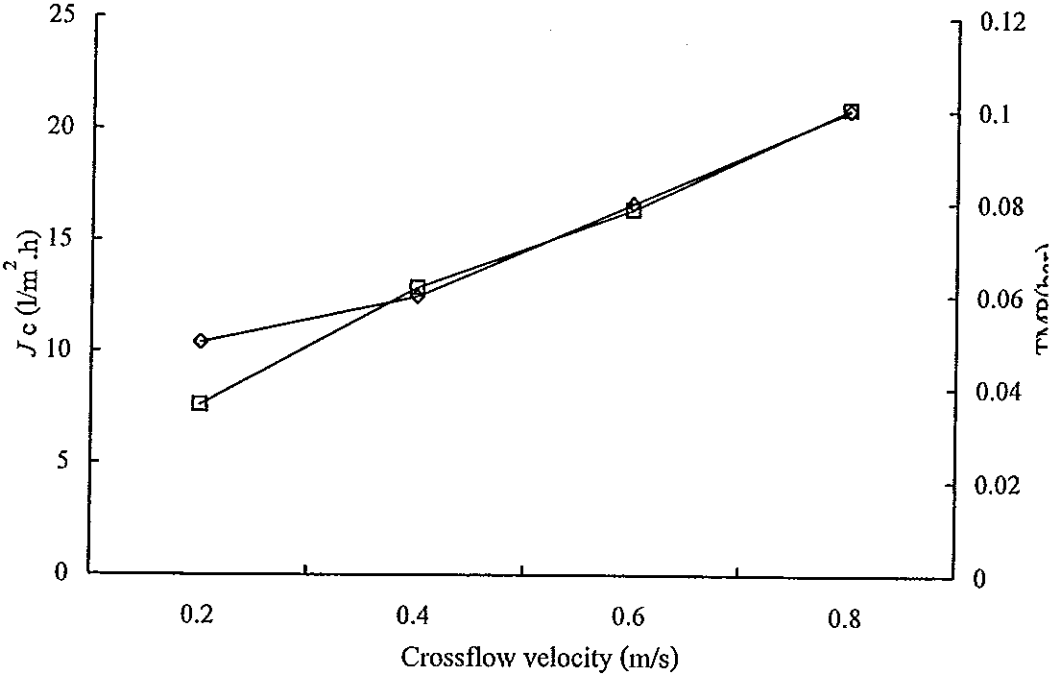

Figure 19. Critical flux (J_c) and corresponding critical TMP versus crossflow velocity in the total recycle CFMF (◇ TMP; □ critical permeate flux).

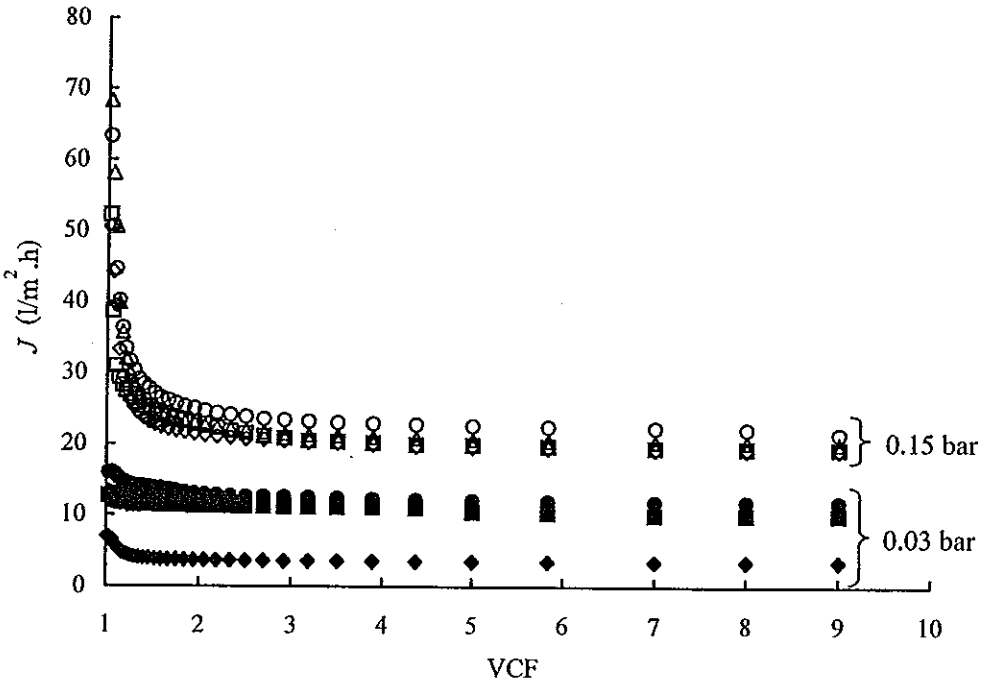

Figure 20. Effect of crossflow velocity (υ) on permeate flux in SBC-CFMF at TMP of 0.03 and 0.15 bar (◇, ◆ υ is 0.2 m/s; □, ■ υ is 0.4 m/s; △, ▲ υ is 0.6 m/s; ○, ● υ is 0.8 m/s).

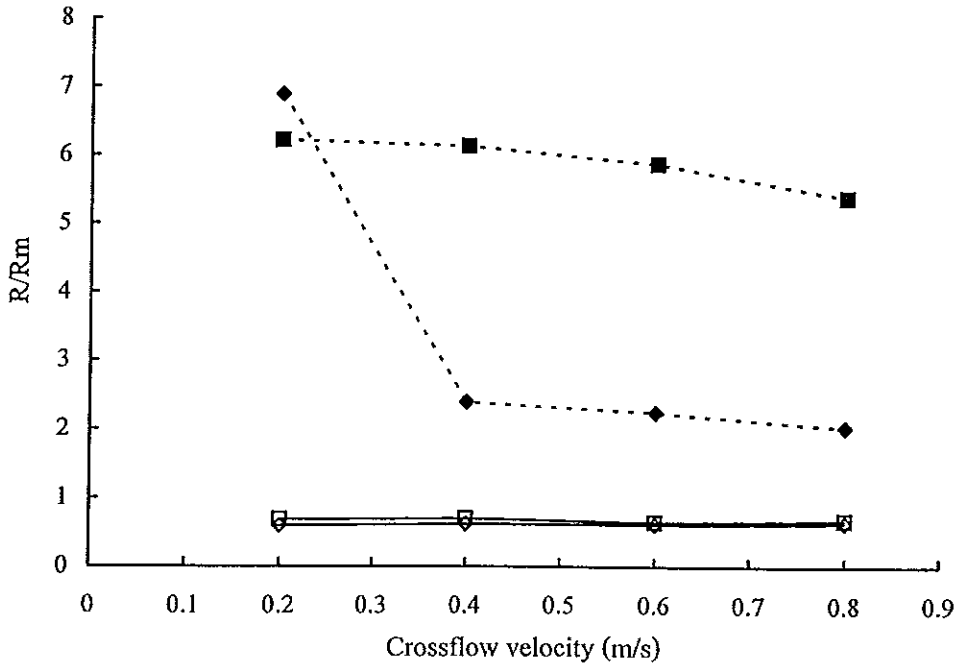

Figure 21. The profile of normalized resistance (R/R_m) in SBC-CFMF (◇, ◆ TMP 0.03 bar; □, ■ TMP 0.15 bar; ····· R_{tot}/R_m; ——— R_{ir}/R_m).

Table 4 Average transmissions (T_r) of soluble protein and enzymes in SBC-CFMF at different crossflow velocities (υ) and TMPs.

TMP	Solute	T_r			
		υ 0.2 m/s	υ 0.4 m/s	υ 0.6 m/s	υ 0.8 m/s
0.03 bar	protein	0.61	0.64	0.68	0.67
	trypsin	0.87	0.85	0.84	0.82
	chymotrypsin	0.82	0.76	0.80	0.78
0.15 bar	protein	0.54	0.58	0.61	0.65
	trypsin	0.80	0.80	0.81	0.78
	chymotrypsin	0.68	0.69	0.65	0.67

4.4.2 Single-batch concentration crossflow microfiltration

Figure 20 shows the flux during SBC-CFMF. When the TMP was constant at 0.03 bar, although it was lower than critical TMP range measured from total recycle CFMF, the decrease of flux at the initial filtration stage was still observed. The decrease of flux indicated the formation of fouling. Total recycle CFMF based on constant feed property could provide a rough range of operational parameters, such as TMP and flux, but it couldn't be applied to SBC-CFMF directly. During SBC-CFMF, the feed bulk was concentrated in the feed tank, thus, the viscosity and concentration of feed bulk increased continuously during the process and these changes of feed property could change the flow pattern and critical operation condition. For example, it has been demonstrated by a number of researches that critical flux would decrease with increasing solute concentration in the feed (Metsamuuronen *et al.*, 2002; Kwon *et al.*, 2000; Manttari and Nystrom, 2000). Once the concentration process begun, the critical condition with neglectable fouling could be exceeded in an initial short period of filtration, then the fouling occurred. In another operation with a high TMP 0.15 bar which was above the critical TMP range, decrease of flux was more significant. The resistance analysis (Figure 21) indicated that the difference in R_{if} between high TMP and low TMP was not significant and R_{if} was very low comparing to R_{tol}.

Therefore most fouling could be classified to reversible fouling. Because of the low R_{if} and its non-significant change with increasing crossflow velocity, it could be assumed that R_{if} was due to protein adsorption on the membrane surface and internal wall of the membrane pore in a short period. R_{if} couldn't be eliminated while most R_{rf} could be limited by increasing crossflow velocity (Vadi and Rizvi, 2001). In addition, regardless of a higher R_{rf} in high pressure operation, TMP 0.15 bar was still considered for further operation since it provided a high permeate flux. R_{rf} at high TMP could be reduced by introducing other techniques such as gas bubbling to CFMF. On the other hand, although a microfiltration membrane with pore size 0.1 μm was used, the transmission of enzymes was lower than 1. These transmissions were about 0.60 to 0.80 in SBC-CFMF (Table 4). Generally, the transmission of solute would be improved by crossflow since concentration polarization and formation of fouling on membrane surface should be more severe in dead-end microfiltration. Thus the loss of transmissions in SBC-CFMF was due to not only concentration polarization and formation of fouling but also denaturation of enzyme. It was hypothesized that the denaturation of enzyme was induced by the mechanical shear stress when fluid flow was repeatedly pumped and circulated with a long holding time in the SBC-CFMF plant loop (Meireles *et al.*, 1991; Bowen and Gan, 1992). No significant improvement of enzyme transmission with increasing crossflow velocity in SBC-CFMF (Table 4) confirmed the negative effect of mechanical shear stress. A possible method to protect enzyme activity was to reduce the shear stress and the holding time of the feed bulk in the filtration loop. Therefore, a different batch operation named continuous-batch concentration CFMF (CBC-CFMF) was employed with a relatively low crossflow velocity 0.2 m/s.

4.4.3 Continuous-batch concentration crossflow microfiltration without gas bubbling

An operation of CBC-CFMF at crossflow velocity of 0.2 m/s and TMP of 0.15 bar without gas bubbling showed that transmission of enzyme was improved in CBC-CFMF (Table 5). As mentioned above, CBC-CFMF could reduce holding time of each batch of feed bulk in system by adding feed bulk into feed tank continuously during operation. The negative effect of shear stress was reduced by using a low crossflow velocity of 0.2 m/s. Unfortunately, even though TMP of 0.15 bar was applied, CBC-CFMF without gas bubbling provided a low steady

state permeate flux (Figure 22) which was close to the flux in the SBC-CFMF with low TMP of 0.03 bar (Figure 20). The reason was probably that more severe concentration polarization induced by faster increase of feed concentration occurred in CBC-CFMF than SBC-CFMF. The faster increase of concentration was due to the small feed volume in the feed tank during CBC-CFMF.

Table 5. Average transmissions (T_r) of soluble protein and enzymes in CBC-CFMF at circulation flow velocity 0.2 m/s and TMP 0.15 bar.

Gas injection factor	T_r		
r	Protein	Trypsin	Chymotrypsin
0	0.51	0.97	0.92
0.16	0.79	1.01	0.94
0.38	0.81	0.99	0.95
0.56	0.71	0.77	0.62
0.65	0.64	0.54	0.47

4.4.4 Continuous-batch concentration crossflow microfiltration aided by gas bubbling

Gas bubbling has been accepted as a simple and promising method to reduce concentration polarization and external fouling on the membrane surface. The results showed that gas bubbling improved the permeate flux and different *r* gave different effect on flux (Figure 22). Varying *r*

from 0 to 0.65 could improve permeate flux up to 386%. The most significant enhancement of flux was observed at r of 0.38. The flux was increased by 300% with r of 0.38 comparing to the flux without gas injection. An increase of flux was still detected when a higher r of 0.56 was applied. The enhancement of flux with gas could be explained by both shear related enhancement effect and heavily weighted flow reversal effect (Smith and Cui, 2004).

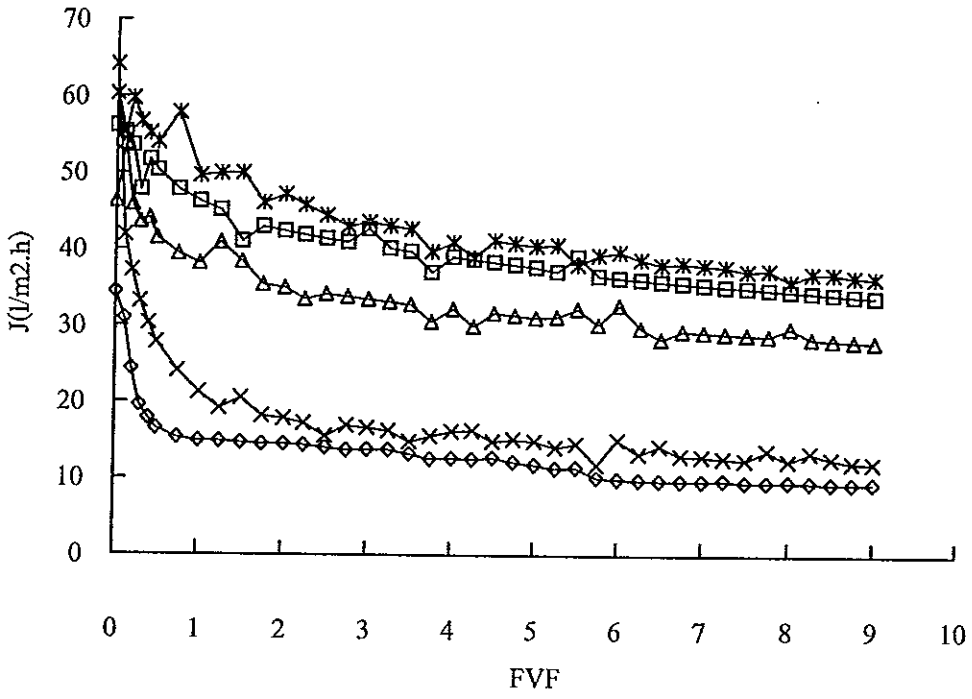

Figure 22. Effect of gas bubbling on the permeate flux in CBC-CFMF at the crossflow velocity of 0.2 m/s and TMP of 0.15 bar (◇ No gas; × r of 0.16; △ r of 0.38; □ r is 0.56; * r of 0.65).

However, no further increase of flux was observed when r was increased from 0.56 to 0.65. It was expected that gas bubbling was more effective in a system where flux decline was dominated by concentration polarization than that coupled with fouling (Li *et al.*, 1998). A

relatively low gas flow successfully limited concentration polarization, leaving less room for further enhancement by higher gas flow. Hence, the lack of enhancement of flux by gas bubbling was seen in the stage of high *r*. on the other hand, the dual flow pattern might be changed with increasing viscosity of feed in a concentration process even though the superficial velocities of gas and liquid were controlled at the fixed values. In the case of relatively low superficial velocity of gas ($\leq$ 2.5 m/s), Furukawa and Fukano (2001) observed that the gas-liquid dual flow pattern did not reach the annular regime regardless of increasing the viscosity of liquid flow to 15 times of that of water. These researchers concluded that higher superficial velocity of gas (i.e. higher gas injection factor) was required for transition from slug flow to annular flow with increasing the viscosity of liquid flow (Furukawa and Fukano, 2001). In our work, the viscosity of feed increased less than 15 times (data not shown) while VCF or FVF was 9 and maximum superficial velocity of gas was just 0.37 m/s. Hence the dual flow pattern was still in slug regime when the viscosity of feed increased in this work.

The effect of gas bubbling on transmission of solutes was also studied. At low *r* ($\leq$ 0.38), the transmission of enzyme was kept close to 1 and transmission of protein was increased while the flow pattern changed from bubble flow to slug flow (Table 5). The reason was clearly that gas bubbling reduced concentration polarization and formation of fouling layer on the membrane surface. But a drastic decrease of transmissions of both enzyme and protein occurred when *r* was higher than 0.38 even though higher flux was still attained at these *r*. It was supposed that an increase in wall shear stress due to high gas injection factor decreased the cake thickness and porosity by preferential removal of larger particles away from the membrane, then caused a formation of fouling layer thinner but more tightly packed (Mercier-Benin and Fonadeb, 2002). This tighter and less porous layer prevented the passage of solutes. In addition, the local mixing generated by gas bubbling transported the solutes rejected by membrane back to the bulk solution, then decreased the local concentration near membrane surface (Li *et al.*, 1997b). Decreasing local concentration reduced the diffusion driving force which was one of the driving forces for solutes to pass through the membrane. Once reduction of the diffusion driving force by increasing *r* achieved a certain level, the decrease of transmission of solutes was seen. It should be noted that shear stress generated by gas bubbling not only provided an enhancement to permeate flux but also gave a negative effect on enzyme activity once it achieved a certain level.

Thus, the decrease of transmission of enzyme at high r was due to the loss of enzyme activity as well. When r of 0.56 and 0.65 were applied, a significant losses of enzyme activity in both permeate and feed bulk in the tank was observed while neglectable loss of enzyme activity occurred at low r ($\leq$ 0.38). The loss of enzyme activity caused a poor yield of enzyme even though an apparent transmission could be still calculated. Considering both maintaining a high permeate flux and protecting enzyme activity, the optimal gas injection factor (r) in this given system was about 0.38 which indicated a low gas velocity in the region of slug flow. Furthermore, once concentration polarization and formation of fouling layer on the membrane surface were limited by gas bubbling technique, the major factors affecting transmissions of protein and enzymes were protein aggregation and maintenance of enzyme activity which were mainly dominated by hydrodynamic conditions (i.e. TMP and liquid flow velocity, gas flow velocity). Therefore, the variation of transmissions as the function of time was not significant during CBC-CFMF with fixed hydrodynamic conditions.

After CBC-CFMF, a clear permeate with slight yellow colour was obtained. The size of solid particles possibly presenting in this permeate was below the capacity of the Laser Particle Size Analyzer used in this study, thus made a failed measurement. Considering the precision of 0.04 μm of this analyzer, it could be concluded that all suspended solids were removed after CBC-CFMF. Since the transmissions of about 1 for both trypsin and chymotrypsin could be attained in CBC-CFMF while the crossflow velocity of 0.2 m/s, the TMP of 0.15 bar and r of 0.38 were applied, a yield of 90% for trypsin and chymotrypsin could be achieved when FVF was 9.

4.5 Conclusion

Microfiltration could be applied to remove suspended solids from yellowfin tuna spleen extract successfully. After investigation of critical TMP range in total recycle CFMF, SBC-CFMF and CBC-CFMF were applied to remove suspended solids from yellowfin tuna spleen extract. Most fouling in SBC-CFMF was classified to be reversible. In order to improve permeate flux and reduce the negative effect on enzyme activity by shear induced denaturation and long holding time, CBC-CFMF operation combined with gas bubbling was designed. A gas

injection factor of 0.38 was found to be optimal while CBC-CFMF was performed at TMP of 0.15 and crossflow velocity of 0.2 m/s in this study. All visible solids in yellowfin tuna spleen extract were removed by CBC-CFMF with given conditions in this study. The clear permeate from CBC-CFMF would be used as feed in further UF process to recover and purify trypsin and chymotrypsin.

CHAPTER 5

SEPARATION OF PROTEASE FROM YELLOWFIN TUNA SPLEEN EXTRACT BY ULTRAFILTRATION: EFFECT OF HYDRODYNAMICS AND GAS SPARGING ON FLUX ENHANCEMENT AND SELECTIVITY

5.1 Abstract

The pretreated yellowfin tuna spleen extract was used as feed in ultrafiltration for recovery of protease. The combined impact of crossflow rate and gas sparging on critical flux, limiting flux and selectivity was studied by a total recycle mode using a hollow fiber membrane with molecular weight cut-off 30 kDa. The critical flux varied from 28.8 to 44.2 l/m^2.h and limiting flux varied from 34.3 to 52.4 l/m^2.h while crossflow rate increased from 17.55 to 69.98 l/h without gas sparging. A low gas injection factor of 0.15 could improve critical and limiting flux significantly comparing to that without gas sparging. Higher gas injection factors varied from 0.30 to 0.61 did not give remarkable improvement of both critical and limiting flux. The benefit of increasing crossflow rate to enhance critical and limiting flux was great when gas sparging was not applied. Selectivity of ultrafiltration process was increased with increasing permeate flux at sub-critical condition and critical flux condition. It became insensitive to the flux and crossflow rate at limiting flux condition when gas sparging was not applied. Gas sparging gave negative effect on soluble protein and peptide transmission and resulted in the decline of selectivity at sub-critical condition and critical flux condition. The selectivity at limiting flux condition with gas sparging was not sensitive to gas injection factor.

5.2 Introduction

Protease is an important commercial enzyme, which is widely used in the food industry, such as protein removal from bones, protein hydrolysate production, meat tenderization, clarification, and fermentation (Haard, 1998). The applications of enzyme have demanded economical, efficient and large-scale enzyme purification techniques. Ultrafiltration process is a

cost-effective method giving a high productivity and reasonable product purity. Ultrafiltration process is also easier to scale-up in comparison to other techniques such as chromatography and electrophoresis. In recent years, ultrafiltration has been used for separation of a variety of compounds, such as lysozyme from chicken egg white (Ghosh and Cui, 2000), proteases from surimi wash water (Dewitt and Morrissey, 2002a), native protein from potato fruit juice (Zwijnenberg *et al.*, 2002) and plasma proteins from chicken blood (Torres *et al.*, 2002). On the other hand, the demand of fishery product has increased significantly in recent years. As one of the largest exporters of canned tuna in the world, Thailand produces more than 311,070 tons of canned tuna annually (National Food Institute, 2005). Since the major part of aquatic animal for fishery processing is only meat, a large amount of wastes has been generated from the fishery industry. In tuna canning process, there are 23-25% solid waste (e.g. head, skin, viscera) and about 35% liquid waste (e.g. blood, tuna condensate, oil) (Prasertsan *et al.*, 1988). The utilization of fishery waste has caught more attention. In previous studies, the protease can be recovered from yellowfin tuna spleen by ultrafiltration. But severe membrane fouling was observed (Li *et al.*, 2006).

It is well known that concentration polarization and fouling are the major problems causing the functional decay of membrane separation process. For maintaining a sustainable operation with negligible fouling and reducing total cost of process, such as purchasing membrane, energy and cleaning, the critical flux has been introduced and investigated by a number of researchers (Howell, 1995; Espinasse *et al.*, 2002; Howell *et al.*, 2004). The critical flux is defined as the flux below which membrane fouling is negligible. It could be measured by a 'step by step' technique in which flux with increasing transmembrane pressure (TMP) was monitored (Chiu and James, 2005). There are two types of critical flux. The strong form of critical flux exists if the flux of a suspension is identical to the flux of clean water at the same TMP. The weak form of critical flux exists if the relationship between TMP and flux is linear, but the slope of the line is lower than that for clean water (Wu *et al.*, 1999). Besides critical flux, limiting flux is related to TMP as well. Limiting flux is the maximum stationary flux obtained with increasing TMP. Critical flux is a criterion for initial formation of fouling whereas limiting flux is reached when the whole membrane surface operates above the critical flux, i.e. when a further increase in flux at any point on the membrane surface leads to another layer

deposit fully compensating the increased TMP (Bacchin, 2004). Both critical flux and limiting flux therefore are physically related to the formation of concentration polarization and membrane fouling depending on numerous factors, such as feed property and hydrodynamic conditions. On the other hand, gas sparging is a novel hydrodynamic method to enhance membrane process by introducing gas into module to promote local mixing near the membrane surface. It was observed that injection of gas increased the permeate flux by 7% to 50% in HSA and IgG ultrafiltration process with flat sheet membrane (Li *et al.*, 1998). A 75% increase in permeate flux for microfiltration of yeast using a polymeric membrane module with gas injection was also observed (Cui *et al.*, 2003). Comparing to other methods, gas sparging is simple and economical. Gas bubbles pose less risk to the membrane and are easily separated from the process stream. According to its advantages, gas sparging technique is expected to be helpful for improvement of critical flux and limiting flux.

The aim of present work is to study the combined impact of hydrodynamics (i.e. crossflow rate) and gas sparging on critical flux, limiting flux and selectivity during recovery of proteases from yellowfin tuna spleen extract by ultrafiltration.

5.3 Materials and methods

5.3.1 Preparation of tuna spleen extract

The yellowfin tuna spleen was provided by Chotiwat Manufacturing Co. Ltd (Thailand), Hat Yai. The extract was prepared by homogenizing yellowfin tuna spleen with Tris-HCl buffer (Li *et al.*, 2006) and simply filtered through nylon net, cotton pad and cloth in series to remove large particles, then the extract was pretreated by a microfiltration process using a hollow fiber membrane with pore size 0.10 μm (CFP-1-E-5A, Amersham Biosciences, UK) at crossflow rate 96 L/h and TMP 0.15 bar. The permeate from microfiltration process was collected and used as feed.

5.3.2 Ultrafiltration process setup

gauge (2419-2C-P, CKD, Japan). The gas-liquid two-phase flow pattern corresponds to the gas injection factor (r) which equals $U_g / (U_g + U_l)$. U_g and U_l are the superficial gas and liquid flow velocity, respectively (Chiu and James, 2006). The main generally reported gas-liquid two-phase flow pattern are bubble flow ($r < 0.25$), slug flow ($0.25 < r < 0.9$) and annular flow ($r > 0.9$) (Cabassaud *et al.*, 2001). Gas injection factors of 0, 0.15, 0.30, 0.46, and 0.61 were applied in this study. J_{crit} and J_{lim} during ultrafiltration aided by gas sparging were determined by the 'step by step' method mentioned above. The feed tank was designed as a liquid-gas separator. In order to minimize foaming problems, a nylon net was put in the feed tank to extend the holding time of the foam in the tank and allow the transformation of the foam to liquid in the tank.

5.3.5 Determination of selectivity during ultrafiltration

The selectivity was expressed by the equations from Ghosh and Cui (2000). The transmission of partially rejected solute during ultrafiltration process was expressed by the apparent sieving coefficient (S_a):

$$S_a = \frac{C_p}{C_b} \tag{28}$$

where C_p is solute concentration in permeate and C_b is solute concentration in the bulk feed.

The efficiency of solute fractionation was expressed by selectivity (Ψ) which was calculated as:

$$\Psi = \frac{S_{a1}}{S_{a2}} \tag{29}$$

where 1 stands for the preferentially transmitted solute, i.e. soluble protein and 2 stands for the preferentially retained solute, i.e. protease.

Selectivity was detected at three different conditions including sub-critical condition (sampling when flux was lower than the critical flux), critical flux condition (sampling when the critical flux was reached) and limiting flux condition (sampling when the limiting flux was reached).

5.3.6 Analytical methods

Major compositions of the feed were analyzed by the methods of AOAC. Soluble protein concentration in the permeate and feed was measured by the Lowry method. Since the best detection range of protein concentration by Lowry method is 5 to 100 μg/ml (Yaobo, 2002), all samples were appropriately diluted before detection by Lowry method. Protease activity was measured by using casein as substrate at 50°C and pH 8.0. One unit of protease activity was expressed as the amount of enzyme that liberated 1μg of tyrosine equivalent in 1 minute (Li *et al.*, 2006).

5.4. Results and discussion

Microfiltration following simple filtration was applied to pretreat the raw extract. The permeate from microfiltration was used as feed in this study. This feed was clear and showed slight yellow colour. The major compositions analyzed in this feed were protein (8.72 mg/ml), protease (54.85 U/ml), salt (0.89 mg/ml) and crude fat (5.72 mg/ml).

5.4.1 Critical flux and limiting flux pattern during ultrafiltration without gas sparging

During determination of J_{crit} and J_{lim} by the 'step by step' method, the permeate flux was constant over the course of the time before J_{crit} was reached and the relation between permeate flux and TMP was linear. Once J_{crit} was reached, the permeate flux became unstable and decreased with time, leading to a non-linearity in the permeate flux versus TMP. Finally a stationary flux with increasing TMP, i.e. J_{lim} was reached.

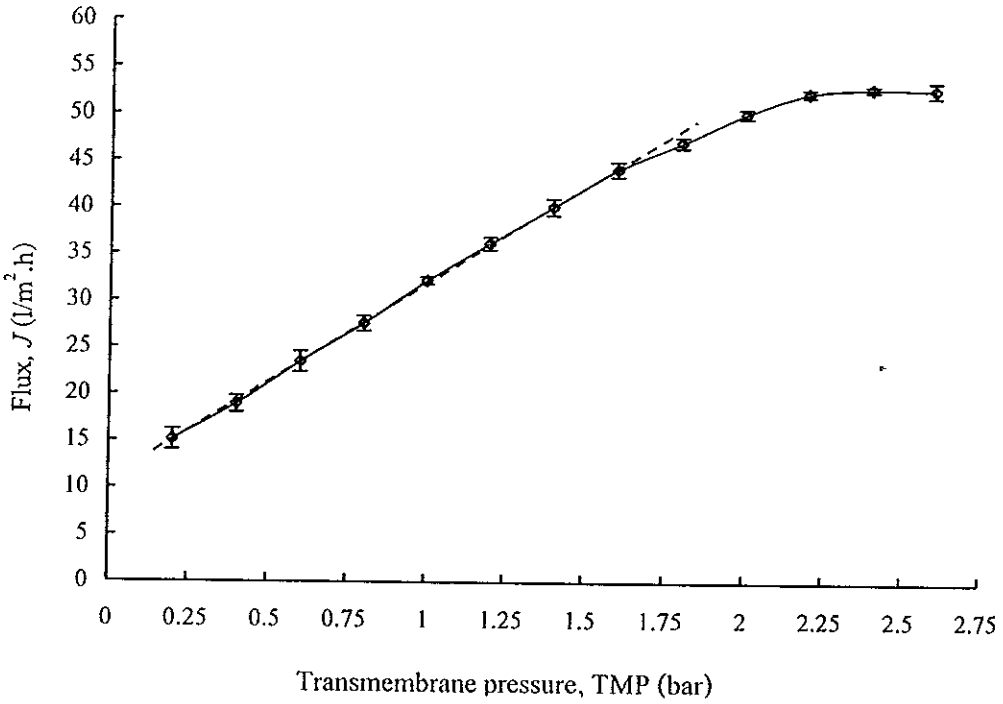

Figure 23. Determination of critical flux (J_{crit}) and limiting flux (J_{lim}) by the 'step by step' method at crossflow rate of 69.98 l/h during UF without gas sparging.

Figure 23 exemplified the determination of J_{crit} and J_{lim} at crossflow rate of 69.98 L/h without gas sparging. Other J_{crit} and J_{lim} were measured by the same method at different crossflow rate. All critical fluxes in this study were weak form. The effect of crossflow rate on J_{crit} and J_{lim} in the operations without gas sparging was illustrated in Figure 24. It was observed that increasing crossflow rate enhanced J_{crit} and J_{lim}. J_{crit} varied from 28.8 to 44.2 L/m^2.h while crossflow rate increased from 17.55 to 69.98 L/h. J_{lim} varied from 34.3 to 52.4 L/m^2.h at same operation conditions. Increasing crossflow rate would enhance wall shear stress on the membrane surface. Higher wall shear stress was helpful to reduce concentration polarization and external fouling on the membrane, thus improved both J_{crit} and J_{lim}. In addition, the values of J_{crit} and J_{lim} were close in this work. J_{crit} was about 80% of J_{lim}. The feed property was considered as the possible reason for this observation. Since the feed was pretreated by simple filtration and microfiltration. Most suspended particle and a part of protein and other large molecules were

removed. The proteins with relatively small molecular weight were the major foulants during ultrafiltration process. On the other hand, the attraction between protein and a surface increases with increasing hydrophobicity of the surface (Lu and Park, 1991; Metsaimuuronen and Nystrom, 2005). Polysulphone membrane used in this study is more hydrophobic in nature and therefore the tendency of proteins on membrane to form fouling is supposed to be more severe than other membranes. Once flux exceeded J_{crit}, i.e. the balance between flux which governs convective mass transport to the membrane and crossflow which rules the transport by erosion to diffuse molecules back from the membrane towards the fluid bulk was broken, these proteins could block the membrane pores and form gel layer onto membrane rapidly and limited the flux. Increasing TMP could accelerate the formation of these fouling. Hence the duration of transfer between J_{crit} and J_{lim} was short and the values of J_{crit} and J_{lim} were close.

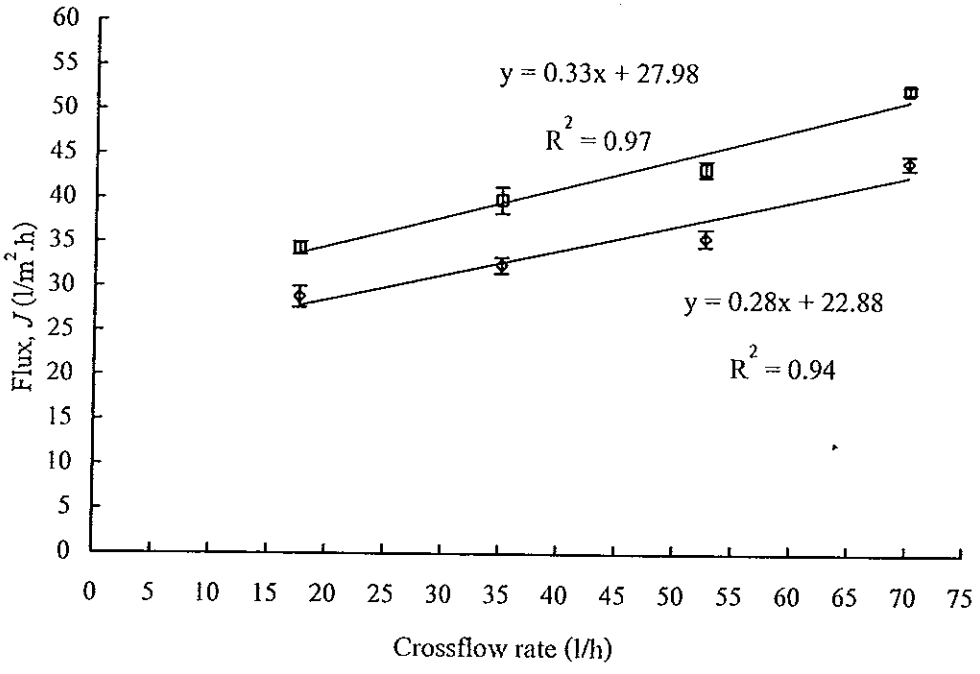

Figure 24. Effect of crossflow rate on critical flux (J_{crit}, ◇) and limiting flux (J_{lim}, □) during UF without gas sparging.

5.4.2 Effect of gas sparging and crossflow rate on critical flux and limiting flux

When the gas was injected into the system, the foam was found in the outflow of retentate and accumulated in the tank. The amount of foam was not considerable and feed tank worked as a gas-liquid separator successfully. The reason was probably that the feed was pretreated by simple filtration and microfiltration. Most suspended solids and a part of protein were removed. No significant effect of foam on protease was observed by monitoring protease activity in the tank. Through whole process, the protease activity in the tank was close to the original activity (i.e. 54.85 U/ml). The gas injection factor of 0.15 to 0.61 indicated gas-liquid two-phase flow pattern varying from bubble flow to slug flow according to the generally reported gas-liquid two-phase patterns (Chiu and James, 2006). However, since the small inner diameter (1 mm in this study) of hollow fiber, the capillary effect may modify the flow. It has been proved that the gas-liquid two-phase flow pattern was only slug flow when gas injection factor varies from 0.17 to 0.67 in tubes with inner diameter varying between 1 and 4 mm (Laborie *et al.*, 1999). Similar phenomena have been reported in some other works (Cheng and Lin, 2001). Therefore, all gas-liquid two-phase flow patterns in this study were considered as slug flow even though gas injection factor was lower than 0.25. The enhancement of flux with gas could be explained by both shear related enhancement effect and heavily weighted flow reversal effect (Smith and Cui, 2004). Fouling including adsorption and deposition of solutes onto membrane, particularly in or around the pore is linked to concentration polarization. The greater concentration polarization, the greater fouling (Ghosh, 2006). Bubble-induced secondary flows play an important role by promoting local mixing in the bubble wake to minimize accumulation of solutes and molecules on the membrane (Cui and Wright, 1996). Hence, gas sparging could be applied as a simple method to reduce membrane fouling. For slug flow in hollow fiber membrane systems, physical displacement of the mass-transfer boundary layer and high shear stresses are thought to be the main reasons for the flux improvement (Muriel *et al.*, 2000). Figure 25, 26 and 27 illustrated the combined influence of crossflow rate and gas injection on J_{crit}, J_{lim} and the ratio of J_{crit} to J_{lim}, i.e. J_{crit}/J_{lim}. The vertical dotted line in Figure 25, 26 and 27 indicated the operation conditions (i.e. gas injection factor and crossflow rate). It was found that the enhancement of both J_{crit} and J_{lim} was more significant at low gas injection factor and gas injection was more effective at low

crossflow rate. For example, J_{crit} was increased 69% (from 28.8 to 48.8 l/m^2.h) while gas injector factor was varied from 0 to 0.15 at crossflow rate of 17.55 l/h. This improvement was 10% (from 48.8 to only 53.7 l/m^2.h) with gas injector factor varied from 0.15 to 0.30 at the same crossflow rate. J_{crit} varied from 44.2 to 57.8 l/m^2.h (i.e. 31% increase) at crossflow rate of 69.98 l/h when gas injection factor varied from 0 to 0.15. The reason was probably that gas sparging was more effective in a system where flux decline was dominated by concentration polarization than that coupled with fouling (Bacchin, 2004). In addition, for controlled gas sparged ultrafiltration process, it was found that gas flow rate required for substantial improvements in permeate flux was very small (Taha *et al.*, 2006). A relatively low gas flow already successfully limited concentration polarization, leaving less room for further enhancement by higher gas flow. Higher gas injection factor may not provide advantageous over lower gas injection factor in enhancing flux. Hence gas injection factor varied from 0.30 to 0.61 did not show the significant enhancement on both J_{crit} and J_{lim} in this study.

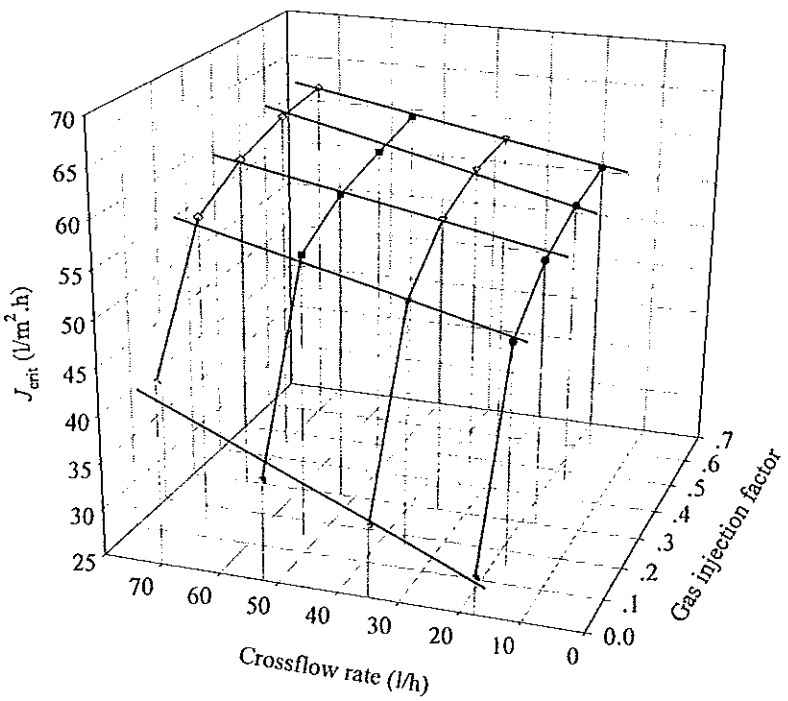

Figure 25. Effect of gas injection factor on critical flux, J_{crit} (●, crossflow rate 17.55 l/h; ▽, crossflow rate 34.99 l/h; ■, crossflow rate 52.49 l/h; ◇, crossflow rate 69.98 l/h).

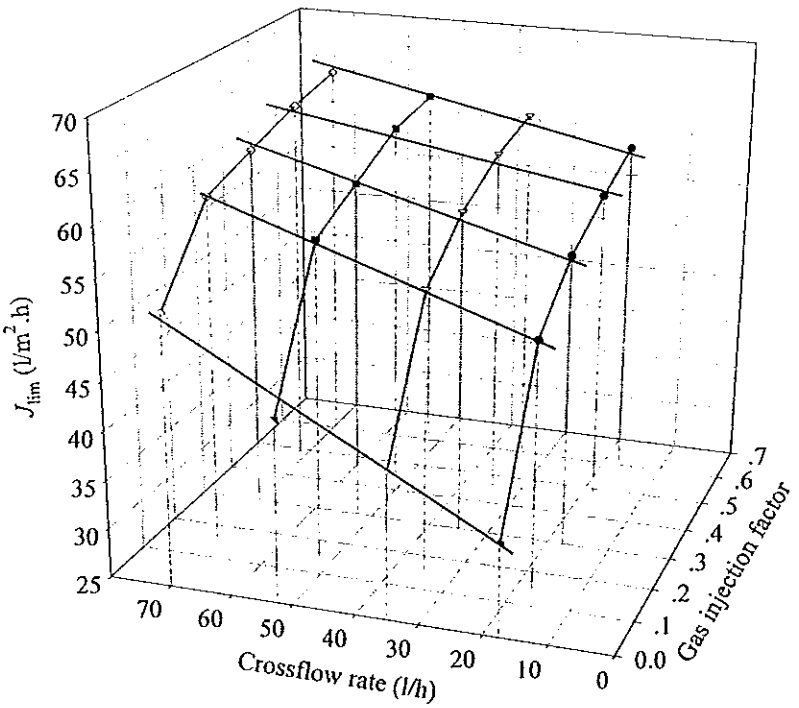

Figure 26. Effect of gas injection factor on limiting flux, J_{lim} (●, crossflow rate 17.55 l/h; ▽, crossflow rate 34.99 l/h; ■, crossflow rate 52.49 l/h; ◇, crossflow rate 69.98 l/h).

On the other hand, the benefit of increasing crossflow rate to enhance J_{crit} and J_{lim} was greater at the conditions without gas sparging. For example, when crossflow rate was increased from 17.55 to 69.98 l/h, enhancement of J_{crit} was 53% (from 28.8 to 44.2 l/m^2.h), 18% (from 48.8 to57.8 l/m^2.h) and 10% (from 57.3 to 63.2 l/m^2.h) at gas injection factor of 0, 0.15 and 0.61, respectively. Similar results were obtained from detection of J_{lim}. It has been found that liquid flow velocity at membrane surface has less effect on permeate flux during gas sparging (Cui and Wright, 1994; Cui *et al.*, 1997).

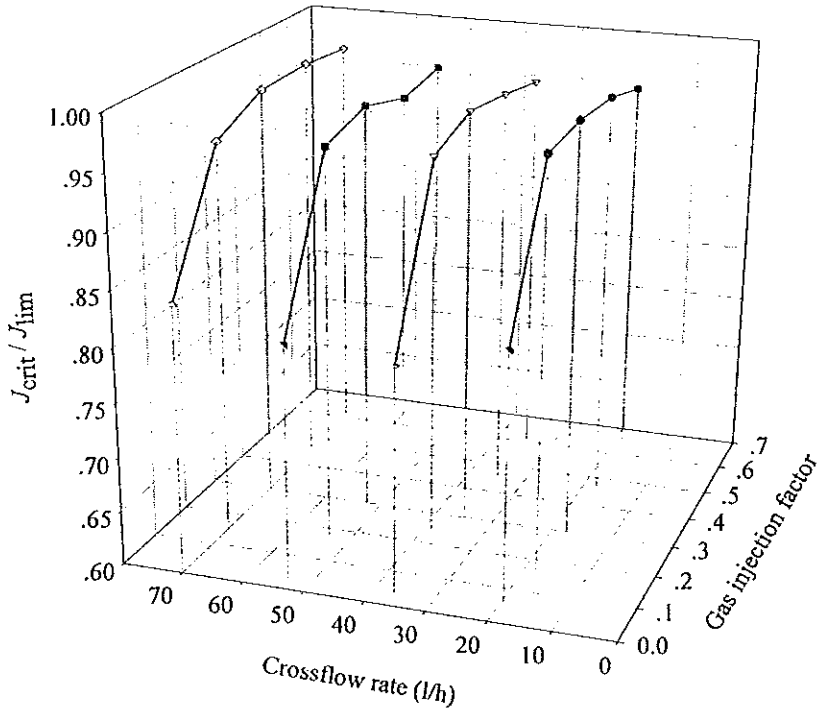

Figure 27. Effect of gas injection factor on ratio of critical flux to limiting flux (J_{crit}/J_{lim}) (●, crossflow rate 17.55 l/h; ▽, crossflow rate 34.99 l/h; ■, crossflow rate 52.49 l/h; ◇, crossflow rate 69.98 l/h).

Both J_{crit} and J_{lim} presented linear relation to crossflow rate. It has been concluded that an increase in the strength of the hydrodynamics is synonymous with a critical flux rise. The reason is probably the distribution of critical fluxes (and then of fouling) along a membrane surface (Bacchin *et al.*, 2006). Similar observations have been reported. For example a linear variation of critical flux and wall shear stress was shown from the experimental results on ultrafiltration of skimmed milk (Gesan-Guiziou *et al.*, 1999). The relation between J_{crit}, J_{lim} and crossflow rate was demonstrated in Table 6.

Table 6. Effect of gas injection factor (r) on the relation between critical flux (J_{crit}), limiting flux (J_{lim}) and crossflow rate (Q).

r	J_{crit} vs. Q	J_{lim} vs. Q
0	$J_{crit} = 0.28Q + 22.88$ ($R^2 = 0.94$)	$J_{lim} = 0.33Q + 27.98$ ($R^2 = 0.97$)
0.15	$J_{crit} = 0.17Q + 45.74$ ($R^2 = 0.99$)	$J_{lim} = 0.20Q + 46.74$ ($R^2 = 0.99$)
0.30	$J_{crit} = 0.13Q + 51.69$ ($R^2 = 0.98$)	$J_{lim} = 0.14Q + 52.89$ ($R^2 = 0.98$)
0.46	$J_{crit} = 0.12Q + 54.29$ ($R^2 = 0.96$)	$J_{lim} = 0.12Q + 56.09$ ($R^2 = 0.99$)
0.61	$J_{crit} = 0.11Q + 55.44$ ($R^2 = 0.99$)	$J_{lim} = 0.11Q + 58.31$ ($R^2 = 0.97$)

Injection of gas did not change the linear relation between J_{crit}, J_{lim} and crossflow rate. Gas could alter the slopes of these linear lines. It was observed that the slopes of the lines with gas were lower than those without gas (i.e. from 0.28 to 0.11 for J_{crit} in Figure 25 and from 0.33 to 0.11 for J_{lim} in Figure 26 at gas injection factor varying from 0 to 0.61). There was an intersection between each line from operation with gas and the line from operation without gas at higher crossflow rate. Exceeding this intersection, flux aided by gas sparging would be lower than

flux without gas sparging. Gas would provide negative effect on J_{crit} and J_{lim}. Since gas flow rate should be increased with increasing crossflow rate to keep a constant gas injection factor. It could be supposed that there is a critical gas flow rate at a given gas injection factor. Above this critical gas flow rate, gas might show negative effect on flux. It was hypothesized that gas injection could take both positive and negative effect on flux. Bubble could increase turbulence leading to flux enhancement. However, too much injected gas could decrease effective membrane area because of replacing liquid mass by bubble contacting to membrane surface (Mi-Jung *et al.*, 2001). This hypothesis may not applicable for given membrane filtration system in this study since the hollow fiber membrane required operations in laminar flow region which limited crossflow rate. However, it should be proved by further works and might be valuable for the membrane module (such as tubular membrane) with large operation range from laminar flow to turbulent flow.

Referring to J_{crit}/J_{lim}, gas injection factor of 0.15 gave the strongest effect. Higher gas injection factors did not show advantages. Gas injection factor of 0.15 indicated a gas-liquid two-phase flow pattern as slug flow in this study. In slug flow, most of gas is located in large bullet shaped bubbles which have a diameter almost equal to the pipe diameter (Yehuda *et al.*, 1980). The local turbulence generated by intermittent succession of Taylor bubbles and liquid slug could delay the formation of concentration polarization and partial external fouling layer and enhanced J_{crit} consequently. However, both external fouling (e.g. cake layer and gel layer) and internal fouling (e.g. pore blocking) dominated J_{lim}. Gas injection only partially reduced external fouling and showed less benefit on enhancement of J_{lim}. Therefore J_{crit}/J_{lim} increased by operation with a low gas injection factor. Higher gas injection factors, as mentioned above, could not improve flux significantly. Change of J_{crit}/J_{lim} with changing gas injection factor was not seen at higher gas injection factor region ($r \geq 0.30$). From the results of Figure 27, it was observed that crossflow rate did not affect J_{crit}/J_{lim}. For the operation without gas sparging, increasing crossflow rate enhanced J_{crit} and J_{lim} simultaneously, thus did not change J_{crit}/J_{lim}. However, the effect of crossflow rate on J_{crit}/J_{lim} was still not seen even the process was aided by gas injection. It could be due to that the secondary flow induced by the bubbles was more dominant within laminar flow region (Cheng *et al.*, 1998). The result agrees with previous work which concluded that permeate flux with gas sparging was insensitive to the actual liquid flow over much of the laminar flow

region (Li *et al.*, 1998). Liquid crossflow rate had little effect on the permeate flux in gas sparged ultrafiltration (Ghosh, 2006).

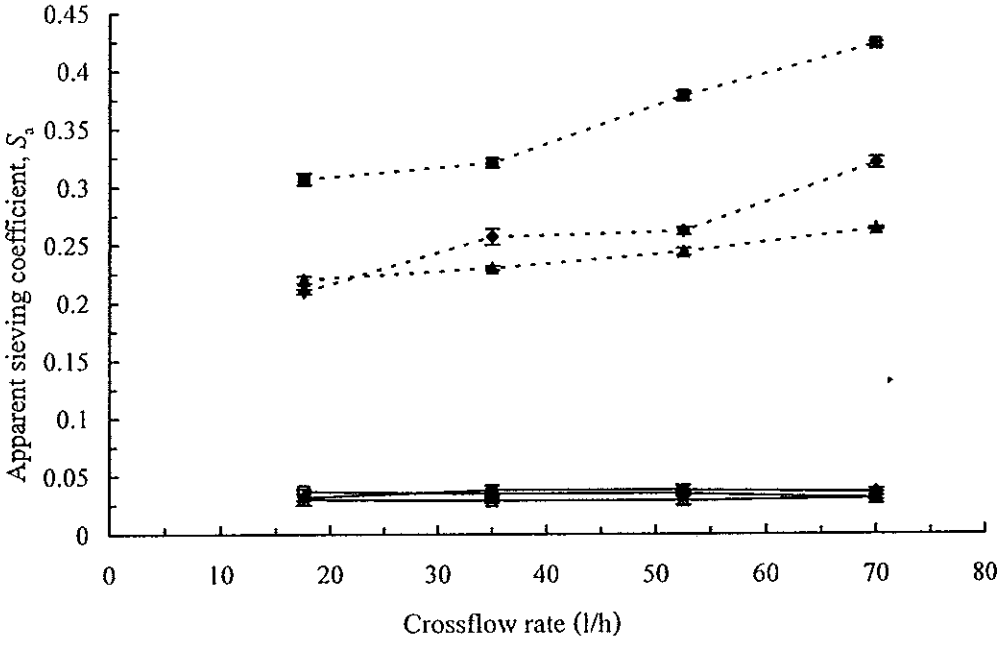

Figure 28. Effect of crossflow rate on apparent sieving coefficient (S_a) during UF without gas bubbling (◇, ◆ sub-critical condition at TMP of 0.8 bar; □,■ critical flux condition; △, ▲ limiting flux condition; - - - - - protein; ——— protease).

5.4.3 Selectivity during ultrafiltration without gas sparging

The selectivity was measured from three operation conditions, i.e. sub-critical condition, critical flux condition and limiting flux condition. Since the lowest corresponding TMP for critical flux was 1.0 bar at crossflow rate of 17.55 l/h, TMP of 0.8 bar was selected as sub-critical condition for all operations with different crossflow rates. Based on Equation (28) and (29), the effect of crossflow rate on the apparent sieving coefficient and selectivity during

ultrafiltration without gas sparging was shown in Figure 28 and 29. It was found that selectivity is proportional to the permeate flux in both sub-critical condition and critical flux condition.

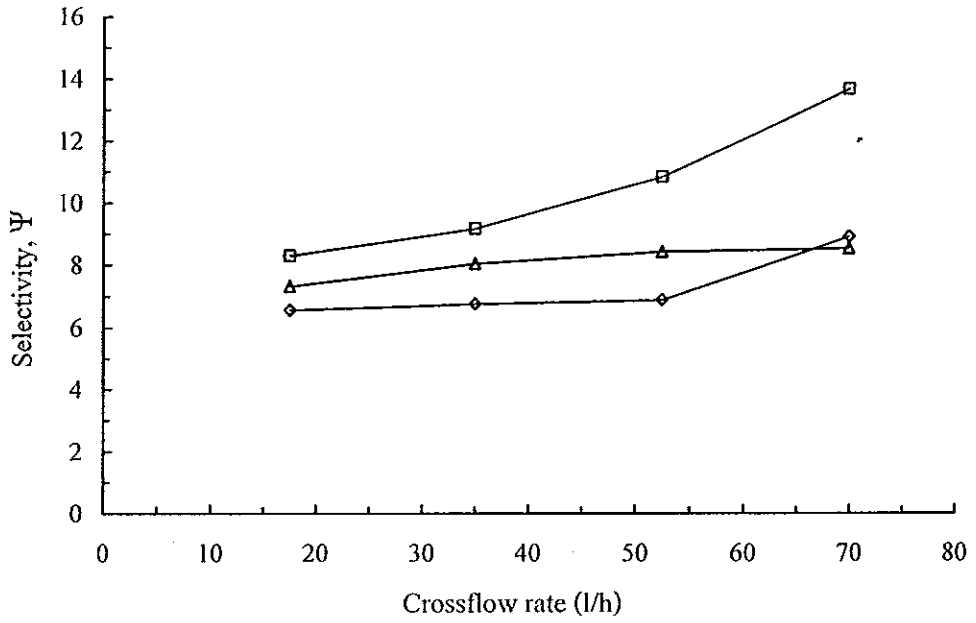

Figure 29. Effect of crossflow rate on selectivity (Ψ) during UF without gas bubbling (◇, sub-critical condition at TMP of 0.8 bar; □, critical flux condition; △, limiting flux condition).

At sub-critical or critical flux condition, severe fouling is negligible. However, TMP drove solution to pass membrane and still made a concentration gradient onto membrane. The higher concentration near membrane surface limited the mass transport. The increase in crossflow rate resulted in a reduction in concentration polarization and leaded to an increase in the permeate flux (Ghosh *et al.*, 2000). Higher permeate flux took more small soluble protein molecules and peptides to permeate while more than 95% protease molecules were rejected by membrane (Figure 28). As a result, the selectivity was promoted. Hence, selectivity was increased while permeate was increased by crossflow rate and higher increase of permeate flux leaded to higher increase of selectivity. However, selectivity was not sensitive to permeate flux or

crossflow rate at limiting flux condition and it was lower than selectivity under critical flux condition even though the limiting flux was higher than critical flux. It was due to the formation of concentration polarization and external gel layer on membrane surface which could prevent the passage of solutes. Both permeate and selectivity was limited by these two factors. Even increasing crossflow rate partially reduced concentration polarization and improved limiting flux, the selectivity was still limited by both external and internal fouling.

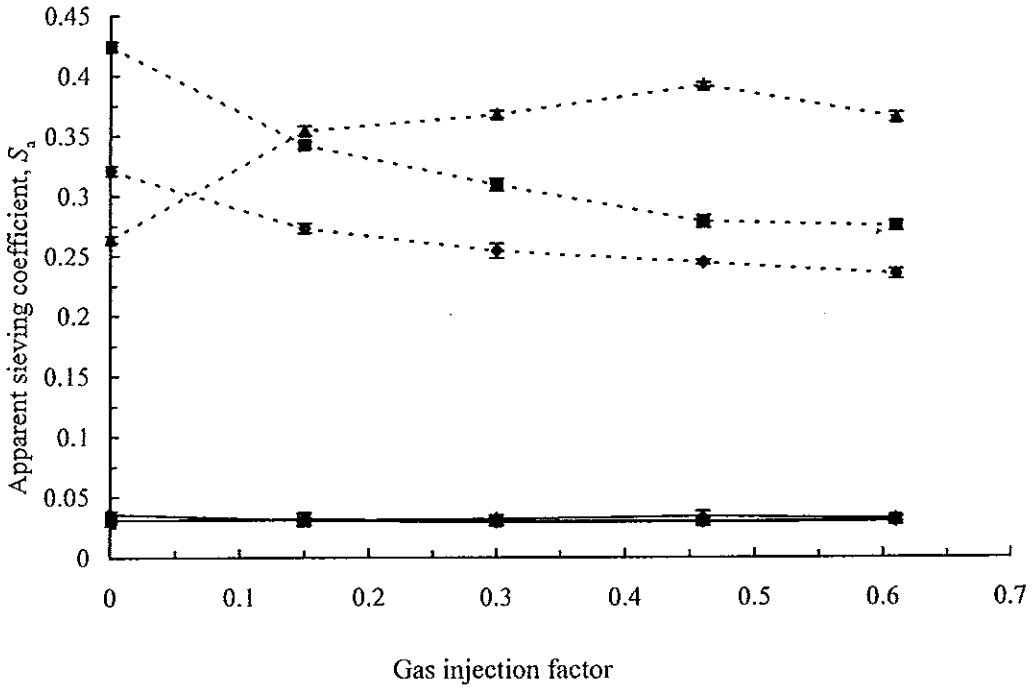

Figure 30. Effect of gas injection factor on apparent sieving coefficient (S_a) during UF with gas sparging at crossflow rate 69.98 L/h (◇, ◆ sub-critical condition at TMP of 0.8 bar; □,■ critical flux condition; △, ▲ limiting flux condition; - - - - - protein; ——— protease.

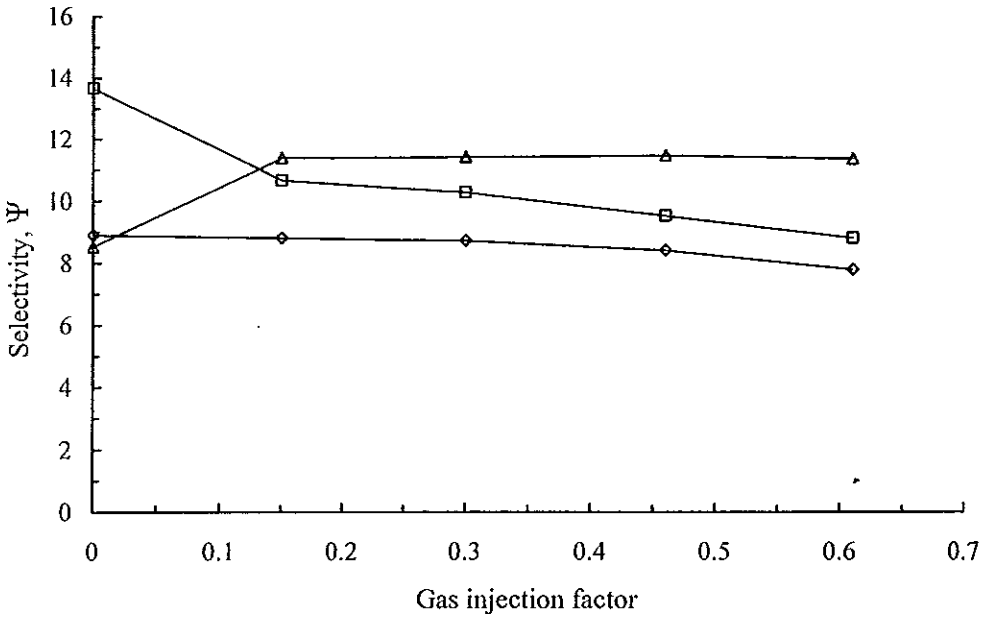

Figure 31. Effect of gas injection factor on selectivity (Ψ) during UF with gas sparging at crossflow rate 69.98 l/h (◇, sub-critical condition at TMP of 0.8 bar; □, critical flux condition; △, limiting flux condition).

5.4.4 Effect of gas sparging on selectivity

Some researchers have been mentioned that the increase of gas flow rate could noticeably deteriorate the selectivity of system regardless of solute concentration in the solution and the upgrading of transport property of ultrafiltration membrane is generally concomitant with the deterioration of selectivity as well (Majewska-Nowak *et al.*, 1999). In this study, Sub-critical condition (at TMP 0.8 bar), critical flux condition and limiting flux condition were applied to determine the apparent sieving coefficient and selectivity during operation with gas sparging as well. A fixed crossflow rate of 69.98 l/h was applied while gas injector factor was varied from 0 to 0.61. Results were shown in Figure 30 and 31. It was indicated that selectivity decreased with increasing gas injection factor at sub-critical condition and critical flux condition although fouling was negligible at these conditions (Figure 31). The reason was considered as reduction of soluble protein and peptide transmission due to gas sparging (Figure 30). It has been observed that gas

sparging can effectively reduce protein transmission with or without fouling (Li *et al.*, 1998). The enhanced local mixing near the membrane surface and secondary flow generated by gas sparging drove protein molecules back to feed bulk solution, decreased protein concentration at membrane wall, then reduced protein molecules transmission (Li *et al.*, 1997a). This reduction of membrane wall concentration is independent of membrane fouling (Li *et al.*, 1998). While the most target enzyme (i.e. protease) was barred by membrane, the decrease of transmission of other soluble proteins and peptides caused the decay of selectivity. The selectivity at limiting flux condition with gas sparging operation was not sensitive to gas injection factor and it was higher than selectivity at limiting flux condition without gas sparging. The reason for initial increase of selectivity with gas injection factor varying from 0 to 0.15 is probably that gas sparging helped to eliminate concentration polarization, thus enhanced limiting flux and mass transportation. However, under limiting flux condition, the fouling occurred. In this study, according to the feed property, gel layer and internal fouling of protein were considered as major factors dominating transportation during ultrafiltration process. Gas-liquid two-phase flow could not able to completely disrupt deposited fouling which was created by protein with strong physico-chemical and mechanical interaction, even with increasing proportions of gas injection (Mercier-Bonin *et al.*, 2000). Therefore the significant improvement on selectivity at limiting flux condition by increasing gas injection factor could not be observed.

5.5 Conclusion

Ultrafiltration was applied for recovery of protease from yellowfin tuna spleen extract. The effects of crossflow rate and gas sparging on critical flux, limiting flux and selectivity were studied. The crossflow rate showed positive effect on critical flux and limiting flux since it could enhance wall shear stress then reduce concentration polarization and external fouling on membrane. The critical flux varied from 28.8 to 44.2 $l/m^2.h$ and limiting flux varied from 34.3 to 52.4 $l/m^2.h$ while crossflow rate increased from 17.55 to 69.98 l/h without gas sparging. A low gas injection factor of 0.15 could enhance both critical flux and limiting flux

significantly. The enhancement of critical flux and limiting flux by gas sparging was more significant when low gas injection factor was applied with a low crossflow rate. Under sub-critical condition and critical flux condition without gas sparging, selectivity was enhanced by increasing crossflow rate since soluble protein and peptide transmission was promoted. However gas sparging gave negative effect on selectivity at sub-critical condition and critical flux condition. The reason was due to reduce of soluble protein and peptide transmission caused by bubble. At limiting flux condition, gas injection factor of 0.15 increased selectivity while crossflow rate and other gas injection factors showed little effect on selectivity.

CHAPTER 6

PURIFICATION OF PROTEASE FROM PRE-TREATED TUNA SPLEEN EXTRACT BY DIAFILTRATION: AN ALTERED OPERATIONAL MODE INVOLVING CRITICAL FLUX CONDITION

6.1 Abstract

Diafiltrations by altered operational modes were applied to improve protease purity during ultrafiltration of pre-treated tuna spleen extract. The performance of mode 1 (pre-diafiltration followed by post-concentration) and mode 2 (pre-concentration followed by post-diafiltration) were compared. A critical flux operation was involved in mode 1. Severe fouling was avoid in mode 1 while12-fold purification of protease was achieved after initial diafiltration volume of 3 and final volume concentration factor of 2 with a total operational time of about 4 hours. The conventional operation, i.e. mode 2 provided 2-fold purification of protease after initial volume concentration factor of 2 and final diafiltration volume of 3. Fouling was much more severe in mode 2 than those in mode 1. Consequently low flux caused by fouling led to a long operational time of about 9 hours in mode 2. The internal membrane fouling was due to adsorption of molecules onto membrane pore wall in both mode 1 and mode 2. The difference of resistance between mode 1 and mode 2 was mainly due to concentration polarization and external membrane fouling. It was proved in this work that operation of diafiltration with critical flux concept could provide more efficient purifying process than conventional diafiltration operation. The reduction of fouling could not only improve process efficiency and protease purity but also possibly reduce further cost of membrane cleaning.

6.2 Introduction

Membrane filtration process is an approach to separate different solutes by semi-permeable membranes which allow the passage of one or more of the solutes much more readily than the others. Pressure normally plays a role as driven force. According to its separation

capability varying from below10 kDa to about 1,000 kDa (Brummer and Gunzer, 1987), ultrafiltration has been widely applied for protein processing, such as whey fractionation (Zydney, 1998), concentration of blood plasma proteins (Torres *et al.*, 2002) and recovery of protein from poultry processing wastewater (Lo *et al.*, 2005).

In order to obtain a product with desirable purity, a specialized operation of membrane filtration, called diafiltration has been applied (Wang *et al.*, 2002; Henderson *et al.*, 2004; Li *et al.*, 2006; Molinari *et al.*, 2008). In diafiltration, the feed volume is kept constantly by adding fresh solvent (e.g. water or buffer). Feed is diluted with added solvent to reduce the concentration of permeable components and remove them by passing through membrane. Therefore, the purity of retained components could be further increased. The overall process of diafiltration generally includes three steps, i.e. a pre-concentration step, a diafiltration step and a post-concentration step (Dutre, and Tragardh, 1994). The main purification process is performed by diafiltration step. The major consideration of applying two steps of concentration is probably that the pre-concentration step may reduce the feed volume, then save solvent volume used in the diafiltration and the post-concentration could yield a desirable concentration of final product. However, due to the fouling potential of feed solution and increased concentration of solute, membrane may be severely fouled after the pre-concentration step. Consequently, the performance of diafiltration and post-concentration could be limited by the membrane fouling. Severe fouling may also increase the cost of membrane cleaning and reduce the membrane lifetime.

Reducing fouling is always a major challenge for the applications of membrane filtration. A number of methods have been investigated. The concept of critical flux firstly introduced by Field *et al.* (1995) is a hydrodynamic manipulation method to avoid severe fouling onto membrane. The applications of critical flux theory during membrane filtration have been reviewed by Pollice *et al.* (2005) and Bacchin *et al.* (2006). At critical flux condition, the drag forces on solute molecules concentrated over membrane surface are equal to the dispersive forces by flow shear stress near membrane surface, leading to a nearly constant long-term flux with negligible fouling. It has been proved by a number of researches that critical flux may increase with enhancing crossflow rate (i.e. Reynold number or shear stress) and decrease with increasing feed concentration (Manttari and Nystrom, 2000.; Chiu *et al.*, 2006; Metsamuuronen *et al.*, 2002).

Since feed concentration could be diluted by adding solvent during diafiltration, it is possible to operate diafiltration at critical flux to remove most permeable solutes without severe fouling, and diluted feed could also reduce fouling load during post-concentration.

Previous work showed that ultrafiltration could be applied to separate protease from tuna spleen extract and the purity of protease could be improved by diafiltration (Li *et al.*, 2006). However, fouling due to feed property limited process efficiency and scale up for industrial application. The aim of present work was to apply critical flux operation in diafiltration during separation of protease from tuna spleen extract by ultrafiltration and to compare process performance between operation of critical flux condition and conventional operation.

6.3 Materials and Methods

6.3.1 Preparation of tuna spleen extract

The yellowfin tuna spleen was provided by Chotiwat Manufacturing Co. Ltd (Thailand), Hat Yai. The extract was prepared by homogenizing yellowfin tuna spleen with Tris-HCl buffer (Li *et al.*, 2006) and simply filtered through nylon net, cotton pad and cloth in series to remove large particles, then the extract was pretreated by a microfiltration process using a hollow fiber membrane with pore size 0.10 μm (CFP-1-E-5A, Amersham Biosciences, UK) at crossflow rate of 96 l/h, TMP of 0.15 bar and nitrogen gas injection factor of 0.38 (Li *et al.*, 2008). The permeate from microfiltration process was collected and used as feed.

6.3.2 Ultrafiltration setup

Membrane used for ultrafiltration was a polysulphone hollow fiber membrane (UFP-30-E-3MA, Amersham Biosciences, UK) with molecular weight cut-off of 30 kDa, fiber diameter of 1 mm, flow length of 30 cm and effective area of 0.01 m^2. The set-up of ultrafiltration unit in this study is described in previous work (Li *et al.*, in press). A peristaltic pump (Model 7524-45, Masterflex, USA) was used in this work. All filtration processes were operated with transmembrane pressure of 1.6 bar and circulation crossflow rate of 69.98 l/h at ambient

temperature (28°C). The real feed temperature in the feed tank during ultrafiltration was measured as 31°C due to the heat generated by pump. Therefore the operational temperature was considered as 31°C. 500 ml of pre-treated tuna spleen extract was used as feed in each run. Both feed and permeate were sampled for analysis of protein and protease content. The time of filtration and volume of permeate were monitored for evaluation of flux.

Two operational modes were employed. The first mode (mode 1) included an initial pre-diafiltration process and a post-concentration process. The second mode (mode 2) included a pre-concentration process followed by a post-diafiltration process. In all runs, the retentate was recycled to the feed tank while permeate was collected and removed from the system. During diafiltration process, the fresh buffer was added into feed tank to replace the loss of volume by permeate, and then maintain a constant feed volume in feed tank. During concentration process, the feed volume in the tank was reduced by removing permeate without adding buffer.

The stage of pre- and post-diafiltration process was defined as diafiltration volume (DV) by Equation (30) (Simon *et al.*, 2002):

$$DV = \frac{V_b}{V_0} = \frac{V_p}{V_0} \tag{30}$$

where V_b is the volume of newly added buffer, V_0 is the initial volume in feed tank and V_p is the collected permeate volume.

The stage of pre- and post- concentration process was expressed as volume concentration factor (VCF) by Equation (31):

$$VCF = \frac{V_0}{V_f} = \frac{V_0}{V_0 - V_p} \tag{31}$$

where V_0 is the initial feed volume; V_f is the feed bulk left in the tank during concentration process and V_p is collected permeate volume.

The transmission of solute during ultrafiltration process was expressed by the apparent sieving coefficient (S_a):

$$S_a = \frac{C_p}{C_f} \tag{32}$$

where C_p is solute concentration in permeate and C_f is solute concentration in the feed.

The transmission of protease or protein was expressed as the ratio of either protease activity (U/ml) or protein concentration (mg/ml) in permeate to those in the feed (Muller *et al.*, 2003).

The observed rejection (R_{obs}) of solute is usually expressed by Equation (33)

$$R_{obs} = 1 - \frac{C_p}{C_f} = 1 - S_a \tag{33}$$

6.3.3 Membrane resistance analysis

The types of fouling in both mode 1 and mode 2 were classified as concentration polarization, external fouling and internal fouling. The resistance during ultrafiltration was divided into membrane resistance (R_m), resistance caused by concentration polarization (R_{cp}) and resistance caused by fouling (R_f). R_f was further divided into resistance caused by external fouling ($R_{f\text{-}ex}$) and resistance caused by internal fouling ($R_{f\text{-}in}$). The resistances caused by fouling were evaluated by a further hydrodynamic cleaning process of the membrane after ultrafiltration process.

The total resistance to the fluid flow during ultrafiltration was defined by Darcy's law:

$$J = \frac{TMP}{\mu R_t} = \frac{TMP}{\mu(R_m + R_{cp} + R_f)} = \frac{TMP}{\mu(R_m + R_{cp} + R_{f\text{-}ex} + R_{f\text{-}in})} \tag{34}$$

where J is the permeate flux across the membrane (m/s), TMP is the transmembrane pressure (Pa), μ is the permeate viscosity (Pa.s).

R_t could be calculated by Equation (34). R_m and resistances caused by fouling were evaluated by measuring water flux of clean membrane or fouled membrane. All water flux was measured at TMP of 0.10 bar for 5 minutes. R_{cp} was detected as the resistance which could be eliminated by a mild cleaning with water flow. After ultrafiltration process, fouled membrane was flushed by deionized water at circulation crossflow rate of 69.98 l/h and TMP of 0.10 bar for 10 minutes to remove concentration polarization. After flushing with water, the residual resistance was classified as R_f. R_f was calculated from water flux of flushed membrane. In order to determine $R_{f\text{-}ex}$ and $R_{f\text{-}in}$, the water flushed membrane was further washed by circulating NaOH solution (0.5 N, 50°C) at circulation crossflow rate of 69.98 l/h for 1 hour while the permeate side was closed to prevent cleaning reagent from passing through membrane. After chemical cleaning, $R_{f\text{-}in}$ could be evaluated from the water flux of chemically cleaned membrane.

6.3.4 Analytical methods

Protein concentration was measured by the Lowry method (Lowry *et al.*, 1951). Protease activity was measured by using casein as substrate at 50°C and pH 8.0. One unit of protease activity was expressed as the amount of enzyme that liberated 1 μg of tyrosine equivalent in 1 minute (Munilla-Moran and Saborido-Rey, 1996). The purity of protease was expressed as specific activity (Sp.Act.) in Equation (35).

$$\text{Sp.Act. (U/mg)} = \frac{\text{protease activity (U/ml)}}{\text{protein concentration (mg/ml)}} \qquad (35)$$

6.4 Results and discussion

The pre-treated tuna spleen extract was clear and showed slight yellow colour. It contained protein of 8.72 mg/ml and protease of 54.85 U/ml.

6.4.1 Effect of operational mode on flux profile

As mentioned above, critical flux condition could be applied during diafiltration process. Previous study indicated that a critical flux could be attained in total recycle ultrafiltration with the same pre-treated tuna spleen extract and membrane at TMP of 1.6 bar and circulation crossflow rate of 69.98 l/h (Li *et al.*, in press). Therefore, the transmembrane pressure of 1.6 bar and circulation crossflow rate of 69.98 l/h were applied at the beginning of ultrafiltration and maintained through the whole process in this work. The operational condition according to critical flux could be classified to sub-critical zone, critical zone and supra-critical zone (Yu *et al.*, 2003). Both sub-critical and critical zone undergo filtration process with negligible fouling. Fouling could form in supra-critical zone. Therefore, if TMP and circulation crossflow rate are set for critical zone and maintained, decreasing feed concentration could lead to an alteration from critical zone to sub-critical zone while increasing feed concentration could result in an opposite direction, i.e. from critical zone to supra-critical zone. Figure 32 and 33 showed the flux profiles in mode 1 (pre-diafiltration followed by post-concentration) and mode 2 (pre-concentration followed by post-diafiltration), respectively. DV of 3 and VCF of 2 were applied in both mode 1 and 2.

In mode 1, the ultrafiltration started with a pre-diafiltration step. The flux was nearly constant during pre-diafiltration (Figure 32). The value of flux was close to the critical flux of 44.2 $l/m^2.h$ detected from a total recycle mode in same operational condition in previous work (Li *et al.*, in press). In pre-diafiltration, the feed concentration decreased with time or DV by adding buffer. Since the initial operational condition (TMP and circulation crossflow rate) was set for critical flux, the operation during pre-diafiltration could be maintained in a sub-critical zone when feed concentration was reduced and the flux could be constant with a constant TMP. When DV of 3 was achieved, adding buffer was terminated while retentate was still recycled to feed tank and permeate was collected and removed from the system. The operation was switched to post-concentration. Since the feed volume was constant in pre-diafiltration, the initial volume of feed for post-concentration of mode 1 was 500 ml. The post-concentration was stopped when VCF of 2 was achieved. Figure 32 showed that the flux decreased with time. About 24.6% decline of flux (from 44.3 $l/m^2.h$ at the end of pre-diafiltration to 33.4 $l/m^2.h$ at the end of post-

concentration) was observed. The decrease of flux during post-concentration was probably due to the formation of concentration polarization with increasing VCF.

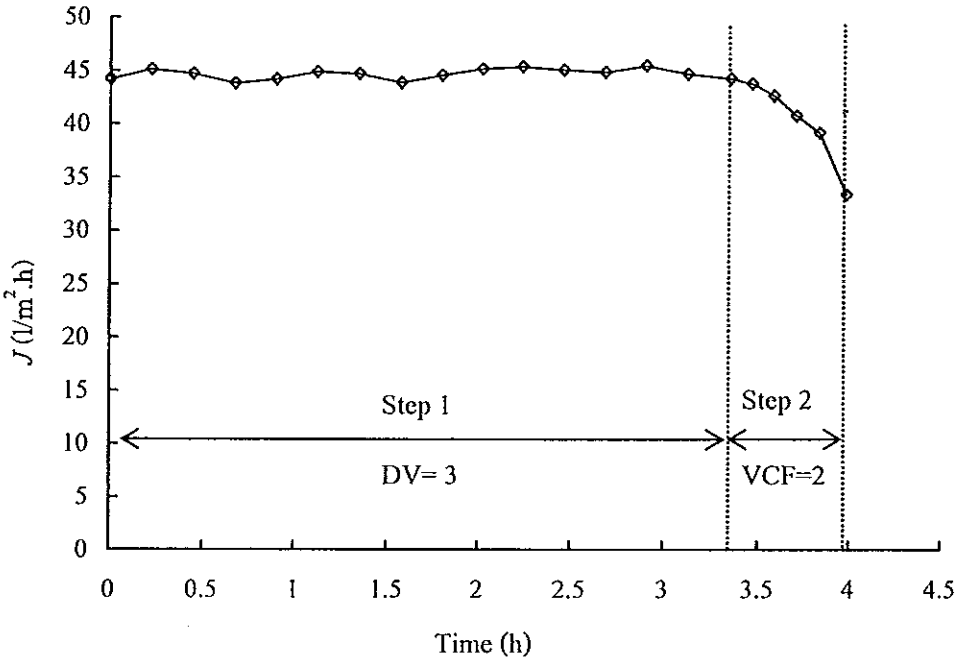

Figure 32. Permeate flux profile in mode 1 (pre-diafiltration followed by post-concentration).

In mode 2, a pre-concentration was initially performed. The initial feed volume for pre-concentration was 500 ml as well. The flux at the initial point of pre-concentration (i.e. VCF of 1 or time of 0) could be considered as 44.2 l/m^2.h according to the operational condition for critical flux. It was observed that the flux decreased significantly with time during pre-concentration in mode 2 (Figure 33). It was due to the formation of fouling with increasing feed concentration. The reason was the fact that the solute deposition at higher feed concentration would be higher than that at lower feed concentration (Kwon *et al.*, 2000). In order to maintain condition in critical zone, TMP should be reduced if feed concentration is increased while circulation crossflow rate is constant (Manttari and Nystrom, 2000). However, a constant TMP of

1.6 bar was applied in whole process in this study. Consequently, when feed concentration increased with increasing VCF, the filtration condition was moved to supra-critical zone where operation cannot be maintained for a long term and flux decreased sharply. About 59% decline of flux (from 44.2 to 18.1 l/m^2.h) occurred after pre-concentration with VCF of 2. When VCF of 2 was attained by pre-concentration of mode 2, buffer was added into feed tank continuously to keep a constant feed volume. The operation was switched to post-diafiltration. The initial volume for post-diafiltration was 250 ml. In contrast to pre-diafiltration of mode 1, it was observed that flux still decreased with time in the post-diafiltration of mode 2 (Figure 33). Even though adding buffer could dilute the feed in post-diafiltration process, it might not improve the flux because of the previously formed fouling. The fouling formed in pre-concentration could not be redispersed by diluting feed in post-diafiltration In addition, at supra-critical zone, pressure drove flux through the membrane still make a convection of solutes towards membrane and the negative effect of concentration polarization on flux still occurred. Therefore, the decrease of flux was still observed in post-diafiltration. The flux was 5.15 l/m^2.h when DV of 3 was achieved.

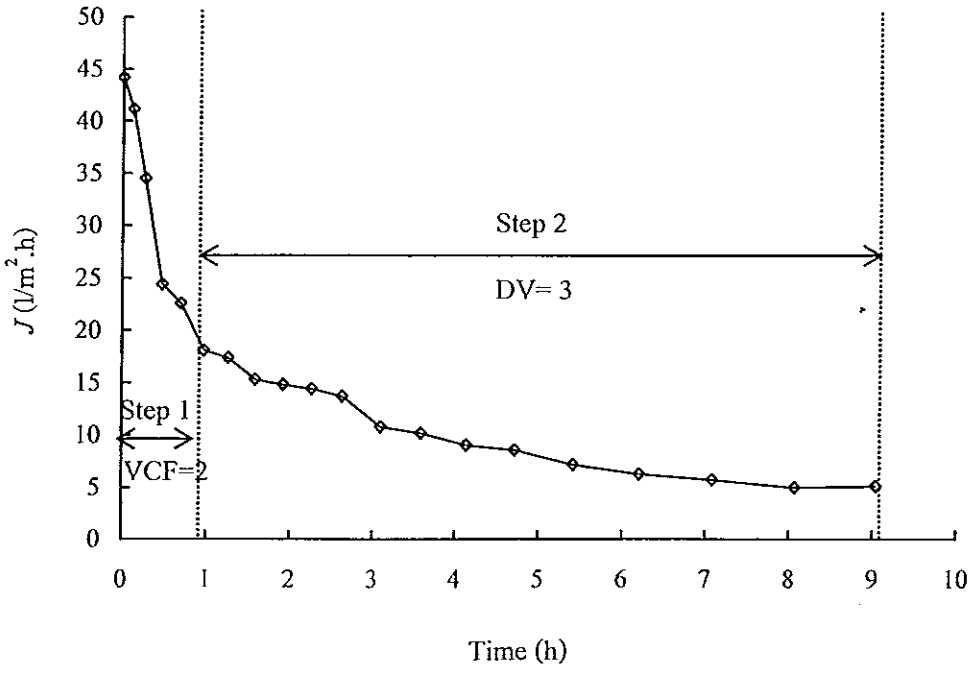

Figure 33. Permeate flux profile in mode 2 (pre-concentration followed by post-diafiltration).

The total running time was 3.98 hours for mode 1 and 9.04 hours for mode 2. The operation of mode 1 showed its benefit to save operational time. However, mode 1 required larger volume of buffer (1.5 liters) for pre-diafiltration step while 0.75 liter buffer was added for post-diafiltration step in mode 2.

6.4.2 Effect of operational mode on purification of protease

The variation of solute concentration generally modeled by Equation (36) and (37) for concentration process and diafiltration process, respectively (Wang *et al.*, 2002).

$$\frac{C_{f,i}}{C_{f,i0}} = \left(\frac{V_0}{V_f}\right)^{R_{obs,i}} \tag{36}$$

(for pre- or post-concentration process)

where $C_{f,i}$, is the concentration of solute i in feed during concentration process; $C_{f,i0}$ is the initial concentration of solute i in feed before concentration process; $R_{obs,i}$ is observed rejection of solute i.

$$\frac{C_{f,i}}{C_{f,i0}} = \mathrm{Exp}\left[-\frac{V_b}{V_0}(1 - R_{obs,i})\right] = \mathrm{Exp}\left(-\frac{V_b}{V_0} S_{a,i}\right) \tag{37}$$

(for pre- or post-diafiltration)

where $S_{a,i}$ is the apparent sieving coefficient of solute i.

In this work, solute concentration (i.e. protein or protease) during process was determined by sampling feed and permeate. The experimental data from samples were compared to theoretical data from Equation (36) and (37). The transmission of protein and protease at critical zone and supra-critical zone in a total recycle mode with the same feed and membrane were determined in previous work (Li *et al.*, in press). S_a of protein and protease were about 0.40 and 0.03 respectively at sub-critical zone and critical zone. S_a were 0.26 and 0.03 for protein and protease, respectively at supra-critical zone. However, the protein concentration in feed during pre-diafiltration of mode 1 in this work showed a significant difference from those evaluated by Equation (37) with S_a of 0.40. The practical S_a of protein was determined as about 0.76 by protein

content in permeate and feed during pre-diafiltration of mode 1. The reason for higher S_a of protein obtained in mode 1 was probably that concentration polarization was reduced by adding buffer in mode 1. Thus, 0.76 and 0.26 were accepted as S_a of protein in mode 1 and mode 2 , respectively while S_a of protease was 0.03 in both modes. These S_a were applied in Equation (36) and (37). Experimental data obtained by measuring solute content in the feed were in acceptable agreement with theoretical data (Figure 34 to 37). In mode 1, it can be seen that protein concentration decreased 88% (from 8.72 to 1.05 mg/ml) when DV of 3 was achieved in pre-diafiltration (Figure 34a). When operation switched to post-concentration, protein concentration increased with VCF (Figure 34b). Protein concentration was 1.05 mg/ml at the end of pre-diafiltration and became 1.29 mg/ml at VCF of 2 after post-concentration. Since S_a of protease was just about 0.03, most protease was rejected by membrane. In pre-diafiltration, the decrease of protease concentration was 9% (from 54.85 to 49.89 U/ml) (Figure 35a) which was much lower than decease of protein concentration. And then protease was concentrated in post-concentration. The final protease concentration at the end of post-concentration (VCF of 2) was 96.50 U/ml (Figure 35b). The specific activity of protease varied from 6.29 before ultrafiltration to 74.81 U/mg after ultrafiltration. About 12-fold of purification was achieved. In mode 2, considering the possibility of fouling formation and referring to previous work mentioned above, the theoretical S_a of 0.26 for protein and 0.03 for protease were applied to evaluate theoretical protein and protease content in feed during process. The agreement between experimental data and theoretical data indicated that fouling might play major role for solute transmission at supra-critical zone. After pre-concentration, the increasing rate was 171% (from 8.72 to 14.89 mg/ml) for protein concentration while it was 184% (from 54.85 to 101.11 U/ml) for protease. In following post-diafiltration, protein concentration varied from 14.89 to 6.83 mg/ml. Decreasing rate of protein concentration was 54%. Comparing to pre-diafiltration in mode 1 (decreasing rate of 88%), the efficiency to remove protein by diafiltration was lower in mode 2. The severe fouling formed in pre-concentration probably reduced transmission of solute in following post-diafiltration. The variation of protease content in post-diafiltration was similar to that in mode 1. A minor decrease (7%) of protease concentration was observed in post-diafiltration of mode 2. At the end of post-diafiltration, protease concentration was 94.35 U/ml. The specific activity of protease was 13.81

U/mg after process of mode 2. The purity of protease increased 220% (i.e. about 2-fold purification).

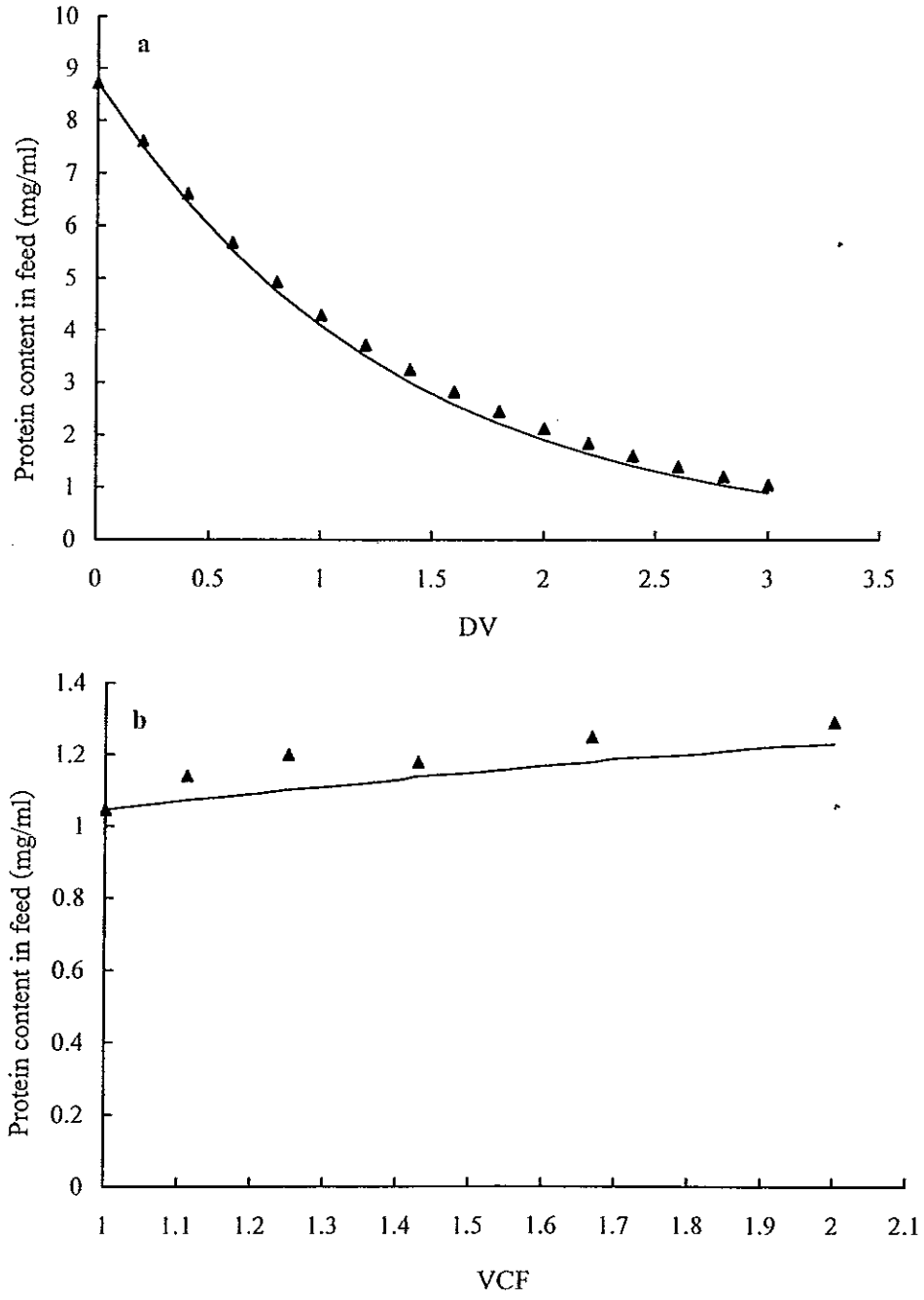

Figure 34. Variation of protein content in feed during process of mode 1 (a, pre-diafiltration; b, post-concentration).

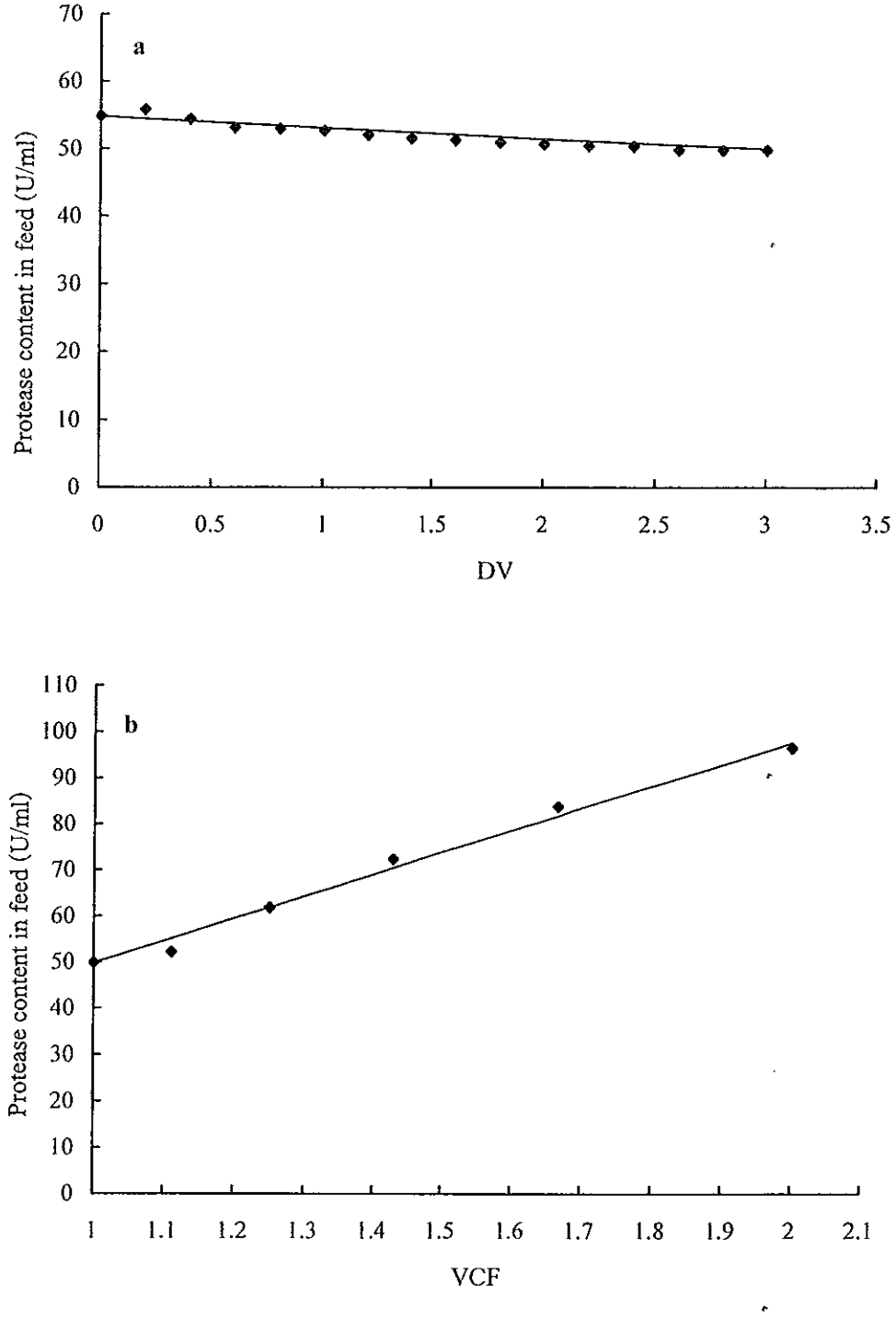

Figure 35. Variation of protease content in feed during process of mode 1 (a, pre-diafiltration; b, post-concentration).

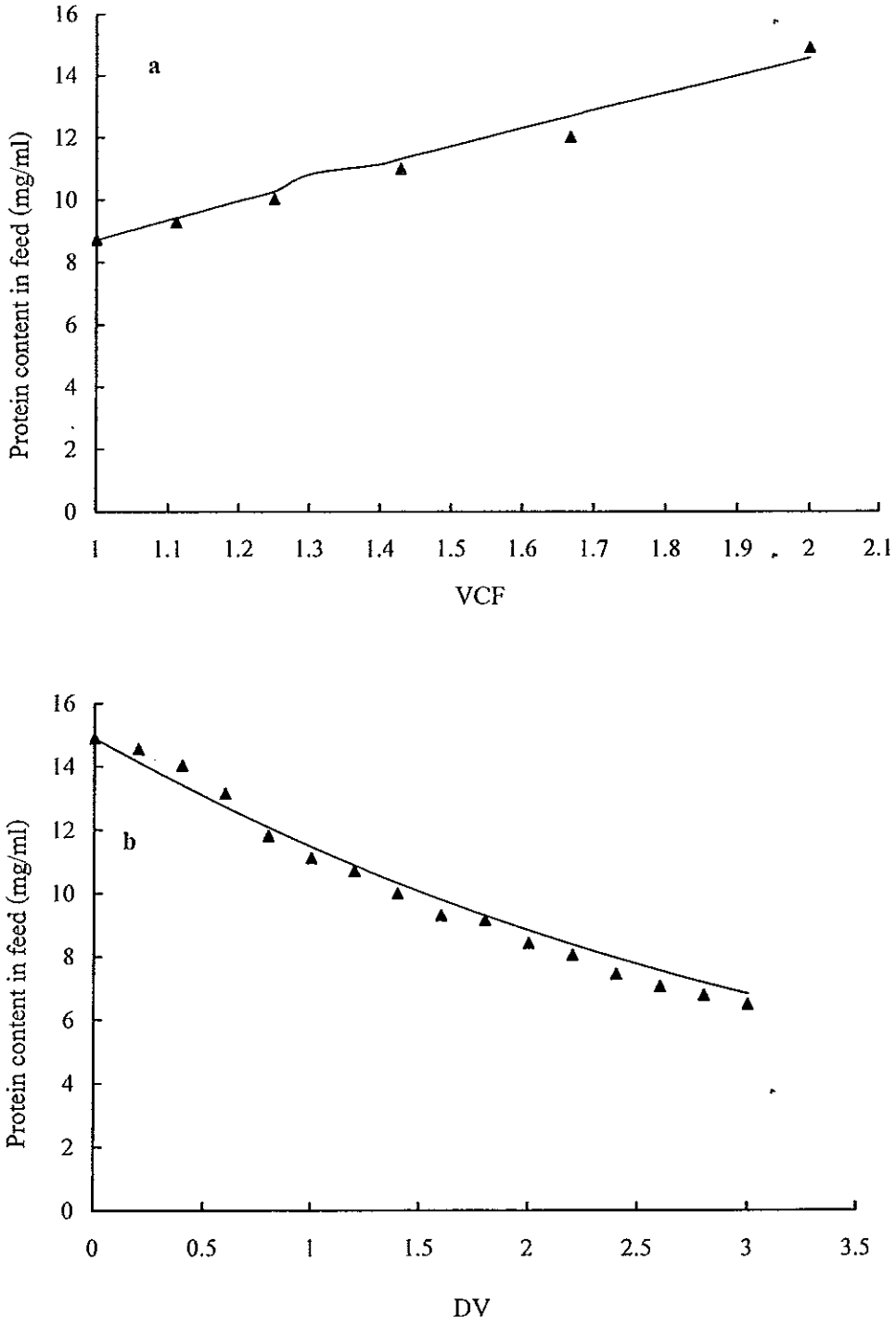

Figure 36. Variation of protein content in feed during process of mode 2 (a, pre-concentration; b, post-diafiltration).

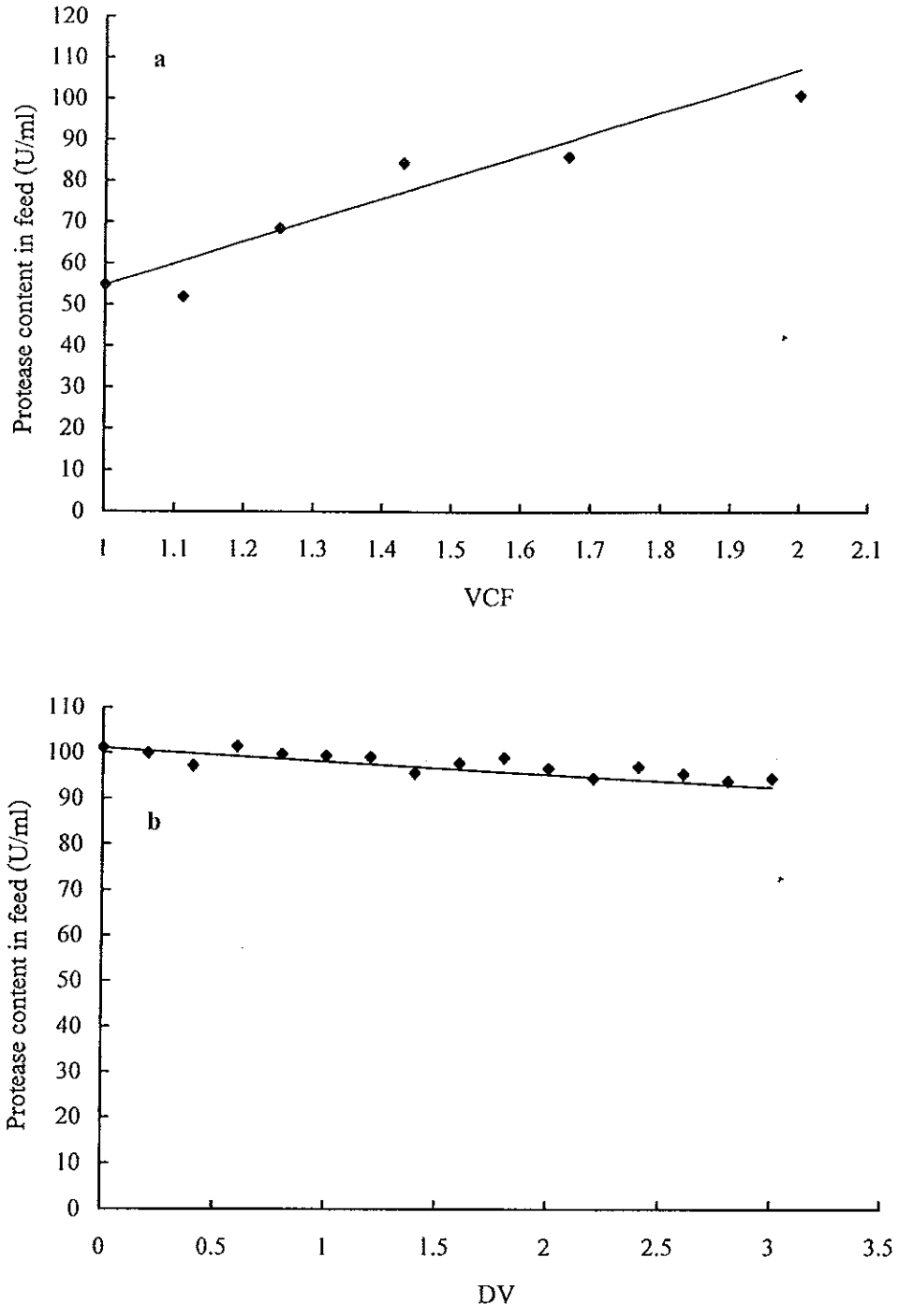

Figure 37. Variation of protease content in feed during process of mode 2 (a, pre-concentration; b, post-diafiltration).

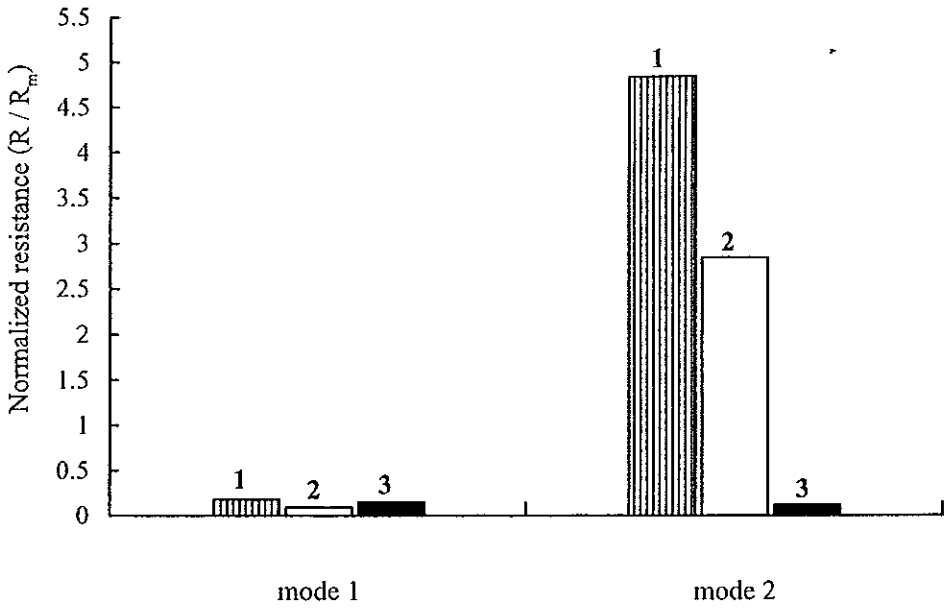

Figure 38. Various filtration resistances during mode 1 and mode 2 in terms of normalized resistance (column 1, R_{cp} / R_m, normalized resistance caused by concentration polarization; column 2, $R_{f\text{-}ex}$ / R_m, normalized resistance caused by external fouling; column 3, $R_{f\text{-}in}$ / R_m, normalized resistance caused by internal fouling).

6.4.3 Resistance analysis of different modes

The types of fouling in both mode 1 and mode 2 were analyzed by a further hydrodynamic cleaning of the membrane after ultrafiltration process.

Figure 38 illustrated the normalized resistances in mode 1 and mode 2. It was found that the resistance caused by concentration polarization and fouling (i.e. sum of R_{cp}, $R_{f\text{-}ex}$ and $R_{f\text{-}in}$) in mode 1 was much lower than those in mode 2. As mentioned above, the operational condition was controlled at critical zone at the beginning of ultrafiltration. In mode 1, condition could be kept in sub-critical zone until the end of pre-diafiltration. Thus, severe fouling was avoided in the first step of mode 1. When the operation was switched to post-concentration, the feed was concentrated. However, the feed concentration was still low at the end of post-

concentration process since most permeable solutes was removed in pre-diafiltration. VCF of 2 could not make a high concentration which might cause heavy fouling. The highest resistance in mode 1 is R_{cp}. Since it was due to concentration polarization, R_{cp} in mode 1 was easily to be eliminated by water flushing. Both R_{cp} and $R_{f\text{-}ex}$ in mode 2 was much higher than those of mode 1. The reason could be related to difference of operation mode. Mode 2 started from a pre-concentration. Generally, fouling increases with feed concentration for a given operation regime. In pre-concentration of mode 2, a high concentration gradient towards membrane formed soon. The extent of concentration polarization was much higher in mode 2 than mode 1. Therefore higher R_{cp} was observed in mode 2. Furthermore, with increasing feed concentration, the balance between convective mass transportation to the membrane by pressure drove flux and erosion by wall shear stress to diffuse molecules away from the membrane was broken. Thus convection dominated the mass transport process. Protein fouling observed in convection condition was found to be severe (Czekaj *et al.*, 2000). It should be noted that apparent sieving coefficient of protein in mode 2 (about 0.26) was lower than that in mode 1 (about 0.76). Macromolecular transmission rates are also useful to characterizing membrane fouling. External fouling was accompanied by a significant drop in macromolecular transmission (Mueller and Davis, 1996). On the other hand, protein fouling does not depend on the size of individual proteins, but on their ability to form aggregates (Guell. and Davis *et al.*, 1996.). It has been concluded that the increase in protein concentration could cause the increase in observed aggregation (Iulek and Carcamo, 2001; Mary *et al.*, 2006). Thus extent of fouling could be enhanced with a high feed concentration in pre-concentration of mode 2. Protein aggregates deposited on the membrane surface and additional molecules added to the initial deposits leading to the formation of a multi-layer fouling on membrane surface. Due to the colloid property of protein layer, the fouling could not be washed away by water flushing and the fouling was classified as external fouling. $R_{f\text{-}in}$ in both mode 1 and 2 were low. And $R_{f\text{-}in}$ in mode 1 was slightly higher than that in mode 2. Internal fouling in ultrafiltration of protein solution in this work was probably due to the adsorption of protein molecules onto the membrane pore wall. During membrane filtration of biological fluid, such as protein solution, membrane fouling, especially colloidal fouling occurs in two stages, i.e. internal and external stage (Belfort *et al.*, 1994). External fouling is caused by deposition of molecules and their aggregates onto pore mouth and membrane surface. The internal fouling is

caused by adsorption or deposition of small particles or macromolecules within the internal structure of membrane. This adsorption occurs immediately as soon as the fluid passes through the membrane. However, the resistance of internal fouling is not high due to the limited internal space of membrane structure. Once external fouling layer forms, it could acts as secondary membrane for filtration and dominates the fouling mechanism. The slight difference of $R_{f\text{-}in}$ between mode 1 and mode 2 was probably due to amount of molecules passed through membrane. In mode 1, higher flux and less fouling make it possible that more molecules passed through before formation of external fouling layer. Consequently, internal fouling could be enhanced in mode 1.

6.5 Conclusion

The performance of different operational modes of diafiltration was studied during ultrafiltration of pretreated tuna spleen extract for purification of protease. There was an acceptable agreement on solute content during ultrafiltration between experimental and theoretical data. The operation of mode 1 (pre-diafiltration followed by post-concentration) could achieve higher purity of protease in a shorter time while flux showed a minor decrease. The operation of mode 2 (pre-concentration followed by post-diafiltration) showed a significant drop of flux. A lower purity of protease was attained in mode 2 with a long operational time. The yield of protease was about 88% in operation of mode 1 while it was 86% in operation of mode 2. Extent of fouling was limited at low level in mode 1 combining with critical flux condition at beginning of pre-diafiltration while a severe fouling occurred in mode 2. However, the volume of buffer used for diafiltration process in mode 1 was double of that in mode 2. For further industrial application, all factors including product quality, process efficiency, cost of solvent and cost of membrane cleaning should be taken in consideration.

CHAPTER 7

PROTEIN HYDROLYSIS BY PROTEASE ISOLATED FROM TUNA SPLEEN BY MEMBRANE FILTRATION: A COMPARATIVE STUDY WITH COMMERCIAL PROTEASES

7.1 Abstract

Industrial applications of animal pancreatic proteases are limited by the high cost of these enzymes. A multi-stages membrane filtration process including microfiltration and ultrafiltration was applied for recovery and purification of trypsin-like serine protease from the extract of yellowfin tuna spleen and reduced the cost of enzyme for industrial applications. The behaviors of yellowfin tuna spleen protease (TSP) and commercial proteases (Alcalase and Delvo-Pro) with respect to the hydrolysis of casein and soybean protein isolate were compared. Degree of hydrolysis of 43% was reached after hydrolysis of casein by Alcalase and TSP while Delvo-Pro showed lower degree of hydrolysis after hydrolysis for 180 minutes. In the case of soybean protein isolate as substrate, TSP provided lowest capacity to hydrolyse soybean protein isolate. The reason was probably due to the residual activity of soybean trypsin inhibitor in soybean protein isolate. The present work showed that a trypsin-like serine protease with low-cost and qualified hydrolysis efficiency could be obtained from tuna canning waste by membrane filtration. Protein hydrolysis could be achieved by this protease instead of the commercial proteases.

7.2 Introduction

Protein plays an important role in the human diet for providing essential amino acids. It is possible to enhance their use in different food and non-food applications with diversified and improved functional properties by hydrolysis. Hydrolysis processes have been used to increase the biological value of proteins, such as improving their digestibility and

reducing allergenicity (Lahl and Braum 1994; Asselin *et al.*, 1988). Protein hydrolysates are mixtures of oligopeptides, polypeptides and free amino acids. They may be a source of easily available protein in remedies used to cure metabolic diseases or in dietary/medicinal preparations (Schmidl *et al.*, 1994).

Both chemical and enzymatic methods have been adopted for protein hydrolysis. Chemical methods involve alkali, such as NaOH and acid, such as HCl. However, enzymatic hydrolysis is greatly preferred because chemical hydrolysis may oxidize cysteine and methionine, destroy some serine and threonine, convert glutamine and asparagine to glutamate and aspartate, respectively, then reduce protein quality and biological value (Bucci and Unlu, 2000). Enzymatic hydrolysis involves minimum side reactions and leads to limited hydrolysis of selected peptide bonds. The hydrolysis is more specific and can eliminate many of the problems associated with chemical hydrolysis (Kumar *et al.*, 2004). A number of commercially available proteases have been applied for specific modification of protein and production of peptides, such as trypsin, Alcalase and Neutrase (Karamac *et al.*, 2002; Abdul-Hamid *et al.*, 2002; Shahidi *et al.*, 1995;).

A large amount of processing discards and by-products is created from slaughter or fishery industry annually. For example, approximately 30% of the total fish landing can be considered as under-utilized, by-catch, unconventional or unexploited in fisheries and fish processing. Only a small part of these is used for human consumption (Venugopal and Shahidi, 1995). Because of increasing demand for protein hydrolysates, a cheaper resource and simple method for preparation of enzymes are required. Enzymes contained in industrial wastes have caught more attention recently. Utilization of these wastes could not only provide a large and cheap resource for production of enzymes but also reduce the pollution loading to the environment. Studies of separation and utilization of enzymes in the waste from slaughter or fishery industry have been reported, such as pepsin from stomach of Atlantic cod (Gildberg, 1992), chicken intestine proteases (Raju *et al.*, 1997) and serine proteases from pyloric caeca of Atlantic salmon (*Salmo salar*) (Kristinsson and Rasco, 2000).

As the largest producer and exporter of tuna canning products in the world, Thailand produces more than 311,070 tons canned tuna annually (National Food Institute, 2005). Consequently, the utilization of wastes from tuna canning could be a challenge in Thailand. Previous works proved that tuna viscera are the potential resource of enzymes (Klomklao *et al.*,

2004, 2005). The proteases in yellowfin tuna spleen could be isolated and partially purified by membrane filtration (Li *et al.*, 2006; 2007). So far, few results have been reported about hydrolysis efficiency of purified protease from tuna spleen extract. The reason is probably that the amount of enzyme separated by conventional method (e.g. chromatography or affinity purification) for characterization research is very small. Membrane filtration provides a way to purify these enzymes on a relatively larger scale. Therefore, the aim of present work was to study the hydrolysis of casein and soybean protein isolate by the protease isolated from yellowfin tuna spleen by membrane filtration. The results were compared with commercial proteases. The information from this research can aid in utilization of wastes from tuna canning industry.

7.3 Materials and Methods

7.3.1. Proteins and proteases

Casein from bovine milk (Fluka, Switzerland; 87.3% w/w protein content) and soybean protein isolate (Supro 670IP, Abbra Co Ltd, Thailand; 81.9% w/w protein content) were used as substrates for protein hydrolysis. 10% casein or soybean protein isolate was prepared in 0.1 N NaOH, then pH was adjusted to pH 8.0 by 1N HCl. The protein solutions were heated in a water bath with continuous stirring at 37°C for 12 hours to achieve maximum dissolution. Then undissolved particles were removed by filtration with filter paper (No.1, Whatman). Final protein concentration was standardized to be 10.0 mg/ml. (modified method of Dziuba *et al.*, 2004).

Alcalase® 0.6L (Novo Nordisk, Denmark) was donated by The East Asiatic (Thailand) Co Ltd. It is commonly used in the food as well as the detergent industry. The main enzyme component is subtilisin. The declared activity by the manufacturer is 0.6 AU/g.

A less used commercial protease, called Delvo-Pro (DSM Food Specialties, The Netherlands) was kindly provided by Songkhla Canning Public Co Ltd (Songkhla, Thailand). It is a protease preparation for food use containing alkaline protease derived from a selected GRAS strain of *Bacillus licheniformis*. The declared activity by manufacturer is 580,000 DU/g. This protease is currently using in Songkhla Canning Public Co Ltd for production of protein concentrate from the waste of fish canning processing.

Procedure of isolation and purification of proteases from yellowfin tuna spleen was described in previous study (Li *et al.*, 2006, 2007 and 2008).

7.3.2 Hydrolysis process

In order to enable a comparison of results, hydrolysates were prepared by enzymatic hydrolysis with three different enzymes at pH 8.0. Temperature of 60°C was applied for hydrolysis by Alcalase hydrolysis while 50°C for hydrolysis by Delvo-Pro and TSP. In order to simplify the operations in industrial applications, enzyme/substrate (E/S) ratio is often expressed as volume to volume (v/v), weight to weight (w/w) or volume to weight (v/w) (Sarabok and H-Kittikun, 1999; Guerard *et al.*, 2002; Bhaskar *et al.*, 2008). In this work, the enzymes were added into protein solution in the range of 0.1-0.4% (v/v). Control experiments were performed without enzyme addition. All experiments were carried out in triplicate (modified method of Sarabok and H-Kittikun, 1999).

An initial 150 ml protein solution for each hydrolysis reaction was prepared with adjusting temperature to the desired value. The protease was then added and the reaction allowed to be proceeded for 180 minutes in temperature controlled water bath under shaking (200 rpm). The hydrolysis was carried out using the pH-stat method (Adler-Nissen, 1977). The volume of NaOH solution (2N) needed to keep the pH constant during hydrolysis was recorded for calculation of degree of hydrolysis (DH). Reaction was terminated by heating the solution to 95°C for 20 minutes.

7.3.3 Analytical methods

Protein concentration in solution was measured according to the Lowry method (Lowry *et al.*, 1951) using bovine serum albumin (BSA) as the standard. Protein in dry materials was measured by Kjeldahl method (AOAC, 1999).

Protease activity based on casein digesting unit (CDU) was assayed by the method described in previous works (Li *et al.*, 2006). One unit of protease activity was expressed as the amount of enzyme that liberated 1 μg of tyrosine equivalent in 1minute at pH 8.

Temperature of 50°C was applied for TSP and Delvo-Pro while 60°C for Alcalase. Protease purity was expressed as the specific activity (Sp.Act.), i.e. the ratio of protease activity units to protein content (U/mg protein).

Degree of hydrolysis (DH) was defined as the percentage ratio between the number of peptide bonds cleaved (h) and the total number of peptide bonds in the substrate studied (h_{tot}). DH was evaluated by pH-stat method which allowed the estimation of DH based on the consumption of alkali to maintain a constant pH at the desired value. The degree of hydrolysis was determined by Equation (38) (Adler-Nissen, 1986).

$$\mathrm{DH}(\%) = \frac{h}{h_{tot}} \times 100 = \frac{BN_b}{M_p \alpha h_{tot}} \times 100 \qquad (38)$$

where B is the amount of alkali consumed (ml); N_b is the normality of alkali; M_p is the mass of the substrate (protein in grams, %N×6.25); α is the average degree of dissociation of α -NH_2 groups released during hydrolysis.

The electrophoresis was performed according to the method of Laemmli (1970). Both SDS-PAGE and native-PAGE were employed. Silver staining (Yaobo, 2002.), activity staining (Carcia-Carreno *et al.*, 1993) and Coomassie blue staining were applied to present proteases from tuna spleen extract, protease activity and protein hydrolysates, respectively. For sample loading, each well of gels contained 20 μg protein.

7.4 Results and discussion

Raw tuna spleen extract was prepared by homogenizing spleen and Tris-HCl buffer (0.02 N, pH 8.0). The raw extract was filtered in series by microfiltration (ploysulphone hollow fiber membrane with pore size 0.1μm) for removal of suspended particles and ultrafiltration (polysulphone hollow fiber membrane with molecular weight cut-off 30 kDa) for recovery and purification of protease (Li *et al.*, 2006, 2007 and 2008). The retentate after ultrafiltration was used as tuna spleen protease (TSP) solution for protein hydrolysis in this study.

7.4.1 Preliminary characterization of proteases isolated from tuna spleen by electrophoresis

General properties of proteases used in this study were described in Table 7. Both Alcalase and Delvo-Pro were dark brown liquids with strong odor. TSP was a clear liquid with slight yellow colour. The odor of TSP was negligible fish smell. TSP showed the highest specific activity which indicated the highest protease purity based on CDU.

Table 7. General description of proteases used for protein hydrolysis.

	Activity[1] (U/ml)	Protein (mg/ml)	Sp.Act. (U/mg protein)	Colour	Odor
Alaclase	57.3	9.62	6.0	dark brown	strong
Delvo-Pro	79.6	71.90	1.1	dark brown	strong
TSP	96.5	1.29	74.8	slight yellow	negligible

1. Based on casein digesting unit.

Figure 39 showed partially purified TSP from yellowfin tuna spleen extract. The electrophoretic pattern showed only one major dark band (lane 3) after microfiltration and ultrafiltration. Most other proteins were removed. It has been proved that major proteases in spleen of all tuna species were trypsin-like serine proteases (Klomklao *et al.*, 2004). The major dark band at lane 3 could be attributable to trypsin-like serine protease, according to its molecular weight (24 kDa). Due to genetic variation among species, the reported molecular weights of trypsin separated from various species are different t(Torrissen, 1984). For example, the molecular weight of trypsin separated from Greenland cod (*Gadus ogac*) was 23.5 kD (Simpson and Haard, 1984) while that separated from anchovy viscera was 25.6 kDa (Heu *et al.*, 1995). Trypsin-like enzyme from pyloric caeca of tambaqui had a molecular weight of 38.5 kDa (Bezerra *et al.*, 2001).

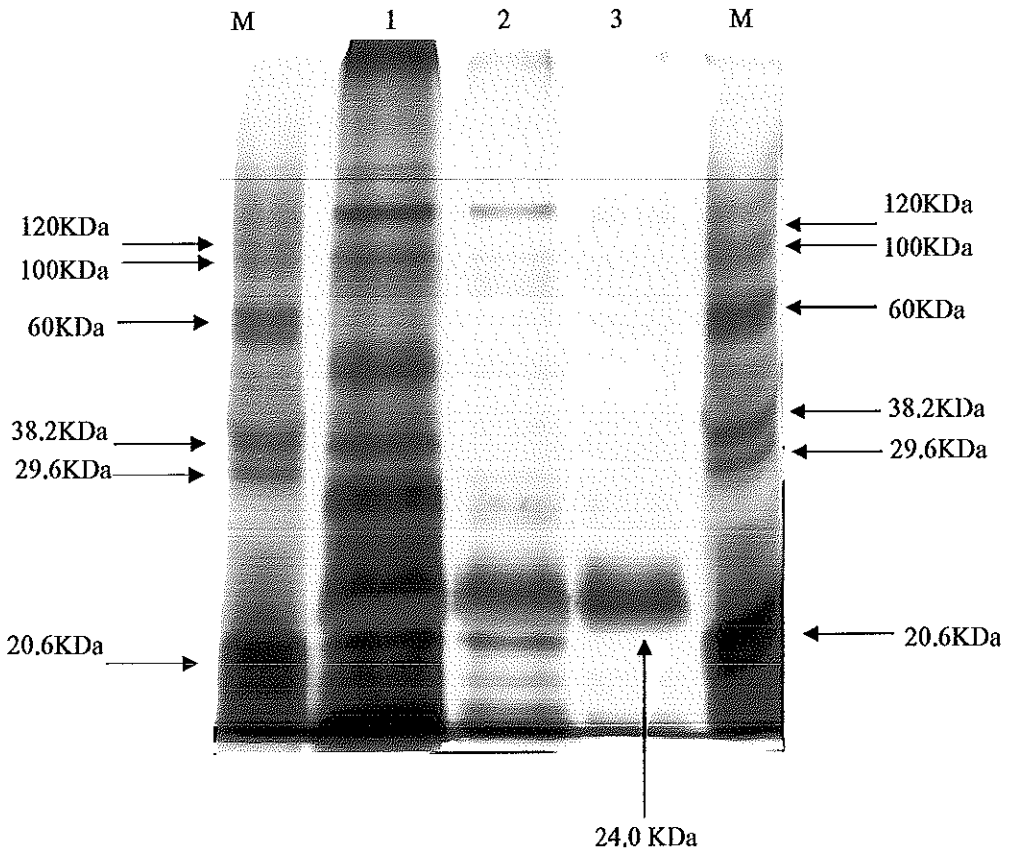

Figure 39. Protease isolated and purified from yellowfin tuna spleen by membrane filtration (SDS-PAGE with 12% gel, stained by silver staining; Lane M, standard marker; Lane 1, raw spleen extract before membrane filtration; Lane 2, spleen extract treated by microfiltration; Lane 3, spleen extract treated by ultrafiltration).

In order to prove protease activity of purified TSP from yellowfin spleen extract, a native-PAGE with activity staining was employed (Figure 40). After electrophoresis, gel was removed to soak in 2% casein in 0.02 N Tris-HCl buffer (pH 8.0) for 1 hour at 4°C. Then the temperature was raised to 50°C and gel was incubated for 90 minutes to digest protein by the active fractions. After incubation, the clear zones on the dark blue background by Coomassie blue staining indicated protease activity. The similar patterns of activity bands were observed from raw extract (Lane 1), the extract treated by microfiltration (Lane 2) and the extract treated by

ultrafilration (Lane 3). It was proved by Figure 39 and 40 that protease activity could be purified and recovered by multi-stages membrane filtration. However, some parallel clear zones were appeared in Native-PAGE (Lane 3 of Figure 40) while a single protein zone was observed in SDS-PAGE (Lane3 of Figure 39) after treatment by ultrafiltration. The reason could be due to the difference between SDS-PAGE and Native-PAGE. The separation of protein relys on only the size of the polypeptide chains by using sodium dodecyl sulfate (SDS). In Native-PAGE, absence of SDS causes a abnormal mobility of protein molecules which is influenced by several factors including molecular size, charge and the attendant frictional resistance (Ninfa and Ballou, 1998)

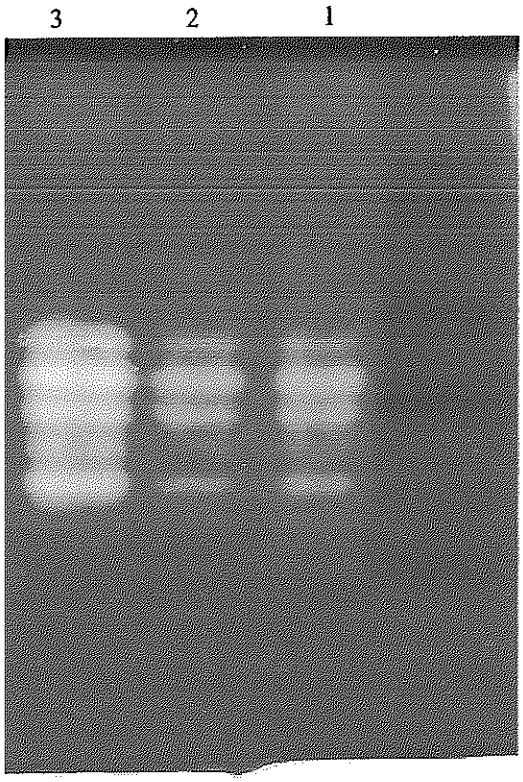

Figure 40. Protease activity of yellowfin tuna spleen extract (Native-PAGE with 14% gel, stained by activity staining with Coomassie blue; Lane 1, raw spleen extract before membrane filtration; Lane 2, spleen extract treated by microfiltration; Lane 3, spleen extract treated by ultrafiltration)

7.4.2 Effect of enzymes and E/S ratio on degree of hydrolysis

Hydrolysis was performed by different proteases with varied E/S ratio. Casein and soybean protein isolate were used as substrates. The variation of DH was dependent on both E/S ratio and the type of enzymes.

The changes of DH during the hydrolysis of casein with Alcalase, Delvo-Pro and TSP were shown in Figure 41. It was possible to reach high DH after enzymatic hydrolysis of casein for 180 minutes. For all proteases applied, DH increased rapidly in initial 30 minutes, and tended towards plateau after hydrolysis for about 120 minutes. In the case of Alcalase, DH of 27.7% was reached after hydrolysis for 180 minutes with E/S ratio of 0.1%. The increase of E/S ratio to 0.2% gave DH of 43.1%. However, further increase of E/S ratio to 0.3% and 0.4% only showed difference of DH in the initial period of hydrolysis. After hydrolysis for 120 minutes, the curves of DH according to E/S ratio of 0.2%, 0.3% and 0.4% were practically identical. After hydrolysis for 180 minutes, DH were 43.4% and 43.8% with E/S of 0.3% and 0.4%, respectively. For Delvo-Pro and TSP, DH was depended on E/S ratio. Hydrolysis of casein for 180 minutes by TSP reached DH of 24.4%, 31.3%, 36.8% and 43.7% with E/S ratio varying from 0.1 to 0.4%. The highest DH by using TSP was 43.7% after hydrolysis of 180 minutes with E/S 0.4% which was close to the value obtained by using Alcalase at same conditions. In the case of Delvo-Pro, after hydrolysis for 180 minutes, DH was 15.8, 25.9, 31.7 and 37.8 with E/S ratio of 0.1%, 0.2% 0.3% and 0.4%, respectively. According to the concentration and volume of protease solution applied, the amount of CDU applied for hydrolysis could be worked out. After hydrolysis for 180 minutes, DH of about 43% could be achieved by Alcalase of 11.5 CDU/g substrate protein (i.e. 0.2% v/v) and by TSP of 38.6 CDU/g substrate protein (i.e. 0.4% v/v). Comparing TSP and Delvo-Pro, DH of about 31% could be achieved by TSP of 19.3 CDU/g substrate protein (i.e. 0.2% v/v) and by Delvo-Pro of 23.9 CDU/g protein (i.e. 0.3% v/v). Therefore, when casein was used as the substrate, Alcalase showed the highest capacity for hydrolysis of casein. TSP showed the hydrolysis capacity on casein between Alcalase and Delvo-Pro.

When soybean protein isolate was used as the substrate (Figure 42), similar profiles of DH as those with casein as substrate were observed when Delvo-Pro and TSP were applied. DH was increased with increasing E/S ratio. After hydrolysis for 180 minutes, DH of

soybean protein isolate reached 19.4%, 21.9%, 26.8% and 29.4% with Delvo-Pro and 8.4%, 12.1%, 18.5% and 20.4% with TSP while E/S ratio varied from 0.1% to 0.4%. The profile of DH with Alcalase using soybean protein isolate as the substrate was different from that using casein as substrate. Each increase of E/S ratio always promoted DH. DH of soybean protein isolate by Alcalase were 22.6%, 28.6%, 30.8% and 33.5% with E/S ratio of 0.1%, 0.2%, 0.3% and 0.4% at hydrolysis time of 180 minutes. When soybean protein isolate was used as the substrate, DH were lower than those using casein as substrate for all three proteases. The reason was probably that the conformation of casein and soybean protein isolate led to the difference on the number of accessible peptide bonds, then altered the hydrolysis rate (Kim *et al.*, 2007). A similar result was reported that the rate and extent of hydrolysis towards casein was substantially higher than those towards soybean protein when Protease As1.398 (from *B. subtilis*) was applied at pH 8.0 and 60°C (Guo *et al.*, 1996). Comparing these three proteases for hydrolysis of soybean protein isolate, Alcalase still showed the highest capacity. Delvo-Pro showed higher capacity than TSP. The results were confirmed by analysis of applied CDU and corresponding DH. After hydrolysis for 180 minutes, Alcalase of 5.7 CDU/g substrate protein (0.1% v/v) gave DH of 22.6% while Delvo-Pro of 8.0 CDU/g substrate protein (0.1% v/v) gave DH of 19.4%. TSP of 9.7 CDU/g substrate protein (0.1% v/v) only gave DH of 8.4%.

It should be noted that more decrease of DH was observed from TSP when the substrate was changed from casein to soybean protein isolate. In addition to the difference of peptide bonds between casein and soybean protein isolate and specificity of proteases led to the change of DH, the reason was also probably due to the residual activity of soybean trypsin inhibitor in soybean protein isolate. As mentioned above, TSP was trypsin-like serine protease. It was proved that soybean protein isolate still reserved soybean trypsin inhibitor of 1 to 30 mg/g depending on processing methods (Peace *et al.*, 1992). Therefore, TSP activity may be partially inhibited in soybean protein isolate solution. On the other hand, both TSP and Alcalase are serine proteases. However, it has been proved that Alcalase could inactivate soybean trypsin inhibitor (Huo *et al.*, 1993). As the result, the activity of Alcalase is not limited by soybean trypsin inhibitor.

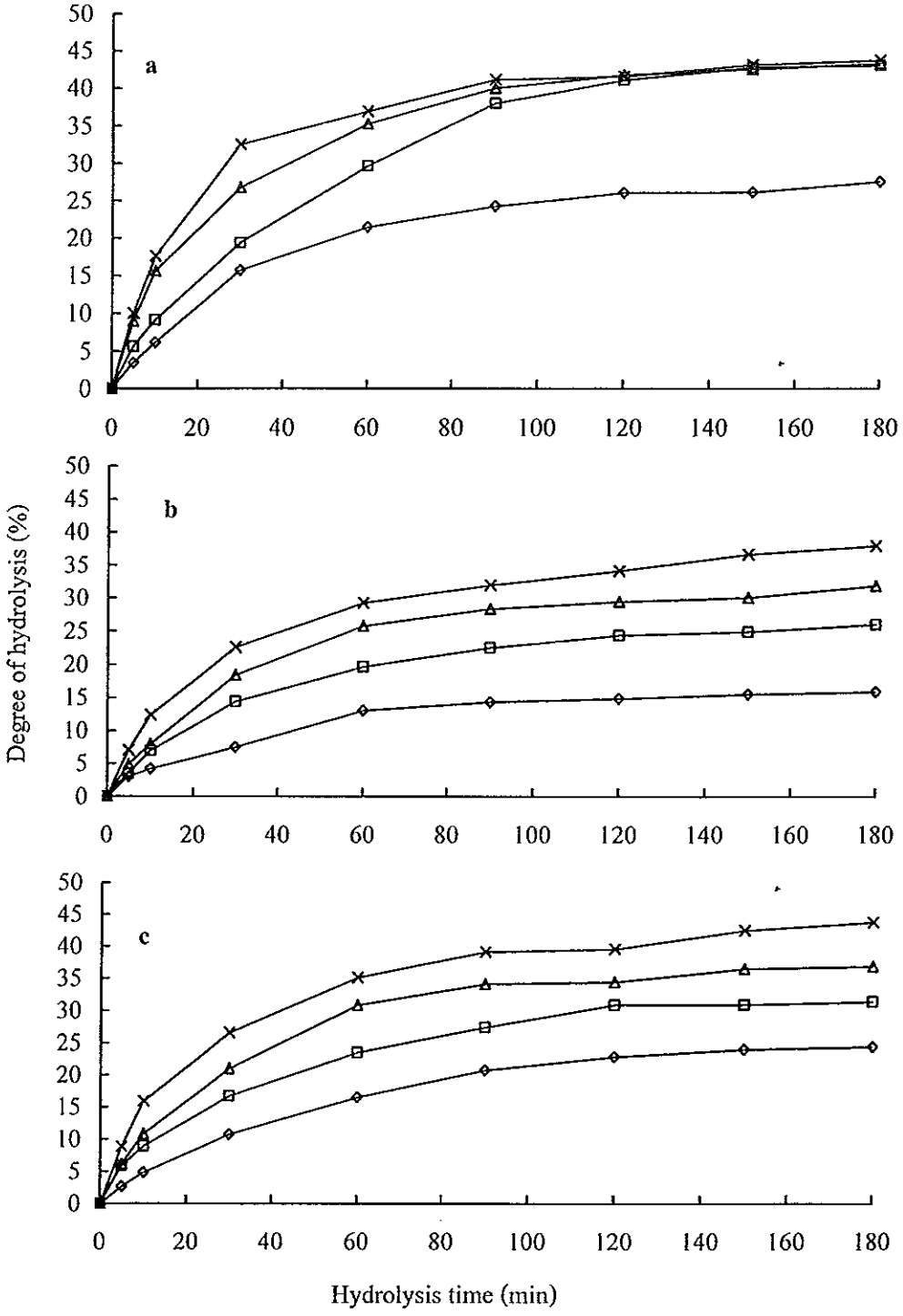

Figure 41. Effect of enzymes and E/S ratios on degree of hydrolysis of casein (a, Alcalase at 60°C and pH 8; b, Delvo-Pro at 50°C and pH 8; c, TSP at 50°C and pH 8; ◇ E/S 0.1%; □, E/S 0.2%; △, E/S 0.3%; ×, E/S 0.4%)

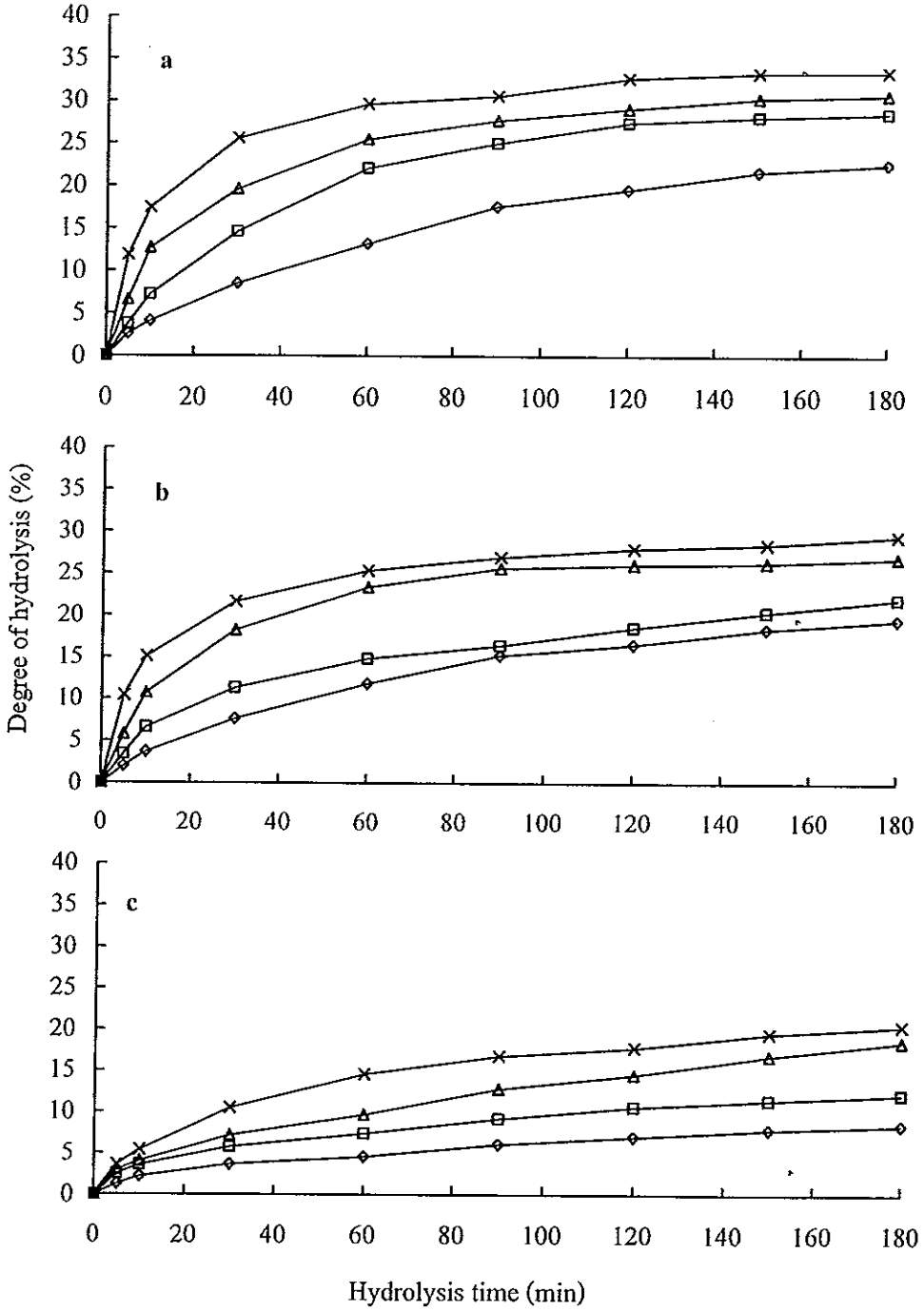

Figure 42. Effect of enzymes and E/S ratios on degree of hydrolysis of soybean protein isolate (a, Alcalase at 60°C and pH 8; b, Delvo-Pro at 50°C and pH 8; c, TSP at 50°C and pH 8; ◇ E/S 0.1%; □, E/S 0.2%; △, E/S 0.3%; ×, E/S 0.4%).

7.4.3 Analysis of hydrolysates by SDS-PAGE

The effect of enzymatic hydrolysis on protein breakdown in casein and soybean protein isolate after hydrolysis with E/S ratio of 0.4% (v/v) for 180 minutes was shown by SDS-PAGE (Figure 43). It can be noticed that the protein profiles were substantially changed in both casein and soybean protein isolate. Electrophoretic patterns indicated hydrolysis of casein was more extensive by Alcalase and TSP than by Delvo-Pro. Casein comprises genetic variants including α-casein, β-casein and κ-casein. The major bands at position with molecular weight about 36 kDa indicated these variants of casein (Lane C1). After hydrolysis for 180 minutes, almost complete degradations of α-, β- and κ-casein were achieved by Alcalase (Lane C2) and TSP (Lane C4). The variants of casein were no longer visible. The peptides formed were visible as some minor bands at the positions lower than the molecular weight of about 19 kDa. The proteolysis of casein was less intensive by Delvo-Pro. After hydrolysis of casein by Delvo-Pro for 180 minutes, the fragment of about 37 kDa was still remained while most fragments of variants of casein were disappeared (Lane C3). Additionally, more fragments at lower part of the gel (Lane C2) were still remained after hydrolysis with Delvo-Pro. The more extensive hydrolysis of casein with Alcalase and TSP may be attributed to their broader specificities in cleaving various peptide bonds. The greater hydrolysis of casein by Alcalase may be ascribed to its hydrolyzing ability on peptide bonds with adjacent aromatic AA residues (Kim *et al.*, 2007). Alcalase is a serine endoproteinase that constitutes a large class of microbial enzymes (Bryan, 2000). It has broad specificity that has been shown to cleave the peptide bonds at Gln^{4}-His^{5}, Ser^{9}-His^{10}, Leu^{15}- Tyr^{16}, and Tyr^{26}-Thr^{27} when incubated with the oxidized B-chain of insulin for 4 hours (Bryan, 2000; Adamson and Reynolds, 1996).. In the case of TSP, its major component was proved to be trypsin-like serine protease and chymotrypsin activity was also observed as well (Li *et al.*, 2006). Trypsin hydrolyses peptide bonds whose carbonyl function is donated by basic amino acids such as lysine or arginine. Chymotrypsin catalyses most effectively the hydrolysis of the peptide bonds on carboxyl side of amino acid residues with large hydrophobic side chains (e.g. phenylalanine, tryptophan or tyrosine) (Kumar *et al.*, 2004).

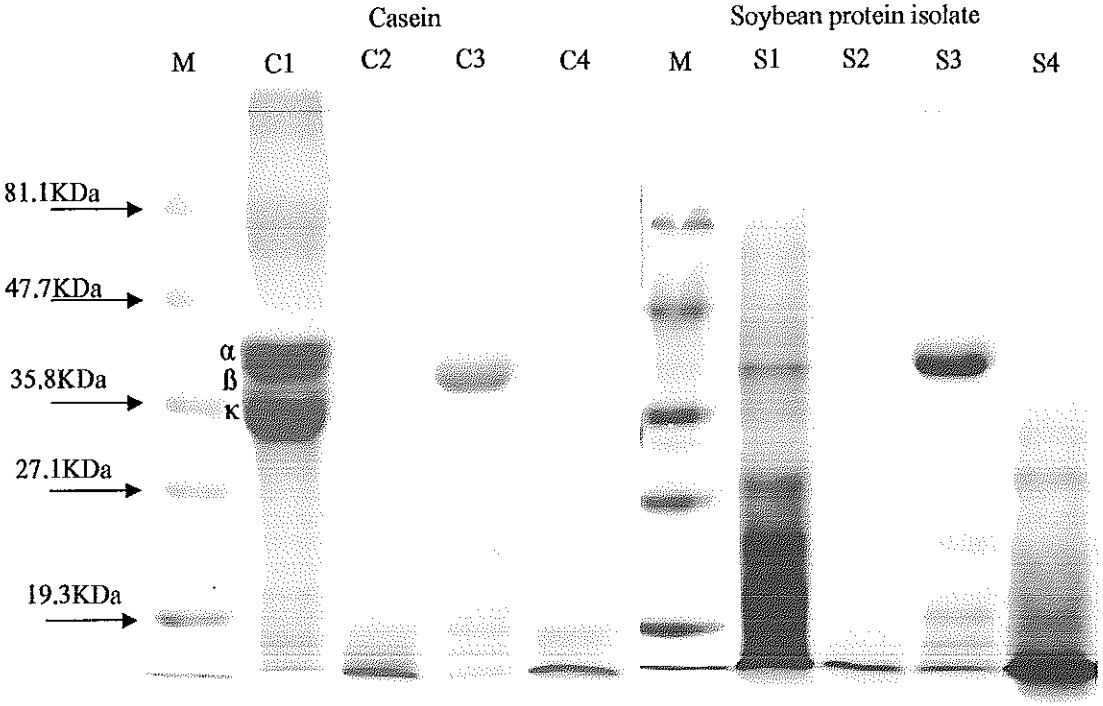

Figure 43. Electrophoretic analysis of hydrolysates with E/S ratio of 0.4% (v/v) after hydrolysis time of 180 minutes (SDS-PAGE with 12% gel and stained by Coomassie blue staining; Lane M, standard marker; Lane C, casein as substrate; Lane S, soybean protein isolate as substrate; Lane 1, protein without enzymatic hydrolysis; Lane2, Alcalase; Lane 3, Delvo-Pro; Lane 4, TSP).

Hydrolysis of soybean protein isolate was also proved by Figure 43. Soybean protein isolate showed one band at the position of molecular weight about 37 kDa. Most other protein in soybean protein isolate showed a group of bands lower than the position of molecular weight of about 29 kDa. As predicted by DH profile, Alcalase showed more intensive hydrolysis. TSP showed less intensive hydrolysis. The digestion of protein with high molecules (the dark area at the upper part of gel in Lane S1) was almost complete by Alcalase and Delvo-Pro. A fragment of about 37 kDa was visible after hydrolysis by Alcalase (Lane S2) and Delvo-Pro (Lane S3). Furthermore, this fragment looked more intense after hydrolysis by Delvo-Pro. It indicated that this fragment could be further digested by Alcalase rather than Delvo-Pro. When TSP was applied

for hydrolysis of soybean protein isolate, most protein at lower part of gel still remained. The hydrolysis of protein with high molecular weight by TSP yielded two fragments of 33.1 kDa and 29.5 kDa (Lane S4). However the fragment of about 37 kDa observed in hydrolysis by Alcalase and Delvo-Pro (Lane S2 and Lane S3) was absent in hydrolysis by TSP (Lane S4). It indicated that this fragment could be completely hydrolyzed by TSP.

7.5 Conclusion

The economical perspective of the process often limits the type and amount of enzymes which can be used in industrial protein hydrolysis. Therefore, microbial enzymes were widely applied for hydrolysis on industrial scale. Isolation and purification of trypsin-like serine proteases from tuna canning by-product provided a possible low-cost resource of animal-derived protease containing trypsin and chymotrypsin activity. The comparative study between commercial enzymes (Alcalase and Delvo-Pro) and the protease (TSP) purified from yellowfin tuna spleen by membrane filtration showed that TSP could provide an equal degree of hydrolysis of casein comparing to Alaclase. Its hydrolysis efficiency on casein was higher than Delvo-Pro. When soybean protein isolate was used as substrate, TSP showed lower hydrolysis efficiency than Alcalase and Delvo-Pro. In addition, membrane filtration is a technology which could further increase both concentration and purification of TSP easily. It is possible to apply membrane filtration to isolate and purify protease from yellowfin tuna spleen for industrial applications.

CHAPTER 8

SUMMARY AND FUTURE WORKS

8.1 Summary

In order to reduce the pollution of tuna canning wastes and improve the economical benefit of tuna canning industry, the viscera of tuna are considered as a potential resource for recovery of valuable compounds. The present work was to develop a multi-stages membrane filtration process to separate and purify proteases from yellowfin tuna spleen.

A dead-end microfiltration was applied initially to select optimal pore size of microfiltration membrane. The effect of pretreatment method (i.e. pre-centrifugation and simple pre-filtration), transmembrane pressure and membrane pore size on fouling mechanism, filtration and separation performances were studied. The short membrane resistance-limited stage occurred at initial period of filtration. 0.10 μm membrane showed shorter pore blocking resistance-limited stage than 0.22 and 0.45 μm membranes. The pore blocking dominated the major part of flux decline while the cake resistance-limited stage dominated most periods of filtration process for all three membranes. 0.10 μm membrane showed the highest steady-state flux. The difference of cake resistance-limited stages among membranes was minimized by higher transmembrane pressure. Recovery rate of protease was more than 90% by using 0.10 μm membranes. Increasing both membrane pore size and transmembrane pressure caused loss of protease activity. According to the permeate flux, fouling extent and recovery of protease, 0.10 μm membrane was selected as optimal pore size of microfiltration membrane. A longer pore-blocking resistance-limited stage and earlier cake resistance-limited stage were observed when pre-centrifugated extract was used. Therefore, the simple pre-filtration could be employed as pre-treatment method before microfiltration process.

A crossflow microfiltation with a 0.10 μm hollow fiber membrane was applied to remove suspended particles in pre-filtered extract. Since the proteases could pass microfiltration, the permeate should be collected for further recovery of proteases. A total recycle crossflow microfiltration was applied to investigate critical flux and corresponding critical transmembrane pressure before concentration process. The critical flux was increased from 7.6 to

20.9 l/m^2.h while crossflow velocity was increased from 0.2 to 0.8 m/s. The critical TMP for each critical flux ranged from 0.05 to 0.1 bar at each given crossflow velocity.

According to the results from total recycle crossflow microfiltration, a conventional concentration process, called single-batch concentration crossflow microfiltration was operated with a sub-critical condition (transmembrne pressure of 0.03 bar) and a supra-critical condition (transmembane pressure 0.15 bar). Comparing to supra-critical condition, sub-critical condition did not show distinct advantage on both fouling and flux in single-batch concentration process. Actually, a higher steady-state flux was observed when 0.15 bar was applied. In addition, a decay of protease activity in feed was observed. It was due to denaturation of protease during long-term recirculation of fluid in the system.

Fouling mechanism was more complex during single batch concentration process than that during total recycle process. The present work intended to model a low fouling condition during single batch concentration process. A low fouling condition was experimentally evaluated based on the definition of critical ratio of flux (J) to wall shear stress (τ_w). Transmembrane pressure of 0.15 bar was fixed through the single batch concentration crossflow microfiltraion. A power relation between volume concentration factor (VCF) and critical J/τ_w was found as $J/\tau_w = 3.29\ (VCF)^{-0.74}$. Once a wall shear stress and a desired concentration rate were decided, a critical flux could be worked out. If the flux was controlled under critical flux, the fouling was negligible. The severe fouling could occur if flux exceeded critical flux. However, this model was based on a single batch concentration process. The high concentration rate would cause a low permeate flux, i.e. a low efficiency of process. This problem could be more severe in the industrial process with a large feed volume. Therefore, more practical method should be studied for further industrial applications.

A newly designed process, called continuous-batch concentration was applied at transmembrane pressure of 0.15 bar and a crossflow velocity of 0.2 m/s. Permeate was removed while the feed volume was kept constantly by adding spleen extract. The holding time of solutes in the system and negative effect of shear stress on protease activity were reduced. Furthermore, a gas sparing technique was applied as well to reduce concentration polarization and fouling. Gas injection factor of 0.38 gave a 300% improvement of flux comparing that of continuous-batch concentration without gas sparging. High gas injection factor (> 0.38) did not show capacity to

improve flux and caused a reduction of transmission of solutes. After continuous-batch concentration with crossflow velocity of 0.2 m/s, transmembrane pressure of 0.15 bar and gas injection factor of 0.38, a clear permeate with slight yellow colour was obtained. Transmission of proteases was about 1 while all suspended particles and some proteins were removed.

Ultrafiltration was applied for recovery of protease from microfiltration pre-treated tuna spleen extract. The effects of hydrodynamics and gas sparging on critical flux (J_{crit}), limiting flux (J_{lim}) and selectivity were studied by a total recycle crossflow ultrafiltration with a hollow fiber membrane. All critical fluxes observed in this study were weak form. It was observed that increasing crossflow rate enhanced J_{crit} and J_{lim}. The benefit of increasing crossflow rate to enhance J_{crit} and J_{lim} was greater at the conditions without gas sparging. It was also found that the enhancement of both J_{crit} and J_{lim} was more significant at low gas injection factor if a fixed crossflow rate was applied and gas injection was more effective at low crossflow rate. J_{crit} varied from 28.8 to 44.2 l/m^2.h and J_{lim} varied from 34.3 to 52.4 l/m^2.h while crossflow rate increased from 17.55 to 69.98 l/h without gas sparging. A low gas injection factor of 0.15 could enhance both critical flux and limiting flux significantly. Both J_{crit} and J_{lim} presented linear relation to crossflow rate. Injection of gas did not change the linear relation between J_{crit}, J_{lim} and crossflow rate but altered the slopes of these linear lines. When gas sparging was not applied, selectivity was proportional to the permeate flux in both sub-critical and critical flux conditions. Selectivity was not sensitive to permeate flux or crossflow rate at limiting flux condition and it was lower than the selectivity under critical flux condition. Gas sparging gave negative effect on selectivity at both sub-critical and critical flux conditions. At limiting flux condition, gas injection factor of 0.15 increased selectivity while crossflow rate and other gas injection factors showed little effect on selectivity. The highest critical flux without gas sparging could be attained at crossflow rate of 69.98 l/h and transmembrane pressure of 1.6 bar. Gas sparging could enhance this critical flux. However, gas sparging gave negative effect on selectivity. An altered method should be considered.

Diafiltration mode was considered as an effective operation for purification process. The diafiltration process could be maintained at sub-critical condition or critical flux condition with decreasing feed concentration if a critical flux condition was applied at the beginning of process. In this study, an altered method, i.e. mode 1 (pre-diafiltration followed by a

post-concentration) and a conventional method, i.e. mode 2 (pre-concentration followed by a post-diafiltration) were tested. A critical flux condition, i.e. crossflow rate of 69.98 l/h and transmembrane pressure of 1.6 bar was applied at the beginning of operation of both modes. Each step was terminated at diafiltration volume of 3 (for pre- or post-diafiltration) or concentration factor of 2 (for pre- or post-concentration), respectively. In the operation of mode 1, the flux was nearly constant during pre-diafiltration. The value of flux was close to the critical flux of 44.2 $l/m^2.h$. About 24.6% decline of flux was observed after post-concentration. In the operation of mode 2, about 59% decline of flux occurred after pre-concentration. The flux at the end of post-diafiltration was 5.15 $l/m^2.h$. The total running time was 3.98 hours for mode 1 and 9.04 hours for mode 2. The operation of mode 1 showed its advantage of filtration efficiency. About 12-fold of purification was achieved by operation of mode 1 while about 2-fold purification was achieved by operation of mode 2.

The protease separated and purified from yellowfin tuna spleen (TSP) was applied for protein hydrolysis. Two commercial proteases including Alcalase@ 0.6L and Delvo-Pro were also applied for protein hydrolysis. For all enzymes applied, degree of hydrolysis increased rapidly in initial 30 minutes, and tended towards plateau after hydrolysis of about 120 minutes. When casein was used as the substrate, about 43% degree of hydrolysis was achieved by Alcalase with E/S ratio of 0.2% (v/v) and TSP with E/S ratio of 0.4%(v/v) after hydrolysis for 180 minutes. Delvo-Pro showed lower capacity to hydrolyse casein than Alcalase and TSP. In the case of soybean protein isolate as substrate, TSP showed lowest capacity for hydrolysis. The reason was probably due to the soybean trypsin inhibitor in soybean protein isolate.

The present study proved that membrane filtration could separate and partially purify proteases from yellowfin tuna spleen. A multi-stages membrane filtration was designed. Optimal parameters of operation were selected step by step. Comparing to the amount and purity of protease in raw tuna spleen extract, a recovery rate of 79% and purification of 13.60-fold of protease were achieved after multi-stages membrane filtration including both microfiltration and ultrafiltration. The capacity of membrane filtration to yield this protease was relatively large and the process is easier to scale up for industrial applications. The protease separated and purified by membrane filtration from yellowfin tuna spleen showed a desirable ability for protein hydrolysis. This protease showed its commercial potential for protein hydrolysis.

8.2 Future works

1. Effect of different membrane modules (such as tubular membrane) on both fouling mechanism and product recovery should be studied.

2. The relation between different membrane materials and fouling should be studied.

3. A process to scale up the process for industrial application should be investigated.

4. An application of membrane bioreactor for continuous hydrolysis of protein and recovery hydrolysates should be studied.

REFERENCES

Abazinge, M.D.A., Fontenot, J.P. and Allen, V.G. 1994. Digestibility, nitrogen utilization, and voluntary intake of ensiled crab waste-wheat straw mixtures fed to sheep. J. Anim. Sci. 72: 565-571.

Abdel-Ghani, M.S. 2000. Cross flow ultrafiltration of an aqueous polymer foam solution produced by gas sparging. J. Membr. Sci. 171: 105-117.

Abdul-Hamid, A., Bakar, J. and Bee, G.H. 2002. Nutritional quality of spray dried protein hydrolysate from Black Tilapia (*Oreochromis mossambicus*). Food Chem. 78: 69-74.

Adamson, N.J., Reynolds, E.C. 1996. Characterization of casein phosphopeptides prepared using Alcalase: determination of enzyme specificity. Enzyme Microbial. Technol. 19:202-207.

Adler-Nissen, J., 1977. Enzymatic hydrolysis of fish proteins. Process Biochem. 12(32), 18–23.

Adler-Nissen, J. 1986. Enzymatic hydrolysis of food proteins. Elsevier Applied Science Publishers Ltd. London.

Afonso, M.D. and Borquez, R. 2002. Review of the treatment of seafood processing wastewater and recovery of proteins therein by membrane separation processes-prospects of the ultrafiltration of wastewaters from the fish meal industry. Desalination 142: 29-45.

AOAC. 1999 *In* Official Methods of Analysis of AOAC. International, 16th ed. (ed. Cunnif, P.). AOAC International, Alrington, VA, USA.

Asselin, J., Amiot, J., Gauthier, S., Mourad, W., Hebert, J. 1988. Immunogenicity and allergenicity of whey protein hydrolysates. J. Food Sci. 53: 1208-1211.

Bacchin, P. 2004. A possible link between critical and limiting flux for colloidal systems: consdideration of critical deposit formation along a membrane. J. Membr. Sci. 228: 237-241.

Bacchin, P., Aimar, P. and Field, R.W. 2006. Critical and sustainable fluxes: theory, experiments and applications. J. Membr. Sci. 281: 42-69.

Bai, R.B. and Leow, H.F. 2001. Microfiltration of polydispersed suspension by a membrane screen-hollow fiber composite module. Desalination. 140: 277-287.

Bake, H.H. and Cadwallader, K.R. 1995. Enzymatic hydrolysis of crayfish processing by-products. J. Food Sci. 60: 929-935.

Belfort, G., Davis, R.H., and Zydney, A.L. 1994. The behavior of suspensions and macromolecular solutions in crossflow microfiltration. J. Membr. Sci. 96: 1-58.

Bellhouse, B.J., Costigan, G., Abhinava, K. and Meny, M. 2001. The performance of helical screw-thread inserts in tubular membrane. Sep. Purif. Technol. 22-23: 89-113.

Beolchini, F., Veglio, F. and Barba, D. 2004. Microfiltration of bovine and ovine milk for the reduction of microbial content in a tubular membrane. Desalination 161: 251-258.

Berge, G.M. and Storebakken, T. 1996. Fish protein hydrolysate in starter diets for Atlantic salmon *Salmo salar* fry. Auqaculture 145: 205-212.

Bezerra, R.S., Ssanto, J.F., Paiva, P.M.G., Correia, M.T.S., Coelho, L.C.B.B., Vieira, V.L.A., Carvalho, L.B. 2001. Partial purification and characterization of a thermostable trypsin from pyloric caeca of tambaqui (*Colossoma macropomum*). J. Food Biochem. 25: 199-210.

Bhaskar, N., Benila, T., Radha, C.and Lalitha, R.G. 2008. Optimization of enzymatic hydrolysis of visceral waste proteinsof Catla (*Catla catla*) for preparing protein hydrolysate using a commercial protease. Bioresour. Technol. 99: 335-343.

Bowen, W.R. and Gan, Q. 1991. Properties of microfiltration membrane: flux loss during constant pressure permeation of bovine serum albumin. Biotechnol. Bioeng. 38: 688-696.

Bowen, W.R. and Gan, Q. 1992. Properties of microfiltration membrane: the effects of adsorption and shear on the recovery of an enzyme. Biotechnol. Bioeng. 40: 491-497.

Brummer, W. and Gunzer, G. 1987. Laboratory techniques of enzyme recovery. *In* Biotechnoogy. 7a (ed. Kennedy, J.F.). p. 255-257, VCH, New York, USA.

Bryan, P.N. 2000. Protein engineering of subtilisin. Biochim. Biophys. Acta. 1543: 203–222.

Bucci, L.R., Unlu, L. 2000. Protein and amino acid supplements in exercise and sport. *In* Energyyielding Macronutrients and Energy Metabolism in Sports Nutrition (eds. Wolinsky, I., Driskell, J.A., Boca Raton, F.L.). p.191-212, CRC Press., FL, USA.

Bustos, R.O., Romo, C.R. and Healy, M.G. 1999. Purification of trypsin-like enzymes from Antarctic krill processing. Process Biochem. 35: 327-333.

Cabassaud, C., Laborie, S. Durand-Bourlier, L. and Laine, J.M. 2001. Air sparging in ultrafiltration hollow fibers: relationship between flux enhancement, cake characteristics and hydrodynamic parameters. J. Membr. Sci. 181: 57-69.

Camera Hawaii, Inc. 1995. http://www.state.hi.us/dbedt/seafood/yellowfin.html.

Cano-Lopez, A., Simpson, B.K. and Haard, N.F. 1987. Extraction of carotenoprotein from shrimp process wastes with the aid of trypsin from Atlantic cod. J. Food Sci. 52:503-506.

Carcia-Carreno, F.L., Dimes, L.E., Haard, N.F. 1993. Substrate-gel electrophoresis for composition and molecular weight of proteinases or proteinaceous proteinase inhibitor. Anal. Biochem. 214, 65-69.

Carvalho, A.P., Escaffre, A.M., Oliva-Teles, A. and Bergot, P. 1997. First feeding of common carp larvae on diets with high levels of protein hydrolysates. Aquacult. Int. 5: 361-367.

Chang, I.S. and Judd, J.S. 2002. Air sparging of a submerged MBR for municipal wastewater treatment. Process Biochem. 37: 915-920.

Cheng, T.W., Yeh, H..M. and Gau, C.T. 1998. Enhancement of permeate flux by gas slugs for crossflow ultrafiltration in tubular membrane module. Sep. Sci. Technol. 33: 2295-2309.

Cheng, T. W. and Lin, T.L. 2001. Characteristics of gas-liquid two-phase flow in small diameter inclined tubes. Chem. Eng. Sci. 56: 6393-6398.

Cheryan, M. and Rajagopalan, N. 1998. Membrane processing of oily streams wastewater treatment and waste reduction. J. Membr. Sci. 151: 13-28.

Chiang, W.D., Shih C.J. and Chu Y.H. 1999. Functional properties of soy protein hydrolysate produced from a continuous membrane reactor system. Food Chem. 65: 189-194.

Chiou, S.H. and Wang, K.T. 1988. Simplified protein hydrolysis with methanesulphonic acid at elevated temperature for the complete amino acid analysis of protein. J. Chromatogr. 448: 404-410.

Chiu, T.Y., Dominguez, M.V.L. and James, A.E. 2006. Critical flux and rejection behaviour of non-circular-channelled membranes Influence of some operating conditions. Sep. Purif. Technol. 50: 212-219.

Chiu, T.Y. and James, A.E. 2005. Critical flux determination of non-circular multichannel ceramic membranes using TiO2 suspensions. J. Membr. Sci. 254: 295-301.

Chiu, T.Y. and James, A.E. 2006. Critical flux enhancement in gas assisted microfiltration. J. Membr. Sci. 281: 274-280.

Cho, C.W., Lee, D.Y. and Kim, C.W. 2003. Concentration and purification of soluble pectin from mandarin peels. Carbohydr. Polym. 54: 21-26.

Chong, A.S.C., Hashim, R., Chow-Yang, L. and Ali, A.B. 2002. Partial characterization and activities of proteases from the digestive tract of discus fish (*Symphysodon aequifasciata*). Aquaculture 203:321-331.

Chutinut, S. 1997. Cultivation of yeast in tuna condensate after protein and fat separation. Master of Science Thesis in Biotechnology, Faculty of Agro-Industry. Prince of Songkla University.

Constenla, D.T. and Lozano, J.E. 1997. Hollow fibre ultrafiltration of apple juice macroscopic approach. Lebensm. Wiss. u. Technol. 30: 373-378.

Cui, Z. F. and Wright, K. I. T. 1994. Gas-liquid two-phase cross-flow ultrafiltration of BSA and dextran solutions. J. Membr. Sci. 90: 183.

Cui, Z.F. and Wright, K.I.T. 1996. Flux enhancement with gas sparging in downwards crossflow ultrafiltration: performance and mechanism, J. Membr.Sci., 117: 109.

Cui, Z. F., Bellara, S. R. and Homewood, P. 1997. Airlift crossflow membrane filtration: a feasibility study with dextran ultrafiltration. J. Membr. Sci. 128: 83-91.

Cui, Z.F., Chang, S. and Fane, A.G. 2003. The use of gas bubbling to enhance membrane processes. J. Membr. Sci. 221: 1-35.

Czekaj, P., Lopez, F. and Guell, C. 2000. Membrane fouling during microfiltration of fermented beverage. J. Membr. Sci. 166: 199–212.

Davis, R.H. 1992. Microfiltration *In* Membrane Handbook (eds. Ho, W.S.W and Sirkar, K.K.). p 457-458, Van Nostrand Reinhold, New York, USA.

Delgado-Zamarreno, M.M., Sanchez-Perez, A., Gomez-Perez, M.C. and Hernandez-Mendez, H. 1995. Directly coupled sample treatment-high-performance liquid chromatography for on-line automatic determination of liposoluble vitamins in milk. J. Chromatogr. 694: 399-406.

Dengxi, W., Howell, J.A. and Field, R.W. 1999. Critical flux measurement for model colloids. J. Membr. Sci. 152: 89-98.

Dewitt, C.A.M. and Morrissey, M.T. 2002a. Parameters for the recovery of proteases from surimi wash water. Bioresour. Technol. 81: 241-247.

Dewitt, C.A.M. and Morrissey, M.T. 2002b. Pilot plant recovery of catheptic proteases from surimi wash water. Bioresour. Technol. 82: 295-301.

Diaz-Lopez, M., Moyano-Lopez, F.J., Alarcon-Lopez, F.J., Garcia-Carreno, F.L. and Toro, M.A.N.D. 1998. Characterization of fish acid proteases by substrate-gel electrophoresis. Comp. Biochem. Physiol. B. 121: 369-377.

Ding, L.H., Al-Akoum, O., Abraham, A. and Jaffrin, M.Y. 2002. Milk protein concentration by ultrafiltration with rotating disk modules. Desalination 144: 307-311.

Diouf, T. and Kothias, J.B.A. 1993. Systematic presentation and geographical Distribution of species. *In* Resources: Fishing and Biology of The Tropica Tunas of The Easterri Central Atlantic (eds. Fonteneau, A. and Marcille, J.). p. 3-5, Food and Agriculture Organization of The United Nations, Rome, Italy.

Dutre, B. and Tragardh, G. 1994. Macrosolute–microsolute separation by ultrafiltration: a review of diafiltration processes and applications. Desalination 95: 227–267.

Dutre, B. and Tragardh, G. 1995. Purification of gelatin by ultrafiltration with a forced solvent stream along the membrane permeate side: an experimental approach. J. Food Eng. 25: 233-244.

Dziezak, J.D. 1986. Biotechnology and flavor development: enzyme modification of dairy products. Food Technol. 40: 114-120.

Dziuba, J., Nalecz, D., Minkiewicz, P., Dziuba, B. 2004. Identification and determination of milk and soybean protein preparations using enzymatic hydrolysis followed by chromatography and chemometrical data analysis. Anal. Chim. Acta. 521: 17-24.

Ehsani, N., Parkkinen, S. and Nystrom, M. 1997. Fractionation of natural and model egg-white protein solutions with modified and unmodified polysulfone UF membrane. J. Membr. Sci. 123: 105-119.

Espinasse, B., Bacchin, P. and Aimar, P. 2002. An experimental method to measure critical flux in ultrafiltration. Desalination 146: 91-96.

Evers, D.J. and Carroll, D.J. 1996. Preservation of crab or shrimp waste as silage for cattle. Anim. Feed Sci. Tech. 59: 233-244.

Ferrer, J., Paez, G., Marmol, Z., Ramones, E., Garcia, H. and Forster, C.F. 1996. Acid hydrolysis of shrimp shell waste and the production of single cell protein from the hydrolysate. Bioresour. Technol. 57: 55-60.

Field, R.W., Wu, D., Howell, J.A. and Gupta, B.B. 1995. Critical flux concept for microfiltration fouling. J. Membr. Sci. 100: 259-272.

Fischer, M., Kofod, L.V., Schols, H.A., Piserma, S.R., Gruppen, H. and Voragen, A.G.J. 2001. Enzymatic Extractability of Soybean Meal Proteins and Carbohydrates: Heat and Humidity Effects. J. Agric. Food Chem. 49: 4463-4469

Fonkwe, L.G. and Singh, R.K. 1996. Protein recovery from mechanically deboned Turkey residue by enzymatic hydrolysis. Process Biochem. 31:605-616.

Food market exchange. 2003. http:// www. Foodmarketexchange.com/

Fountoulakis, M. and Lahm, H.W. 1998. Hydrolysis and amino acid composition analysis of proteins. J. Chomatogr. 826: 109-134.

Frokjear, S. 1994. Use of hydrolysates for protein supplementation. Food Technol. 48: 86-88. Gildberg, A. 1992. Recovery of proteinases and protein hydrolysates from fish viscera. Bioresour. Technol. 39: 271-276.

Furukawa, T. and Fukano, T. 2001. Effects of liquid viscosity on flow patterns in vertical upward gas-liquid two-phase flow. Int. J. Multiphase Flow. 27:1109-1126.

Geasan-Guiziou, G, Boyaval, E. and Daufin, G. 1999. Critical stability conditions in crossflow microfiltration of skimmed milk: transition to irreversible deposition. J. Membr. Sci. 158: 211-222.

Ghosh, R. and Cui, Z.F. 2000. Simulation study of the fractionation of proteins using ultrafiltration. J. Membr. Sci. 180: 29-36.

Ghosh, R., Silva, S.S. and Cui, Z.F. 2000. Lysozyme separation by hollow-fibre ultrafiltration. Biochem. Eng. J. 6: 19-24.

Ghosh, R. 2006. Enhancement of membrane permeability by gas-sparging in submerged hollow fiber ultrafiltration of macromolecular solutions: role of module design. J. Membr. Sci. 274: 73-82.

Gildberg, A. 1992. Recovery of proteinase and protein hydrolysates from fish viscera. Bioresour. Technol. 39, 271-276.

Gildberg, A. 1993. Enzymatic processing of marine raw materials. Process Biohem. 28: 1-15.

Gildberg, A. and Shi, X.Q. 1994. Recovery of tryptic enzymes from fish sauce. Process Biochem. 29: 151-155.

Gildberg, A. and Stenberg, E. 2001. A new process for advanced utilization of shrimp waste. Process Biochem. 36: 809-812.

Girard, B and Fukumoto, L.R. 2000. Membrane processing of fruit juices and beverages. Crit. Rev. Food Sci. 40: 91-157.

Guell, C. and Davis, R.H. 1996. Membrane fouling during microfiltration of protein mixtures, J. Membr. Sci. 119: 269–284.

Guerard, F., Dufosse, L., Broise, D.D.L. and Binet, A. 2001. Enzymatic hydrolysis of proteins from yellowfin tuna (*Thunnus albacares*) wastes using Alcalase. J. Mol. Catal. B. 11: 1051-1059.

Guerard, F., Guimas, L., and Binet, A. 2002. Production of tuna waste hydrolysates by a commercial neutral protease preparation. J. Mol. Catal. B-Enzym. 19-20: 489-498.

Guo, M.L., Jiang, M.Y., Ma Z.L., Li Y.L. 1996. Hydrolytic characteristics of chitosan-immobilized As 1.398 neutral proteinase (from *B. srcbtilis*) to soybean protein. Food Chem. 55: 373-377.

Gupta, R.K., Chang, A.C., Griffin, P., Rivera, R., Guo Y.Y. and Siber, G.R. 1997. Determination of protein loading in biodegradable polymer microspheres containing tetanus toxoid. Vaccine 15: 672-676.

Haard, N.F. 1998. Specialty enzymes from marine organisms. Food Technol. 52: 64-67.

Hayashi, R. and Suzuki, F. 1985. Determination of methionine sulfoxide in protein and food by hydrolysis with p-toluenesulfonic acid. Anal. Biochem. 149: 521-528.

H-Kittikun, A. 2003. Enrichment of n-3 polyunsaurated fatty acids in tuna oil. In In International Seminar: Effective Utilization of Marine Food Resources. 18th December, 2003. Prince of Songkla University. Hat Yai. Thailand.

Henderson, L.W. Besarab A., Michaels, A. and L.W. Bluemle. 2004. Blood purification by ultrafiltration and fluid replacement (diafiltration). Hemodialysis Int. 8: 10-18.

Hermia, J. 1982. Constant pressure blocking filtration laws: application to power-law non-Newtonian fluids. Trans. Chem. Eng. 60: 183-187.

Heu, M.S., Kim, H.R. and Pyeun, J.H. 1995. Comparison of trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*). Comp. Biochem. Physiol. B. 112: 557-567.

Howell, J.A. 1995. Sub-critical flux operation of microfiltration. J. Membr. Sci. 107: 165-171.

Howell, J.A., Chua, H.C. and Arnot, T.C. 2004. In situ manipulation of critical flux in a submerged membrane bioreactor using variable aeration rates and effects of membrane history. J. Membr. Sci. 242: 13-19.

Huet, J.C. and Penollet, J.C. 1986. Chromatographic separation and determination of tryptophan in foodstuffs after barytic hydrolysis using Fractogel TSK HW 40 S°. J. Chromatogr. 355:451-455.

Huo, G.C., Fowler, V.R., Inborr, J. and Bedford, M.R. 1993. Enzymes to denature antinutritive factors in soybean. *In* Recent Advanced Research in Antinutritional Factors in Grain Legume Seeds (eds. Huisman PJ, Saini H). p. 517-521, EAAP Publication, Wageningen Press, The Netherlands.

Iulek, J. and Carcamo, A.M. 2001.The influence of precipitant and thaumatin concentration on the protein aggregation state at low ionic strength. Biol. Health Sci. 7(1): 91-98.

Jeantet, R., Rodriguez, J. and Garem, A. 2000. Nanofiltration of sweet whey by spiral wound organic membranes: impact of hydrodynamics. Le Lait, 80: 155-163.

Jim, K., Fane, A.G., Fell, C.J.D. and Joy, D.C. 1992. Fouling mechanisms of membranes during protein ultrafiltration. J. Membr. Sci. 68: 79-91.

Kaichang, Y., Xianghua, W., Qingjie, B. and Huang, X. 2003. Critical flux enhancements with air sparging in axial hollow fibers cross-flow microfiltration of biologically treated wastewater. J. Membr. Sci. 224: 69-79.

Karamac, M., Amarowicz, R., Kostyra, H. 2002. Effect of temperature and enzyme/substrate ratio on the hydrolysis of pea protein isolates by trypsin. Czech. J. Food Sci. 20, 1-6.

Keskinler, B., Yildiz, E., Erhan, E., Dogru, M., Bayhan, Y.K. and Akay, G. 2004. Crossflow microfiltration of low concentration-nonliving yeast suspensions. J. Membr. Sci. 233: 59-69.

Kim, S.B., Seo, I.S., Khan, M.A., Ki, K.S., Lee, W.S., Lee, H. J., Shin, H. S., Kim, H. S. 2007. Enzymatic hydrolysis of heated whey: iron-binding ability of peptides and antigenic protein fractions. J. Dairy Sci. 90: 4033–4042.

Kintz P. and Cirimele, V. 1997. Interlaboratory comparison of quantitative determination of amphetamine and related compounds in hair samples. Forensic Sci. Int. 84:151-156.

Klomklao, S., Benjakul, S., Visessanguan, W., 2004. Comparative studies on proteolytic activity of splenic extract from three tuna species commonly used in Thailand. J. Food Biochem.28: 355-372.

Klomklao, S., Benjakul, S., Visessanguan, W., Simpson, B.K., Kishimura, H. 2005. Partitioning and recovery of proteinase from tuna spleen by aqueous two-phase systems. Process Biochem. 40: 3061-3067.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H., Simpson, B.K. and Saeki, H. 2006. Trypsins from yellowfin tuna (*Thunnus albacores*) spleen: Purification and characterization. Comp. Biochem. Physiol. B. 144, 47-56.

Klomklao, S., Benjakul, S., Visessanguan, W., Kishimura, H. and Simpson, B.K. 2007. Purification and characterisation of trypsins from the spleen of skipjack tuna (*Katsuwonus pelamis*). Food Chem. 100, 1580-1589.

Kristinsson, H.G., Rasco, B.A., 2000. Biochemical and functional properties of Atlanticsalmon (*Salmo salar*) muscle proteins hydrolyzed with various alkaline proteases. J. Agric. Food Chem. 48: 657-666.

Kuberkar, V.T. and Davis, R.H. 2001. Microfiltration of protein-cell mixture wih crossflushing or backflushing. J. Membr. Sci. 183: 1-14.

Kulkarni, S.S., Funk, E.W. and Li, N.N. 1992. Ultrafiltration *In* Membrane Handbook (eds. Ho, W.S.W and Sirkar, K.K.). p. 457-458, Van Nostrand Reinhold, New York, USA.

Kumar, R., Choudhary, V., Mishra, S and Varma I.K. 2004. Enzymatically modified soy protein. J. Thermal. Anal. Cal. 75: 727–738.

Kuruzovich, J.N. and Piergiovanni, P.R. 1996. Yeast cell microfiltration: optimization of backwashing for delicate membranes. J. Membr. Sci. 112: 241-247.

Kwon, D.Y , Vigneswaran, S., Fane, A.G. and Aim, R.B. 2000. Experimental determination of critical flux in cross-flow microfiltration. Sep. Purif. Technol. 19: 169-181.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laborie, S., Cabassud, C., Durand-Bourlier, L. and Laine, J.M. 1999. Characterisation of gas-liquid two-phase flow inside capillaries. Chem. Eng. Sci., 54: 5723-5735.

Lahl, W., Braum S. 1994. Enzymic production of protein hydrolysates for food use. Food Technol. 48: 8-71.

Le Berre, O. and Daufin, G. 1999. Skim milk crossflow microfiltration performance versus permeation flux to wall shear stress ratio. J. Membr. Sci. 117: 261-270.

Li P., Wang, X.X., Hardy, R.W. and Gatlin, D.M. 2004. Nutritional value of fisheries by-catch and by-product meals in the diet of red drum (*Sciaenops ocellatus*). Aquaculture 236: 485-496.

Li, Q.Y., Cui, Z.F. and Pepper, D.S. 1997a. Effect of bubble size and frequency on permeate flux of gas sparged ultrafiltration with tubular membranes. Chem. Eng. J. 67: 71-75.

Li, Q.Y., Cui, Z.F. and Pepper D.S. 1997b. Fractionation of HSA and IgG by gas sparged ultrafiltration. J. Membr. Sci. 136: 181-190.

Li, Q.Y., Ghosh, R., Bellara, S.R., Cui, Z.F. and Pepper, D.S. 1998. Enhancement of ultrafiltration by gas sparging with flat sheet membrane modules. Sep. Purif. Technol. 14: 79-83.

Li, Z.Y., Youravong, W. and H-Kittikun A. 2006. Separation of proteases from yellowfin tuna spleen by ultrafiltration. Bioresour. Technol. 97: 2364-2370.

Li, Z.Y., H-Kittikun, A. and Youravong, W. 2007. Separation of Protease from Yellowfin Tuna Spleen Extract by Ultrafiltration: Effect of Hydrodynamics and Gas Sparging on Flux Enhancement and Selectivity. J. Membr. Sci. In press.

Li, Z.Y., H-Kittikun, A. and Youravong, W. 2008. Removal of suspended solids from tuna spleen extract by microfiltration: a batch process design and improvement. Biochem. Eng. J.38: 226-233.

Lim, A.L. and Bai, R.B. 2003 Membrane fouling and cleaning in microfiltration of activated sludge wastewater. J. Membr. Sci. 216: 279-290.

Lo, Y. M., Cao, D., Argin-Soysal, Wang, S., J. and Hahm, T. 2005. Recovery of protein from poultry processing wastewater using membrane ultrafiltration. Bioresour. Technol. 96: 687-698.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with

Folin phenol reagent. J. Biochem. 193:265-275.

Lu, D.R. and Park, K. 1991. Effect of surface hydrophobicity on the conformational changes of adsorbed fibrinogen. J. Colloid Interf. Sci. 144(1): 271.

Lu, W.M., Hwang, K.J. and Ju, S.C. 1993. Studies on the mechanism of crossflow filtration.Chem. Eng. Sci. 48(5): 863-872.

Ma, H.M., Hakim, L.F., Bowman, C.N. and Davis, R.H. 2001. Factors affecting membrane fouling reduction by surface modification and backpulsing. J. Membr. Sci. 189: 225-270.

Mackie, A.M. and Adron, J.M. 1978. Identification of inosine and inosine 5X-monophosphate as the gustatory feeding stimulats for the turbot *Scophthalmus maximus*. Comp. Biochem. Physiol. 60: 79-83.

Madaeni, S.S. 1999. The application of membrane technology for water disinfection. Water Res. 33: 301-308.

Majewska-Nowak, K., Kabsch-Korbutowicz, M. and Winnicki, T. 1999. The effect of gas bubble flow on ultrafiltration efficiency. Desalination. 126: 187-192.

Manttari, M. and Nystrom, M. 2000. Critical flux in NF of high molar mass polysaccharides and effluents from the paper industry. J. Membr. Sci. 170: 257-273.

Marcier, M., Fonade, C. and Lafforgue-Delorme, C. 1997. How slug flow can enhance the ultrafiltration flux in mineral tubular membranes. J. Membr. Sci. 1997: 128: 103-113.

Martin, A.M. 1999. A low-energy process for the conversion of fisheries waste biomass. Renew. Energ. 16: 1102-1105.

Mary E.M.C., Hilario, E. and Jacobson, F. 2006. Protein Aggregation and Bioprocessing. AAPS J. 8 (3): 572-579.

Matthews, D.M. 1975. Intestinal absorption. Physiol. Rev. 55: 537-608.

Meacle, F., Aunins, A., Thornton, R. and Lee, A. 1999. Optimization of the membrane purification of a polysaccharide-protein conjugate vaccine using backpulsing. J. Membr. Sci. 161: 171-184.

Meindersma, G.W., Augeraud, J. and Vergossen, F.H.P. 1995. Separation of a biocatalyst with ultrafiltration or filtration after bioconversion. J. Membr. Sci. 106: 103-121.

Meireles, M., Aimar, P. and Sanchez, V. 1991. Albumin denaturation during ultrafiltration: effects of operating conditions and consequences on membrane fouling. Biotechnol. Bioeng. 38: 528-534.

Mercier-Bonin, M., Lagane, C. and Fonade, C. 2000. Influence of a gas/liquid two-phase flow on the ultrafiltration and microfiltration performances: case of a ceramic flat sheet membrane. J. Membr. Sci. 180: 93.

Mercier-Benin, M. and Fonadeb, C. 2002. Air-sparged microfiltration of enzyme/yeast mixtures: determination of optimal conditions for enzyme recovery. Desalination 148: 171-176.

Metsamuuronen, S., Howell, J. and Nystrom, M. 2002. Critical flux in ultrafiltration of myoglobin and baker's yeast. J. Membr. Sci. 196: 13–25.

Metsaimuuronen, S. and Nystrom, M. 2005. Critical flux in cross-flow ultrafiltration of protein solutions. Desalination. 175: 37.

Mi-Jung, U., Seong-Hoon, Y., Chung-Hak, L., Kun-Yong, C. and Jae-Jin, K. 2001. Flux enhancement with gas injection in crossflow ultrafiltration of oily wastewater. Wat. Res. 35: 4095-4101.

Milla, P.J. 1991. The clinical use of protein hydrolysate and soya formulae. Eur. J.-Clin. Nutr. 45: 128-140.

Millward, H.R., Bellhouse, B.J., Sobey, I.J. and Lewis, R.W.H. 1995. Enhancement of plasma filtration using the concept of the vortex wave. J. Membr. Sci. 100: 121-129.

Molinari, R, Poerio, T. and Argurio, P. 2008. Selective separation of copper(II) and nickel(II) from aqueous media using the complexation-ultrafiltration process. Chemosphere 70: 341-348.

Morbahan, S. and Trumbore, L. 1991. Enteral tube feeling: a clinical perspective on recent advances. Nutr. Rev. 49: 129-140.

Morimura, S., Nagata, H., Uemura, Y., Fahmi, A., Shigematsu, T. and Kida, K. 2002. Development of an effective process for utilization of collagen from livestock and fish waste. Process Biochem. 37: 1403-1412.

Mueller, J. and Davis, R.H. 1996. Protein fouling of surface modified polymeric microfiltration membranes, J. Membr. Sci. 116: 47–60.

Muller, C.H., Agarwal, G.P., Melin Th., Wintgens, Th. 2003. Study of ultrafiltration of a single and binary protein solution in a thin spiral channel module. J. Membr. Sci. 227: 51-69.

Munilla-Moran, R. and Saborido-Rey, F. 1996. Digestive enzymes in marine species. I. proteinase activities in gut from redfish(*Sebastes mentella*), seabream (*Sparus aurata*) and turbot (*Scophthalmus maximus*). Comp. Biochem. Physiol. B. 113: 395-402.

Muriel, M.B., Claude, M., Christine, L. and Christian, F. 2000. Hydrodynamics of slug flow applied to cross-flow filtration in narrow tubes. AIChE J. 46 (3): 476-488.

Murthy, Z.V.P. and Gupta, S.K. 1999. Sodium cyanide separation and parameter estimation for reverse osmosis thin film composite polyamide membrane. J. Membr. Sci. 154: 89-103.

Nagodawithana, T. 1992. Yeast-derived flavors and flavor enhancers and their probable mode of action. Food Technol. 46: 138-144.

Najarian, S. and Bellhouse, B. J. 1996. Effect of liquid pulsation on protein fractionation using ultrafiltration processes. J. Membr. Sci. 114: 245-253.

Nakatsuka, S. and Michaels, A.S. 1992. Transport and separation of proteins by ultrafiltration through sorptive and non-sorptive membranes. J. Membr. Sci. 69: 189-211.

National Food Institute. 2005. Available from: <www.nfi.or.th/statistic.asp>.

Netto, F.M. and Galaezzi, M.A.M. 1998. Production and characterization of enzymatic hydrolysate from soy protein isolate. Lebensm. Wiss. Technol. 31: 624-631.

Ninfa, A.J. and Ballou, D.P.B. 1998. Gel electrophoresis of proteins. *In* Fundamental Laboratory Approaches for Biochemistry and Biotechnology. (eds. Ninfa, A.J. and Ballou, P.B.). P. 125-155, Fitzgerald Science Press, Inc, Bethesda, USA.

Oliva-Teles, A., Cerqueira, A.L. and Goncalves, P. 1999. The utilization of diets containing high level of fish protein hydrolysate by turbot (*Scophthalmus maximus*) juveniles. Aquaculture 179: 195-201.

Peace, R.W., Sarwar, G., Touchburn, S.P. 1992. Trypsin inhibitor levels in soy-based infant formulas and commercial soy protein isolates and concentrates. Food Res. Int. 25: 137-41.

Pedroche, J., Yust, M.M., Lqari, H., Giron-Calle, J., Vioque, J., Alaiz, M. and Millan, F. 2004. Production and characterization of casein hydrolysates with a high amino acid

Fischer's ratio using immobilized proteases. Int. Dairy J.14: 527-533.

Pollice, A., Brookes, A., Jefferson, B. and Judd, S. 2005. Sub-critical flux fouling in membrane bioreactors: a review of recent literature. Desalination 174: 221-230.

Pradanos, P., Hernandez, A., Calvo, J.I. and Tejerina, F. 1996. Mechanisms of protein fouling in cross-flow UF through an asymmetric inorganic membrane. J. Membr. Sci. 114: 115-126.

Prasertsan, P., Wuttjumnong, P., Sophanodora, P. and Choorit, W. 1988. Seafood processing industries within Songkhla-Hat Yai region: the survey of vasic data emphasis on wastes. Songklanakarin J. Sci. Technol. 10: 447-451.

Pruthi, R.S., Haese, A., Huland, E. and Stamey, T. 1997. Use of serum concentration techniques to enhance early detection of recurrent prostate cancer after radical prostatectomy. Urology 49: 404-410.

Psoch, C. and Schiewer, S. 2005. Long-term study of an intermittent air sparged MBR for synthetic wastewater treatment. J. Membr. Sci. 260: 56-65.

Raju, A.A., Rose, C., Rao, N.M. 1997. Enzymatic hydrolysis of tannery fleshings using chicken intestine proteases. Anim. Feed Sci. Technol., 66: 139-147.

Ramakrishna, M., Hultin, H.O. and Atallah, M.T. 1987. A comparison of dogfish and bovine chymotrypsins in relation to protein hydrolysis. J. Food Sci. 52: 1198-1202.

Ramirez, J.A. and Davis, R.H. 1998. Application of cross-flow microfltration with rapid back-pulsing to wastewater treatment. J. Hazard. Mater. 63: 179-197.

Rattanakit, N., Plikomol, A., Yano, S., Wakayama, M. and Tachiki, T. 2002. Utilization of shrimp shellfish waste as a substrate for solid-state cultivation of *Aspergillus* sp. S1-13: evaluation of a culture based on chitinase formation which is necessary for chitin-assimilation. J. Biosci. Bioeng. 93:550-556.

Ravindran, G. and Bryden, W.L. 2005. Tryptophan determination in proteins and feedstuffs by ion exchange chromatography. Food Chem. B. 9: 309-314.

Redkar, S.G. and Davis, R.H. 1995. Enhancement of crossflow microfiltration performance using high-frequency reverse flitration. AIChE J. 41: 501-508.

Reis, R.V. and Saksena, S. 1997. Optimization diagram for membrane separation. J. Membr. Sci. 129: 19-29.

Reis, R.V. and Zyndey, A. 2001. Membranes separation in biotechnology. Curr. Opin. Biotech. 12: 208-211.

Ripperger, S. and Altmann, J. 2002. Crossflow microfiltration-state of the art. Sep. Purif. Technol. 26: 19-31.

Roth, G. Smith, C.E., Schoofs, G.M., Montgomery, T.J., Ayala, J.L., Monica, T.J., Gastillo, F.J. and Horwitz, J.I. 2001. Vortex flow filtration for cell separation in bioreactor operations. *In* Membrane Separations in Biotechnology (ed. Wang, W.K.). p. 70-71, Marcel Dekker, Inc., New York, USA.

Sablani, S.S., Goosen, M.F.A., Al-Belushi, R. and Wilf, M. 2001. Concentration polarization in ultrafiltration and reverse osmosis. Desalination 141: 269-289.

Samuels, W.A. Fontenot, J.P., Allen, V.G. and Abazinge, M.D.A. 1991. Seafood processing wastes ensiled with straw: utilization and intake by sheep. J. Anim. Sci. 69: 4983-4992.

Sarabok, A., H-Kittikun, A. 1999. Enzymatic hydrolysis of tuna condensate for flavour sauce production. Songklanakarin J. Sci. Technol. 21, 491-450.

Schmidl, M.K., Taylor, S.L., Nordlee, J.A. 1994. Use of hydrolysate-based products in special medical diets. Food Technol. 48: 77-85.

Serra, C., Durand-Bourlier, L., Clifton, M.J., Moulin, P., Rouch, J.C. and Aptel, P. 1999. Use of air sparging to improve backwash efficiency in hollow-fibeer modules. J. Membr. Sci. 161: 95-113.

Shahidi, F., Han, X.Q., Synowiecki, J. 1995. Production and characteristics of protein hydrolysates from capelin *(Mallotus villosus)*. Food Chem. 53, 285-293.

Shi, J., Yuan Q. and Gao, C.J. 2001. Materials, production and characteristics of organic membrane. *In* Handbook of Membrane Technology (eds. Shi, J., Yuan Q. and Gao, C.J.). p. 21-70, Chemistry Industry Press, Beijing, China.

Smith, S.R. and Cui, Z.F. 2004. Gas-slug enhanced hollow fibre ultrafiltration: an experimental study. J. Membr. Sci. 242:117–128.

Simon, A., Vandanjon, L., Levesque, G. and Boursean, P. 2002. Concentration and desalination of fish gelatin by ultrafiltration and continuous diafiltration processes. Desalination 144: 313–318.

Simpson, B.K and Haard, N.F. 1984. Purification and characterization of trypsin from the

Greenland cod (*Gadus ogac*). J. Biochem. 62: 894-900.

Silk, D.B.A., Faairglogh, D., Clark, M.L., Hegarty, J.E., Marrs, T.C., Addison, J.M., Burston, D., Clegg, K.K. and Matthews, D.M. 1980. Use of peptide rather than amino acid nitrogen source in chemically defined 'elemental' diets. J. Parenter. Enter. Nutr. 4: 548-553.

Silk, D.B.A. 1987. Towards the optimization of enteral nutrition. Clin. Nutr. 6: 61-74.

Smith, S.R. and Cui, Z.F. 2004. Gas-slug enhancement hollow fibre ultrafiltration-an experimental study. J. Membr. Sci. 242: 117-128.

Taha, T., Cheong, W.L., Field, R.W. and Cui, Z.F. 2006. Gas-sparged ultrafiltration using horizontal and inclined tubular membranes-a CFD study. J. Membr. Sci. 279: 487.

Taddei, C. and Daufin, G. 1989. Role of some whey components on mass transfer in ultrafiltration. Biotechnol. Bioeng. 34: 171-179.

Tansel, B., Bab, W.Y., and Tansel, I.N. 2000 Characterization of fouling kinetics in ultrafiltration systems by resistances in series model. Desalination. 129: 7-14.

Torres, M.R., Marin, F.R., Ramos, A.J. and Soriano, E. 2002. Study of operating conditions in concentration of chicken blood plasma proteins by ultrafiltration. J. Food Eng. 54: 215-219.

Torrissen, K.R. 1984. Characterization of proteases in the digestive tract of Atlantic salmon (*Salmo salar*) in comparison with rainbow trout (*Salmo gairdneri*). Comp. Biochem. Physiol. B. 77: 669-674.

Tracey, E.M., and Davis, R.H. 1994. Protein fouling of track-etched polycarbonate microfiltration membrane. J. Colloid Interface Sci. 167: 104-116.

Truskey, G.A., Gabler, R., Dileo, A. and Manter, T. 1987. The effect of membrane filtration upon protein conformation. J. Parenteral Sci. Technol. 41: 180-193.

Um, M.J., Yoon, S.H., Lee, C.H. and Chung, K.Y. 2001. Flux enhancement with gas injection in cross flow ultrafiltration of oily wastewater. Water Res. 35: 4095-4101.

Vadi, P.K. and Rizvi,S.S.H. 2001. Experimental evaluation of a uniform transmembrane pressure crossflow microfiltration unit for the concentration of micellar casein from skim milk. J. Membr. Sci. 189: 69-82.

Vandanjon, L., Cros, S., Jaouen, P., Quemeneur, F. and Bourseau, P. 2002. Recovery by nanofiltration and reverse osmosis of marine flavours from seafood cooking waters.

Desalination 144: 379-385.

Velasco, C., Ouammou, M. and Calvo, J.I. 2003. A. Hernandez, Protein fouling in microfiltration: deposition mechanism as a function of pressure for different pH. J. Colloid Interface Sci. 266: 148-152.

Ven, C.V.D., Gruppen, H., Bont, D.B.A.D. and Voragen, A.G.J. 2001. Reversed phase and size exclusion chromatography of milk protein hydrolysates: relation between elution from reversed phase column and apparent molecular weight distribution. Int. Dairy J. 11: 83-92.

Venugopal, V., Shahidi, F., 1995. Value-added products from underutilized fish species. Crit Rev. Food Sci. Nutr. 35(5), 431-453. Agric. Food Chem. 48, 657-66.

Viana, M.T., Lopez, L.M., Garcia-Esquivel, Z. and Mendez, E. 1996. The use of silage made from fish and abalone viscera as an ingredient in abalone feed. Aquaculture 140: 87-98.

Wakeman, R.J. and Williams, C.J. 2002. Additional techniques to improve microfiltration. Sep. Purif. Technol. 26: 3-18.

Wang, S.L. and Hwang, J.R. 2001. Microbial reclamation of shellfish waste for the production of chitinases. Enzyme Microb. Tech. 28: 376-382.

Wang, X.L., Zhang, C.H., and Ouyang, P.K. 2002. The possibility of separating saccharides from a NaCl solution by using nanofiltration in diafiltration mode. J. Membr. Sci. 204: 271-281.

Weiss, M., Manneberg, M., Franc, J., Juranville, O., Lahm, H.W. and Fountoulakis, M. 1998. Effect of the hydrolysis method on the determination of the amino acid composition of proteins. J. Chromatogr. 795: 263-275.

Wiesner, M.R., Veerapaneni, S. and Brejchova, D. 1992 Improvement in Microfiltration using Coagulation Pretreatment. *In* Proceedings of the. Fifth Gothenburg Symposium on Chemical Water and Wastewater Treatment II (eds. Klute, R. and Hahn, H.H.). p. 20-40, Springer, New York, USA.

Wronski, S., Molga, E. and Rudniak, L. 1989. Dynamic filtration in biotechnology. Bioprocess Biosyst. Eng. 4: 99-117.

Wu, D.X., Howell, J.A. and Field, R.W. 1999. Critical flux measurement for model colloids. J.

Membr. Sci. 152: 89-98.

Yanez, E., Ballester, D. and Monckeberg, F. 1976. Enzymatic fish protein hydrolysates: chemical composition, nutritive value and use as a supplement to cereal protein. J. Food Sci. 41: 1289-1292.

Yaobo, L. 2002. Lowry method. *In*: Protein Technology Handbook (eds. Jiazheng, W. and Ming, F.). p. 92-97, Science Press, Beijing, China.

Yehuda, T., Dvora, B. and Dukler, A.E. 1980. Modelling flow pattern transitions for steady upward gas-liquid flow in vertical tubes. AIChE J. 26: 345.

Young, E.A., Heuler, N., Russel, P. and Weswe, E. 1975. Comparative nutritional analysis of chemically defined diets. Gastroenterology 69: 1338-1345.

Youravong, W. 2001. Critical flux and fouling during ultrafiltration of milk components: hydrodynamic and physicochemical effects. Doctor of Philosophy Thesis in Food Engineering, School of Food Biosciences. University of Reading.

Yu, K.C., Wen, X.H., Bu, Q.J. and Xia, H. 2003. Critical flux enhancements with air sparging in axial hollow fibers cross-flow microfiltration of biologically treated wastewater. J. Membr. Sci. 224: 69-79.

Yu, S.Y. and Tan, L.K. 1990. Acceptability of crackers with fish protein hydrolysate. Int. J. Food Sci. Tech. 25: 204-208.

Zwijnenberg, H.J., Kemperman, A.J.B., Boerrigter,M.E., Lotz, M., Dijksterhuis, J.F., Poulsen, P.E. and Koops, G.H. 2002. Native protein recovery from potato fruit juice by ultrafiltion. Desalination 144: 331-334.

Zydney, A.L. 1998. Protein separation by UF,new opportunities for whey protein fractionation. Int. Dairy J. 8: 243-250.

VITAE

Name Mr Zhenyu Li

Student ID 4783006

Educational Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Engineering (Food Science and Engineering)	Anhui Univeristy of Technology and Science P.R.China	1999
Master of Science (Biotechnology)	Prince of Songkla University Thailand	2004

Scholarship Awards during Enrolment

Development of excellence in Agro-Industry Fellowship (2004-2007) for PhD candidate, Faculty of Agro-Industry, Prince of Songkla University.

Chinese Government Award for Outstanding Self-financed Students Abroad (2006), Ministry of Education, P.R.China.

List of Publication and Proceeding

Publications

Li, Z.Y., Youravong, W. and H-Kittikun, A. 2006. Analysis of fouling mechanism during dead-end microfiltration of tuna spleen extract. J. Anhui University Technol. Sci. 21, 1-6.

Li, Z.Y., H-Kittikun, A. and Youravong, W. 2008. Separation of Protease from Yellowfin Tuna Spleen Extract by Ultrafiltration: Effect of Hydrodynamics and Gas Sparging on Flux Enhancement and Selectivity. J. Membr. Sci. 311: 104-111.

Li, Z.Y., H-Kittikun, A. and Youravong, W. 2008. Removal of suspended solids from tuna spleen extract by microfiltration: a batch process design and improvement. Biochem. Eng. J. 38: 226-233.

Li, Z.Y., Youravong, W. and H-Kittikun, A. 2008. Removal of suspended particles from extract of tuna spleen by microfiltration: experimental evaluation of a low fouling conditio J. Food Eng. 85: 309-315.

Proceeding

Li, Z.Y., H-Kittikun, A. and Youravong, W. 2006. Effect of crossflow velocity on suspended solid removal, critical flux and limiting flux in microfiltration of tuna spleen extract. Regional Symposium & Workshop on membrane science and technology (MST2006). 27-28 April 2006. Nanyang Technological University, Singapore. *(Platform presentation)*