Separation of Proteases from Viscera Extract of Yellowfin Tuna (*Thunnus albacares*) by Ultrafiltration

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Master of Science Thesis in Biotechnology

Prince of Songkla University

2004

Thesis Title	Separation of Proteases from Viscera Extract of Yellowfin Tuna (*Thunnus albacares*) by Ultrafiltration
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Major Program	Biotechnology

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Abstract

Yellowfin tuna viscera are major waste products of tuna canning industry. Recovery of enzymes from these waste products can not only improve economic value but also reduce environmental pollution. Separation of proteases by ultrafiltration was studied. The membrane with molecular weight cut off (MWCO) 100 kDa had a higher transmission for enzymes than membrane with MWCO 30 kDa. The transmission ranges of enzymes were 0.6 to 0.8 which depended on the type of enzymes and operation conditions by using regenerated cellulose membrane with MWCO 100 kDa and 0.01 to 0.18 by using MWCO 30 kDa membrane. Temperature at 4°C did not show any difference for enzyme separation compare to the room temperature. It was found that pre-incubation of crude extract at 50°C before ultrafiltration enhanced the average permeate flux. Pre-incubation for 1 hour at 50°C provided the highest enzyme activities which were 58.43 U/ml, 14.35 U/ml and 15.73 U/ml and specific activity which were 5.35 U/mg, 1.46 U/mg and 1.44 U/mg in the retentate for general protease, trypsin and chymotrypsin, respectively by ultrafiltration using regenerated cellulose membrane with MWCO 30 kDa. The effects of transmembrane pressure and cross-flow rate during ultrafiltration using regenerated membrane with MWCO 30 kDa and 100 kDa were also studied. Transmembrane pressure (TMP) and cross-flow rate had little effect on protein and enzyme transmission. Increasing cross

flow rate and TMP increased permeate flux. The highest permeate flux of 54.72 L/m^2.h for MWCO 30 kDa membrane by using TMP 3.5 bar and cross flow rate 360 L/h and 68.4 L/m^2.h for MWCO 100 kDa membrane by using TMP 2.5 bar and cross flow rate 360 L/h were achieved. Higher TMP could not further increase the permeate flux. Continuous diafiltration increased purity factor of these enzymes more than ten times by using TMP at 1.5 bar and cross flow rate of 360 L/h for extract of spleen. It also increased purity factor of these enzymes more than five times for extract of mixed viscera. Concentration of purified extract achieved by using dead-end model ultrafiltraion with MWCO 10 kDa membrane. The gel electrophoresis with both silver staining and activity staining proved that the trypsin and chymotrypsin were kept in the retentate after ultrafiltration. All of the results proved that the separation, purification and concentration of these enzymes were achieved by using ultrafiltration.

Acknowledgement

During the past one and half years, I have experienced one of the most wonderful time in my life. Thailand is the first oversea country where I study. I have lived in a warm environment since I have been surrounded by warm friendship and warm smile from everyone I meet.

I am greatly indebted to my advisor Asst. Prof. Dr. Aran H-Kittikun and co-advisor Dr. Wirote Youravong for their advice and encouragement for both my study and my living.

I would like to express my deepest appreciation to every staff and student in Department of Industrial Biotechnology, Faculty of agro-industry. The help from them supported me to overcome culture shock and any other difficulties. I also would like to thank the staffs and students from other departments in Faculty of Agro-Industry. Their kind assistance help me to save time and study better.

I would like to thank Asst. Prof. Dr. Pikul Wanichapichat from Department of Physics, Faculty of Science for her kind suggestions during my study.

I would like to thank Assoc.Prof. Dr. Wallie Suvachittanont and Assoc. Prof. Dr. Poonsuk Prasertsan for spending valuable time for thesis examination.

Thanks also to Chotiwat Manufacturing Co. Ltd. for providing yellowfin tuna viscera used in this study.

Many thanks to Faculty of Agro-Industry for the scholarship provided from Nov. 2002 to Apr. 2004 and also to Graduate School for supporting this thesis.

Special thanks for my Chinese teacher, Prof. Tang Bin. He helped me get chance to study here.

Finally, I would like to thank my family for their support throughout my life.

Li Zhenyu

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List of Abbreviations

BSA	Bovine serum albumin
BTEE	Benzoyl-L-tyrosine ethyl ester
CP	Concentration polarization
D	Dialysis
DF	Diafiltration
ED	Electrodialysis
EDTA	Ethylenediamine tetraacetic acid
GP	Gas permeation
h	Hours
kDa	Kilodaltons
L	Liter
MF	Microfiltration
mg	Milligram
min	Minutes
ml	Milliliter
mM	Millimolar
MW	Molecular weight
MWCO	Molecular weight cut off
nm	Nanometer
°C	Degree celsius
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PV	Pervaporation
RO	Reverse osmosis

List of Abbreviations (continued)

rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
TAME	N-toluenesulfonyl-L-arginine methyl ester
TEMED	N,N,N',N'-tetramethyl ethylenediamine
TMP	Transmembrane pressure
U	Units
UF	Ultrafiltration
VCF	Volume concentration factor
µg	Microgram
µm	Micrometer

Chapter 1

INTRODUCTION

Introduction

Nowadays, there is the fast growth in the field of biotechnology along with rapid commercialization of enzyme products. For example, the application of proteases can be used for a variety of products, especially in the food industry, such as protein removal from bones, protein hydrolysate production, meat tenderization, clarification of wine or juice, and fermentation (Haard, 1998). The applications of enzyme have led to an increase in the demand of efficient processes and large-scale enzyme purification techniques. Techniques used in research laboratories (e.g. chromatography, electrophoresis, and affinity purification) can be used for enzyme purification, but they are suitable for producing small quantity of enzyme. These processes are difficult to scale-up. In addition to scale-up problem these techniques require complex instrumentation, and give low throughput of product at an extremely high cost of producation.

Membranes have always been an integral part of biotechnology processes for fermentation, clarification, purification and concentration (Reis and Zydney, 2001). Ultrafiltration (UF) has become the method of choice for protein concentration and buffer exchange, largely replaceing size-exclusion chromatography in these applications (Reis and Zydney, 2001). UF process is cost effective process an offering a high productivity and reasonable product purity at the same time. UF processes are also much easier to scale-up in comparison to chromatography and

electrophoresis techniques. In addition to these, UF modules are easy to operate and are quite compact in design.

A survey of proteolytic digestive enzymes in various species of fish revealed that serine protease is widely distributed in fish viscera (Heu *et al.*, 1995). Trypsin has been separated and characterized from intestine of crayfish (Kim *et al.*, 1994), anchovy (Martinez *et al.*, 1988), dogfish (Ramakrishna *et al.*, 1988), and so on. Chymotrypsin also has been separated and characterized from pancreas of carp (Heu *et al.*, .1995) , viscera of tuna (Jantaro, 2000), etc.

Membrane technology has been used for recovery of proteases by several researchers. For example, these enzymes have been isolated from Atlantic cod (Gildberg, 1992), clam viscera (Chen, and Zall, 1985) surimi wash water (Dewitt and Morrissey, 2002a).

On the other hand, tuna canning industry is a large industry in Thailand. Thailand is also the largest exporter for tuna canning products in the world. The quantity of canned tuna exported in 1998 and 1999 were 20,000 tons and 24,000 tons, respectively, and about 650,000 tons raw materials were used annually (Economic Agriculture office, 1999). During tuna canning processing, there are about 25-30% solid wastes (e.g. head, skin, viscera, bones) and 35% liquid wastes (e.g. blood, tuna condensate) (Visessanguan *et al.*, 2003). Tuna canning processing provides a large amount of raw material for enzyme production.

This work aimed to recover the protease from yellowfin tuna viscera extract using UF. Operation condition of UF process was researched to improve both efficiency of production and purity of enzymes. Factors affecting process performance and enzyme recovery were studied

Literature review

1. Ultrafiltration (UF)

1.1 Membrane filtration process

UF belongs to the family of membrane filtration processes. 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. Membrane filtration processes provide means of separation and concentration at the molecular and fine-particle level with unique advantages, i.e. processing can be at modest (even at low) temperatures; chemical and mechanical stresses can be minimized; no phase change is involved; energy demand is modest; selectivity is good in many cases; concentration and purification may be achieved in one step; equipment is easily scaled up, is flexible (can be batch-processed or continuous), and provides a closed system (effective containment) (Fane *et al.*, 1990).

The family of membrane filtration processes, reverse osmosis (RO), ultrafiltration (UF), microfiltration (MF), dialysis (D), electrodialysis (ED), gas permeation (GP), pervaporation (PV) and liquid membrane, can be used to separate the wide range of species. Membrane processes have been a recent development in the process industries and became the accepted separation technique for many processes. The membrane processes are used in a whole variety of fields.

Membranes were produced commercially in the late 1920s for bacteriological laboratory use. When the asymmetric membranes were prepared, it is become possible that RO and UF were used in large scale. MF membranes were very popular for cleaning a variety of fluid streams

and MF processes were used widely from the mid 1960s. In 1970s, the rapid development of UF was spearheaded by the dairy industry. Then, an increasing body of research literatures was apparent, the Journal of Membrane Science was started in 1973. At the beginning of the 1980s, the commercial gas separation was announced. Later in the decade, the PV was introduced and full scale plants were built (Howell, 1993).

At present, many membrane processes have been developed and some processes share common features with the sketch in Figure 1. The generalized membrane system needs a source of pressure. The system circulates fluid across the surface of the membrane. The membrane is selective. The fluid may be a solution, a suspension, a mixture of gases or vapours.

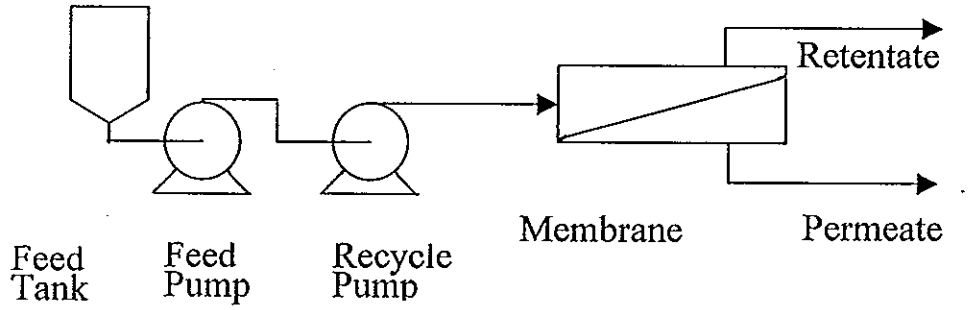


Figure 1 Generalized membrane system

Source: Howell (1993)

1.2. UF and UF membrane

UF is the separation of substances of different molecular dimensions by the use of membranes with small pore sizes. As shown in Figure 2 , the fields of microfiltration (MF), reverse osmosis (RO) and UF are overlapping. UF separates macromolecules in the range of molecular weights from below 10,000 to about 1,000,000 dalton, which corresponds to a particle size of less than 10 nm to about 1μm (Brummer and Gunzer, 1987). Normally, the pressure is used as driven force.

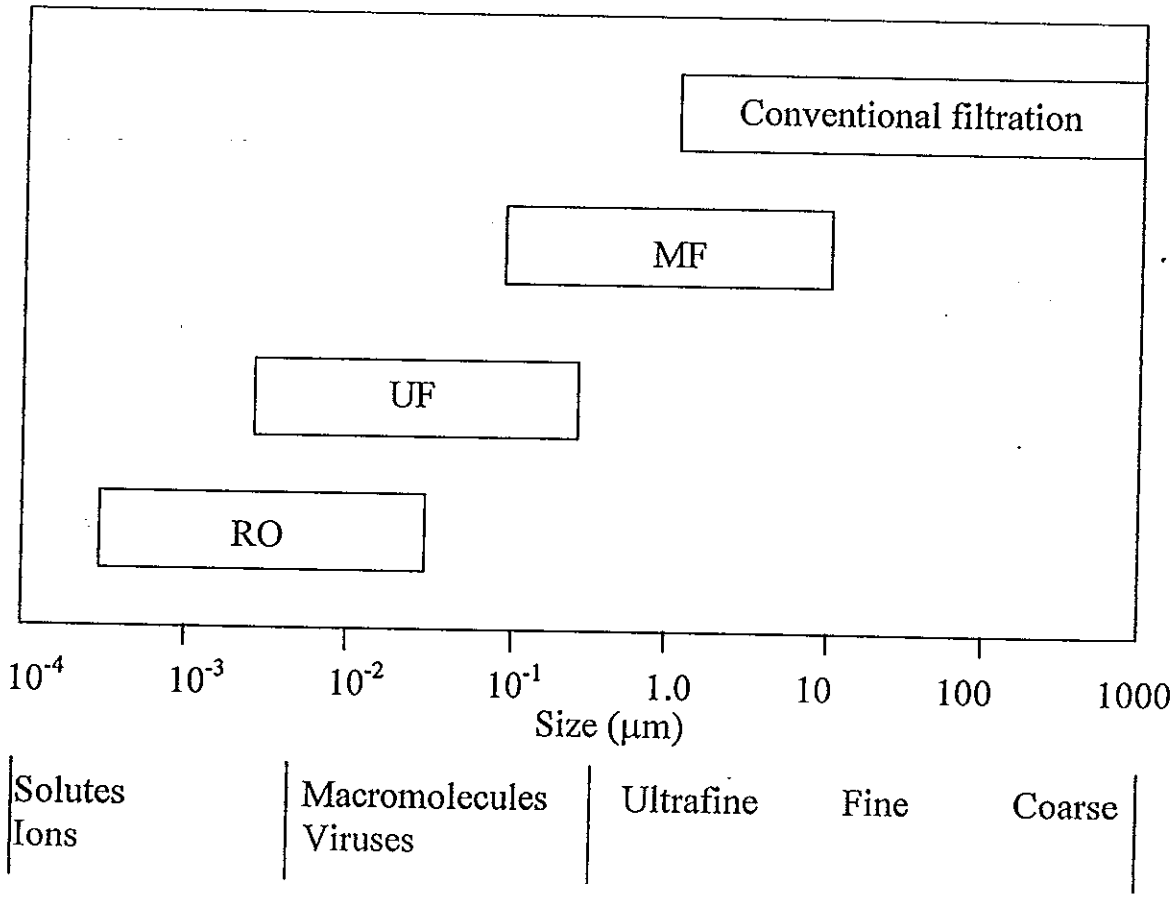


Figure 2 Filtration processes

Source: Brummer and Gunzer (1987)

The key component of the UF processes is the membrane. Membrane is a thin barrier across which physical and/or chemical gradients can be established to produce differential flows of one or more components (Shi *et al.*, 2001). The requirements for membrane are high flux, selectivity, chemical resistance and long lifetime. The cost also should be considered.

All of the membranes are different and have various properties. There are a number of properties for a membrane in the UF process. These properties include pore-size, porosity, phallic/phobic nature, pH tolerances, temperature tolerances, strength, durability and clean-ability. In UF process, there are two main types of membranes used. The first type are asymmetric skinned membranes. These membranes can be produced from a wide variety of synthetic polymers, copolymers and blends. The second type are inorganic membranes. These membranes consist of inorganic materials, such as Zirconium Oxide and Alumina (Mulder, 1993). Normally, membranes used for UF are asymmetric porous and the pore sizes range from 0.05 microns to 1 nanometer. The membranes are characterized by a molecular weight cut-off (MWCO). This is an expression of the membrane in terms of molecules of known size. Retention is rated as that nominal molecular weight cut-off at which 90% of spherical uncharged molecules of that same molecular weight is retained (Brummer and Gunzer 1987).

Commercial membrane materials which are often used for UF and MF are provided in Table1.

Table 1 A survey of materials for commercial polymer membranes

Material	Processes
Polyvinylidenefluoride (PVDF)	MF, UF
Cellulose acetate (CA)	MF, UF
Aliphatic polyamide (Nylon 6, Nylon 6,6)	MF, UF
Aromatic polyamide	MF, UF
Polysulphone (PSP)	MF, UF
Polyethersulphone (PES)	MF, UF
Polymide (PI)	MF, UF
Polyetherimide (PEI)	MF, UF
Polyvinylalcohol (PVA)	MF, UF
Polyacrylonitrile (PAN)	UF
Polyacrylonitrile/polyvinylchloride copolymer (PAN-PVC)	MF, UF
Polyetheretherketone (PEEK)	MF, UF
Zirconium oxide	MF, UF
Alumina	MF, UF

Source : Mulder (1993)

1.3 Theory and terminology of UF

UF is a membranes process with the ability to separate molecules in solution on the basis of size. The separation principle for UF is a sieving mechanism and the driving force is a pressure range of 1-10 bar (Shi and Gao, 2001). The separation is achieved by concentrating the large molecules present in the feed on one side of the membrane, while the solvent and microsolutes are depleted as they pass through the membrane.

Pressure, cross flow rate, solute concentration and temperature are the normal process parameters used in models to describe UF. Other parameters include time, the interaction between solute and membrane, and the characteristics of the solute. Normally, the filtration model describes the flux as a function of the driving force and the total resistance. (Meindersma *et al.*, 1995). The flux formula is:

$$J = \frac{\Delta P}{\eta R} \qquad (1)$$

where ΔP is the differential pressure; η is the dynamic viscosity of the UF; R is total resistance.

Generally, R for UF membrane is about 10^{12}~10^{13} m^{-1}, depending on many factors, e.g. pore size, structure of membrane (Osada and Nakagawa, 1992).

Normally, the process is controlled by adjusting transmembrane pressure (TMP) and cross flow (tangential flow) which can affect on the fouling formation and flux behavior.

TMP is defined as the average applied pressure from the feed to the filtration side of the membrane.

$$TMP = \frac{P_F + P_R}{2} - P_P \qquad (2)$$

where P_F is the feed inlet pressure; P_R is retentate outlet pressure; P_P is the permeate outlet pressure.

TMP is a key operating parameter for pressure driven membrane processes. The whole UF process can be separated to two regions. The first one is pressure-dependent region in which the permeate flux increase with increasing TMP. The second is pressure-independent region in which increasing TMP has no effect on permeate flux (Grandison and Lewis, 1996).

During separation of lysozyme by hollow-fibre UF, Ghosh *et al.* (2000) found that the flux increased with increase in TMP, but at higher TMP values, the permeate flux levelled off. The concentration polarization effect became very significant at high TMP, but TMP had little or no effect on the enzyme transmission. This was due to the fact tat lysozyme was easily transmitted through this membrane and so the extent of concentration polarization of lysozyme molecules did not increase significantly with increase in TMP. They also found that increase in TMP led to a decrease in the effective selectivity. This is due to the fact that the transmissions of the other chicken egg white proteins increased when

there was extensive concentration polarization of these largely retained proteins at higher TMP values.

In the research of transport and separation of proteins by UF through sorptive and non-sorptive membranes, Nakatsuka and Michaeles (1992) found that flux increase about 2-fold when TMP changed from 22 KPa to 100 KPa. The result was consistent with the increased polarization and intrinsic cake resistance which should accompany filtration at higher pressures and transmembrane fluxs. When they decreased TMP from 100 KPa to 22 KPa, 3-fold decrease in flux occurred, with virtually no change in rejection. The phenomena indicated that gel polarization layer formed at the higher pressure was quite stable to disruption by pressure fluctuation.

Torres *et al.* (2002) also found that the limiting flux was attained after a very short time at high TMP when chicken blood plasma protein was separated by UF using polysulfone membrane with MWCO 40 and 100 kDa.

Grund *et al.* (1992) investigated of flux behavior during UF of BSA solution. They observed that hysteresis behavior of the flux-TMP could be detected whenever UF was operated at a TMP lower than some higher value to which the membrane had previously been exposed.

For concentration and purification of gelatin liquor by UF, Chakravorty and Singh (1990) found that flux value remained unaffected on further increase in pressure beyond the gel polarization point. This indicated that the rejected proteins had formed a gel layer at membrane surface which limited the flux, so flux became independent of pressure.

A TMP gradient along a membrane tube during processing at high cross flow velocity also has the effect on a development of fouling.

Daufin *et al.* (1993) found that fouling was greater at higher TMP position (inlet), where the membrane was filtering larger volumes (high permeate flux), causing a rapid increase in fouling at shorter time of operation.

On the other hand, Nakanishi and Kessler (1985) found that reducing TMP during UF of milk protein considerably increased the rate of removal of the deposited layer during rinsing.

Cross flow rate also plays an important role affecting on UF performance, especially for reduce of CP effect or reversible fouling resistance. Cross flow or tangential flow is that the feed solution is pumped to across the surface of the membrane directly. This "sweeping" action helps keep material retained by the membrane from settling, and eventually restricting, permeate flow. Cross flow is characterized by cross flow rate which can be measured directly by fluid exiting the retentate port (Millipore, 1993).

Torres *et al.* (2002) found that fouling was more severe when a solution ultrafiltered at both low feed flow rate and low membrane MWCO.

But 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 work, hence the extent of concentration polarization of lysozyme molecules is expected to be negligible.

Ramachjandra *et al.* (1994) found that increasing cross flow rate or wall shear stress decreased the reversible fouling resulting an increasing in permeate flux in UF of milk.

Cheryan and Chiang (1984) found that cross flow velocity did not have any significant effect on irreversible fouling. But other researchers found that operating at high cross flow velocity not only improved flux but also the recovery of the flux after rinsing which suggested that increasing cross flow velocity reduced irreversible fouling in some cases (Nakanishi and Kessler, 1985).

It was also reported that an increase of flow rate velocity did not improve cleaning results. This indicated that cleaning was mainly limited to removing the uppermost protein layer(s) on the surface (Daufin *et al.*, 1991).

A number of other terms are also useful to characterized the UF process. Such as:

Retention coefficient (R_j) which is given by

$$R_j = 1 - \frac{C_p}{C_b} \qquad (3)$$

where C_p and C_b are the solute concentration in the permeate and in the bulk solution (or feed) respectively.

If the solute is totally rejected by the membrane ($C_p = 0$), R_j is 1 (Youravong, 2001).

Filtration rate at which a solution is filtered is expressed in unit volume / unit time (e.g. ml / min, liters / min) (Millipore, 1993).

Conversion ratio (CR) is defined as the fraction of the feed side flow that passes through the membrane to the filtrate (Millipore, 2003).

Apparent sieving (S_{app}) is defined as a particular protein that passes through the membrane to the filtrate stream based on the measured

protein concentrations in the feed and filtrate streams. A sieving coefficient can be calculated for each protein in a feed stock (Millipore, 2003).

Intrinsic sieving (S_i) is also defined as the fraction of a particular protein that passes through the membrane to the filtrate stream . however, it is based on the protein concentration at the membrane surface. Although it cannot be directly measured, it gives a better understanding of the membrane's inherent separation characteristics (Millipore, 2003)

Throughput is defined as the total volume of fluid processed through the filters before membrane fouling occurs. It is measured in volume of permeate produced (Millipore, 1993).

It is well known that the composition of the feed stream may differ from time to time. Milk, for example, differs from season to season. Very large differences from batch to batch are also found in the composition of biotechnological industry and subjected to rapid degradation with time. This has the consequence that the performance of an UF plant may also differ from time to time. So different parameters according to the performance of the process should be chosen to characterize the process.

1.4 Flux decline during UF

The main problem of UF is the flux decline during the processes. It is due to several phenomena in or on the membrane. These phenomena also cause a loss in selectivity or an additional undesired selectivity (Berg and Smolders, 1990). In some cases, flux decline can reach 90%. The reasons for the decline in flux are different in each case of UF. Normally, it is caused by decreasing driving force or increasing resistance which are motioned in equation (1). In general, the driving force can be controlled as a constant value during operation. So, the resistances are major reasons

causing flux decline. The resistances which can occur during UF processes were represented in Figure.3.

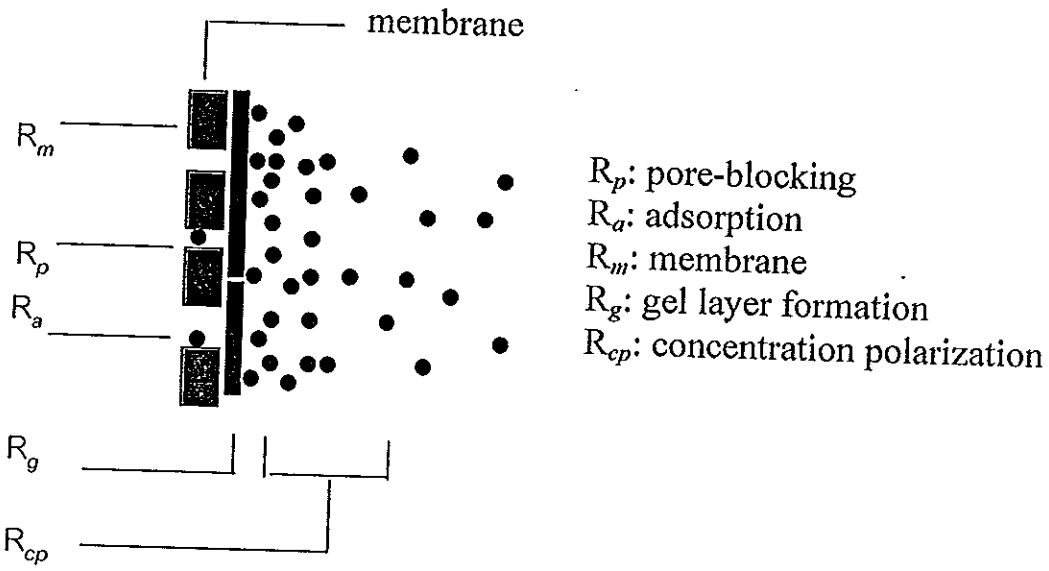


Figure 3 Possible resistances against solvent transport during UF
Source: Berg and Smolders (1990)

In these resistances, the R_m is always present. Other resistances can occur because of pores blocking by solute (R_p), adsorption of the solute onto the membrane inner wall of membranes (R_a), the formation of gel layer (R_g) and concentration polarization (R_{cp}).

According to these resistances, the reasons for decline in flux can be divided into fouling and concentration polarization (CP). Concentration polarization causes a rapid drop in flux, while fouling

causes a gradual, long-term decay. The two flux-decline phenomena were illustrated in Figure 4.

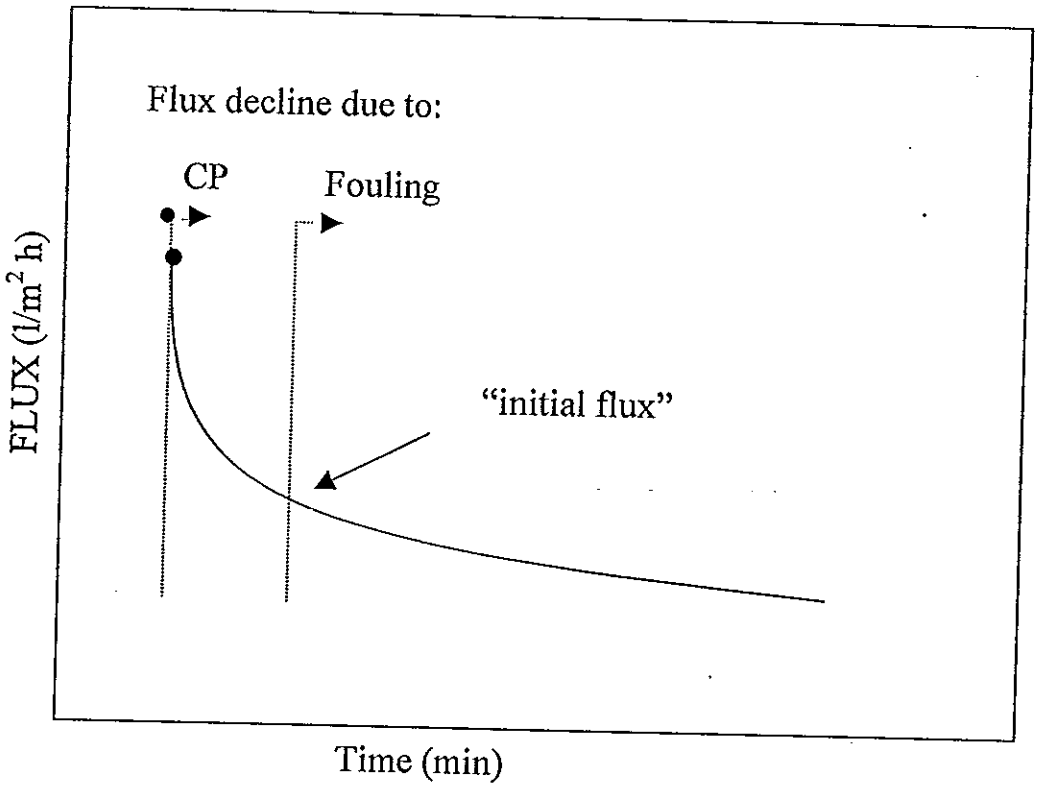


Figure 4 Flux decline is caused by concentration polarization (CP) and fouling. The pure water flux is indicated by (•)

Source: Jonssen *et al.*, (1990)

1.4.1. Concentration Polarization

During the UF processes, the solute is retained by membrane. This retaining can cause accumulation of solute near the membrane surface to form a layer at membrane interface with a high concentration. This layer is less permeable for solvent in feed bulk. This phenomenon is called concentration polarization (Gravatt, 1986).

The drop in flux, caused by concentration polarization, occurs in a very short time, usually in less than 1 minute (Chudacek *et al.*, 1984). This rapid flux decrease is seldom registered, except in scientific experiments.

When talking about the initial flux of an UF membrane one therefore usually refers to the flux measured some minutes after ultrafiltration of the solution has begun (Figure 4). The initial solution flux is typically 5-95% of the pure water flux.

The CP can be described in two ways: cake-filtration theory and film theory.

1.4.1.1. Cake-filtration theory

Cake-filtration theory assumes a constant concentration in the layer near the membrane, which sometimes depends on the applied pressure and increases in thickness with increasing permeate volume (Figure 5).

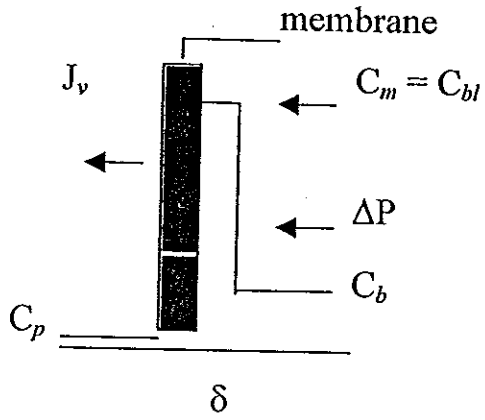


Figure 5 The cake-filtration type of description (ΔP: transmembrane pressure; C_m: concentration at the membrane interface; C_{bl}: constant concentration in the boundary layer; δ: thickness of the boundary layer)

Source: Berg and Smolders (1990)

The flux in cake-filtration theory can be expressed as equation from Berg and Smolders (1990):

$$J_v = \frac{\Delta P}{\eta (R_m + R_{bl})} \qquad (4)$$

where, η is the viscosity of the solvent; R_{bl} is the resistance of the resistance of the concentrated boundary layer; R_m is the hydraulic resistance of the membrane.

1.4.1.2. Film theory

Film theory represents the convective transport towards the membrane and the back-diffusion as a result of the concentration gradient.(Figure 6).

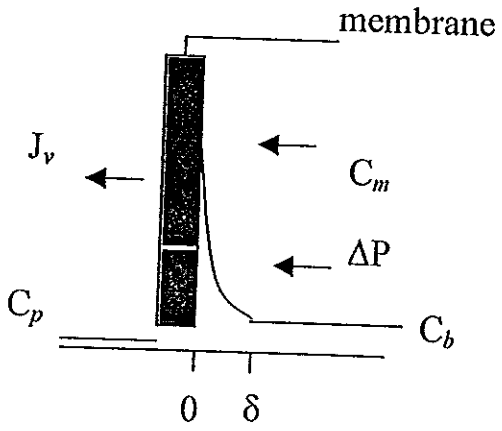


Figure 6. The concentration profile according to the film theory (ΔP: transmembrane pressure; C_m, the concentration at the membrane interface; C_b, the concentration in bulk; C_p, the concentration in the permeate; D, diffusion coefficient; δ: thickness of the boundary layer)

Source: Berg and Smolders (1990)

The well-known film-theory relationship is (Berg and Smolders, 1990):

$$J_v = \left(\frac{D}{\delta}\right) \times \ln\left(\frac{C_m - C_p}{C_b - C_p}\right) \qquad (5)$$

where (D / δ) is the mass transfer coefficient k;

In addition, both for the cake-filtration type of description and for the film theory several different models are also used to explain the effect of the concentration polarization phenomena. These models include resistance model (Baker *et al.*, 1985), gel – polarization model (Trettin and Doshi, 1980) and osmotic pressure model (Berg *et al.*, 1987).

CP is very sensitive to operating parameters, such as cross flow rate, pressure, temperature, nature and concentration of the solute. CP is considered to be reversible and can be controlled by means of cross flow rate adjustment, pulsation, ultrasound or an electric field (Sablani *et al.*, 2001). The influence of CP is thus reduced by an optimal choice of operating parameters. The cross flow rate is especially important.

1.4.2. Fouling

It is very hazardous to make general statements about the influence of different parameters on fouling. Membrane fouling is one of the major factors limiting the use of UF in many applications. It is the irreversible alteration in the membrane caused by specific physical and/or chemical interactions between the membrane and various components present in the process stream (Zemana, 1996).

The obvious consequence of fouling is higher capital expense caused by the lower average flux over a process cycle. In addition, restoring the flux may require powerful cleaning agents, which may increases the cost and reduces the lifetime of the membrane. Rejection and yield may also be affected. If the buildup of solid on the membrane is significant, it may act as a secondary membrane and change the effective sieving and transport properties of the system.

Surface chemical phenomena play an important role in the fouling of membranes. Almost all feed components foul the membranes

to a certain extent. However, It is well known, for example, that hydrophobic solutes are more readily adsorbed onto the membrane surface than hydrophilic solutes. It is also commonly recognized that hydrophobic (e.g. polysulphone) membranes have a larger fouling tendency than hydrophilic (e.g. cellulose acetate) membranes (Shi and Gao, 2001). The solution pH and ionic strength are also known to affect the membrane performance (Osada and Nakagawa, 1992). Changes in fluid management techniques may only increase the flux temporarily or mask the decline for a short period.

As mentioned above, flux may decline in one or more stages, usually rapid in the first few minutes, followed by a more gradual decline in flux (Figure 4). membrane fouling is due to the deposition and accumulation of feed components, e.g., suspended particles, impermeable dissolved solutes, or even normally permeable solutes, on the membrane surface and/or within the pores of the membrane. Membrane fouling is characterized by an "irreversible" decline in flux. The basis evaluation of the degree of the fouling is the water flux of a membrane. Water flux generally represents the best flux that can be obtained with a membrane.

The resistance which appear after fouling (R_f) can be calculated from the water flux after washing with water (Madaeni *et al.*, 2001):

$$R_f = \frac{\Delta P}{\eta J_{ww}} - R_m \qquad (6)$$

where, η is the viscosity of the solvent; ΔP is transmembrane pressure (driving force); J_{ww} is water flux measured after washing; R_m is membrane resistance;

1.4.2.1. Relationship between fouling and feed components

Since membrane fouling occurs because of specific physical or chemical interaction between the various macrosolutes/particles and the membranes, so the components of feed play the important roles in fouling phenomena. Normally, the fouling due to the components in feed, such as protein, fats, oils, grease, carbohydrates, salts and biofoulant (microorganism).

Protein fouling during ultrafiltration remains a very controversial topic, with considerable disagreement over both the mechanisms and rate of fouling as well as its overall importance relative to the concentration polarization effects (Zydney, 1996). Proteins are most soluble at high and low pH and least soluble at pH 4~5 (isoelectric point). But high pH is preferred for protein foulants because of "peptization" (hydrolysis) of the protein, which expedites cleaning. The large increase in hydraulic resistance of the membranes after protein filtration is directly attributable to the formation of a relatively thick protein deposit on the upper surface of the membrane (Kelly and Zydney, 1994). The hydraulic resistance provided by such protein deposits increases with increasing applied pressure (Opong and Zydney, 1991).

Deposits of fat are difficult to remove. High temperature or organic solvents can help removing fatty deposits. But tallow and lard may be initially unresponsive to high temperatures. Fatty deposits have a greater affinity for hydrophobic synthetic polymers than for hydrophilic polymers or inorganic materials.

Low molecular weight carbohydrates, such as low molecular weight sugars, are readily soluble in water and need no special cleaners. But high molecular weight carbohydrates, such as starch,

polysaccharides, fiber and pectin, may need some special treatment (e.g., prefiltration).

The inorganic components, such as salt, come not only from feed, but also from water and even from additives such as emulsifiers. There is a wide range of inorganic species (e.g. calcium carbonate, calcium sulfate, silica, metal oxides and hydroxides) that can cause significant fouling of membranes. Fouling by salts and metal compounds occurs by precipitation of flocculation on or within the porous structure of the membrane. Calcium salts are one of the more prominent inorganic foulants in many systems. Acid and chelating agents such as EDTA, can be used to dissolve salt foulants. Citrates are particularly effective due to their combined detergent and sequestering activities (Suki *et al.*, 1984).

Biofoulant, especially bacteria, may be a major problem in many UF systems, especially those in food industry and biotechnological applications. Many bacterial surfaces have some hydrophobic character (Gristina, 1987), they can attach to many polymeric surfaces by strong hydrophobic interactions (Dumitriu and Meduich, 1994). For instance, marine bacteria attach most readily to hydrophobic plastics, but little number of adherent microorganisms found on negatively charged hydrophilic surface (Fletcher and Loeb, 1979). In addition, many bacteria have specific strategies used to adhere to different surfaces (Baier, 1980). Bacterial fimbriae, flagella or fibrils are long filamentous projections of the cell surface that can act as bridging structures to overcome repulsive electrostatic interactions between negatively charged bacteria and negatively charged surfaces (Gristina, 1987). Once bound to the membrane surface, the bacteria can grow and

multiply using the nutrients present in the process stream. Thus feed streams with high levels of total organic carbon or high total biological oxygen demand are more likely to cause severe biofouling (Zydney, 1996). Some bacteria can even chemically degrade certain polymeric materials. It was reported that bacteria can secrete enzymes that could hydrolyze cellulose acetate membrane, although this type of membrane degradation has not been confirmed under carefully controlled experimental conditions (Flemming, 1993). The biofilm formed by bacteria on membrane surface have dramatic effects on both solute and solvent transport through the fouled membrane. The biofilms formed of different bacterial types can provide very different hydraulic resistance to flow. The retention characteristics of this type of bacterial biofilm could be dramatically reduced upon addition of EDTA due to modification of the extracellular matrix (Hodgson, *et al.*, 1993).

1.4.2.2. Relationship between fouling and membrane properties

Membrane properties also strongly affect on the fouling during UF processes as well as other membrane processes.

Ideal membrane should be hydrophilic. If the membrane is hydrophobic, it can adsorb components that are hydrophobic resulting in fouling (many proteins have hydrophobic region within their structure that can interact strongly with hydrophobic materials). Hydrophobic materials are also tend to attract oil in an oily wastewater stream, but hydrophilizing the membrane can minimize oil fouling. Cellulose membrane can reduce fouling much more than other hydrophobic membranes. The benefits of using cellulose membranes for protein solution is well known (Shi *et al.*, 2001).

The surface topography of membrane is also a reason related to differences of fouling on different membranes. 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).

The pore size of membranes is also very important. Large pore membranes can initially get higher flux than tighter membranes, but eventually (sometimes rapidly) have lower flux. The reason is that the particles in the feed may lodge in the pores without going through them if the sizes of the particles have the same order of magnitude as the range of pore sizes of membranes. This physical blockage of the pores causes a rapid drop in the flux in the first few minutes of process. In contrast, if the pores are much smaller than the particles to be separated, the particles may not be caught within the pores but roll off the surface.

1.5. Methods to enhance permeate flux

The final target of the UF processes is to obtain effective separation of the feed and permeate streams. In order to achieve this target, the effective permeate flux is required. Many different approaches have been taken to combat fouling and concentration polarization.

1.5.1. Hydrodynamic approachs

Increasing the mass transfer away from the membrane can reduce the concentration polarization, and increasing the wall shear rate or scouring the membrane surface can reduce fouling. Both increasing the mass transfer away from the membrane and increasing the wall shear rate or scouring the membrane surface can be achieved by increasing the

cross flow rate (Nystrom and Howell, 1993). Increasing cross flow rate increases the initial flux but also increases the flux reduction rate because the cross flow rate does not have a significant effect on irreversible fouling (Cheryan and Chiang, 1984).

Transmembrane pressure (TMP) is an important parameter for UF process. In some cases (e.g. UF of milk protein), reducing TMP can increase the rate of removal of the deposited layer during rinsing because TMP caused consolidation of the fouling layer. High pressure also may cause a compression of the adsorbed cake and forcing the partially lodged particles to become even more firmly lodged in the pores, making cleaning more difficult.

Backflushing is a method used to improve UF process, especially for hollow fibre model. Backflushing serves to clean the membrane surface by forcing permeate or other fluid such as air back through the fibre. Typically flushing periods of a few seconds every few minutes are found to be the most effective with a trade off between the down time and loss of permeate against increased flux (Nystrom and Howell, 1993).

It has been found that pulsed flow also can reduce fouling. Pulsed flow in the pipes can enhance mass and heat transfer, modify the laminar/turbulent transition, heighten the migration of solid particles away from the wall and shift the maximum velocity under laminar flow conditions towards the wall region (Edwards and Wilkinson, 1971). Pulsed flow may be used to improve membrane performance under experimental conditions when a non-linear relationship between flux and wall shear rate exists (Bauser *et al.,* 1982). Pulsed flow may be induced by many means such as vibration of a porous plate above the membrane

surface, pump vibration or ultrasound. Flux increases of up to 70% have been found with pulsing frequencies up to 1 Hz in reverse osmosis of a 10 wt% sucrose solution in the turbulent or laminar-turbulent transition regimes (Nystrom and Howell, 1993).

In addition, the insertion of rods, wire rings, glass beads, kinetic mixers, doughnut-disc and cone-shaped inserts, baffles or moving balls in the feed channel of UF process has been suggested as a way to minimize fouling

1.5.2. Membrane modification

As mentioned above, the hydrophobic property of membrane is one cause of fouling, especially when protein solution is filtered. Hence, it is believed that introducing more hydrophilic groups in membrane polymer can achieve flux increase and less fouling. Hydrophilic monomers can be grafted into a hydrophobic polymer backbone, e.g. by γ –radiation or by chemical methods (Nystrom and Howell, 1993).

It was reported that the flux was increased 10-fold and the rejection increase 3-fold if the UF membranes was prepared by grafting hydrophobic poly(vinyl chloride) (PVC) with less hydrophobic monomers of vinyl acetate (VAc), hydroxyethyl methacrylate (HEMA) and acrylonitrile (AN) (Vigo and Uliana, 1989). An increase in the hydrophilicity of the membrane can also make it perform better in the fractionation of proteins (Hashimoto and Sumimoto, 1987).

The modification also can be carried out by plasma treatment of the membrane surface in an inert atmosphere if the oxidation of the surface is not desired (Wolff *et al.*, 1988). But it should be noted that

modified versions of membranes may not be as tolerant to aggressive environments as the native materials .

1.5.3. Pretreatment of feed bulk

The fouling ability of a solution may be diminished by suitable pretreatment of the solution. The pretreatment can be mechanical, thermal or chemical. Modifications to the feed solution include adjustment of pH, removal of fibres, fines, etc., and heat treatment of the feed solution.

Some surfactants may interact with the membrane and cause a rapid and irreversible flux decline. The flux of antifoams with cloud points has been shown to be virtually zero for polysulphone membranes (Jonssen *et al.,* 1990). It is advisable to be aware of which antifoam and wetting agents are added to the solution to be processed.

1.5.4. Membrane cleaning.

Fouling necessitate regular cleaning to restore the function of membrane and get a lifetime of membrane as long as possible. In food industry or biotechnological applications, the presence and growth of microorganisms are important factors that we must consider. So disinfection is also needed. Membranes used in the food industry are generally cleaned at least once a day. Normally, a cleaning cycle includes the following stages (Tragardh 1989): (a) product removal from the system; (b) rinsing with water; (c) cleaning in one or more steps; (e) rinsing with water; (f) disinfection.

The product should be rinsed out at the same temperature as that used in the process. This is important for products that tend to form gels at low temperatures. During rinsing, both retentate and permeate should be discharged. The rinsing should be continued until both the retentate and permeate are totally clear and neutral. The chemical and

bacteriological quality of the water used for rinsing and cleaning the membrane is important. The presence of iron, silica, calcium etc. can lead to deposits which are difficult or impossible to remove. The normal water quality required is: (a) iron < 0.005 ppm; (b) manganese < 0.02 ppm; (c) silicate (SiO_2) < 5 ppm; (d) hardness < 20 German degrees; (f) particle < 25μm; (g) total plate count < 1000 per ml; (h) *Coli* count 0 per 100 ml (Tragardh, 1989).

The cleaning also can be done by mechanical, thermal or chemical processes. Mechanical cleaning can be affected by introducing a high shear rate at the membrane surface. Sometime, periodical back flushing, i.e. applying a pressure on the permeate side, thus pushing part of the permeate back through the membrane, can be used. The chemicals used in cleaning process should have the characteristics as following: (a) loosen and dissolve the fouling; (b) keep the foulant in dispersion and solution; (c) avoid new foulings; (d) not attack the membrane and other parts of the system; (e) disinfect all wetted surfaces (Tragardh, 1989).

The types of cleaning agents include alkali, acid, enzyme, surface-active agent, etc. H_2O_2, metabisulphite, hypochlorite and heat treatment are often used for disinfection.

Temperature, time, concentration of solution and type of detergent are important cleaning parameters that vary with foulant and the type of membrane material. As a rule, mineral deposits are removed by acid, and organic compounds are removed by alkaline agent. For some products, it is necessary to use different cleaning approaches simultaneously to obtain a satisfactory cleaning result (Tragardh, 1989).

As mentioned above, fouling can be quantified by the resistance appearing during the filtration, thus cleaning can be specified by the removal of this resistance (Martinez *et al.*, 2001).

Resistance removal (*RR*) which is a tool for cleaning quantification can be estimated from:

$$RR\% = [(R_f - R_c) / R_f] \times 100 \qquad (7)$$

where, R_f is resistance appeared after fouling; R_c is resistance remained after cleaning;

Flux recovery (*FR*) can be estimated from:

$$FR\% = [(J_{wc} - J_{ww}) / (J_{wi} - J_{ww})] \times 100 \qquad (8)$$

where, J_{wc} is water flux after chemical cleaning; J_{ww} is water flux after washing for removal of unbound substances; J_{wi} is the initial water flux;

Both resistance removal and flux recovery have been used for demonstrating the cleaning efficiency (Aguado *et al.*, 1996)

1.6 Applications of UF

The choice of separation process for a specific application is influenced by several factors: the nature of the solutes, the degree of separation required and the volume of the solution to be treated. UF compares favourably with other separation techniques. The most important advantages of UF are its unique fractionation capability, the low energy consumption and the flexibility in operating temperatures. UF

plants can be operated at almost 0°C to about 80 °C, depending on the heat sensitivity of the solution and the membrane material.

As mentioned above, the membranes used for UF are finely microporous, and in many cases, they are asymmetric. Water transport is by viscous flow through the pores, driven by a moderate applied pressure. Small solutes may also pass through the membranes, but macrosolutes, colloids, and some charged species are retained (Fane, 1990).

The UF process offers unique separation possibilities. In some applications UF is an alternative to other separation process. Commercial applications of UF are numerous and found in many different fields. For example, UF is used for product recovery and pollution control in the chemical, biotechnology and other industries. UF has been used for more than 20 years to concentrate protein. It is often used throughout an enzyme-purification scheme that contains many steps. UF is very effective at recovering bioproducts from dilute fermentation broths.

Furthermore, many separation processes effectively reduce the amount of low-molecular weight constituents in solution. However, high-molecular-weight substances in the solution substantially increase the loading on these separation processes. If the high-molecular substances are removed, the loading on the process is appreciably reduced. Therefore, in many cases, more profitable to regard UF (which effectively removes high-molecular-weight compounds) not only as a separation alternative, but also as a complement to processes such as chemical and biological treatment, adsorption, ion exchange, etc.

For concentration application, the low energy consumption of membrane process, compared with thermal separation processes, makes UF an attractive concentration alternative in many applications. When

used for concentration, UF often competes with evaporation. The concentration of milk is already successfully performed by UF. The low operating temperatures of UF allow sensitive solutions to be treated without the constituents being damaged or chemically altered. UF has been found to be a gentle concentration technique for blood serum (Pruthi, *et al.,* 1997) and egg-white (Ehsani, *et al.,* 1997), for example. The concentration of enzyme is another example.

For fractionation, the applications where the unique fractionation capabilities of UF used are, in general, very successful. Applications have the best economy since valuable products are recovered and purified. In food industry, the example of successful fractionation applications is the clarification of fruit juices (Girard and Fukunoto, 2000).

Another valuable advantage of UF is its ability to perform two separations in one step. The example is the clarification of gelatin solutions, where UF replaces both the traditional fine filtration and the evaporation step (Dutre and Gragardh, 1995).

UF is also often used for the recovery and purification of macromolecules. For example, for waste water treatment. The cost of disposal process is reduced, as well as, the high penalties for pollution is avoided.

In addition, Gravatt (1986) gave information on the performance of a flat-sheet membrane system for the recovery of Cephamycin C (an antibiotic) from a Norcardia lactamdurans culture. Furthermore UF was used to harvest cells (Lamparski *et al.,* 2002) and for salt exchange (Jhawar *et al.,* 2003). Many more downstream processing applications of UF are regularly being reported.

2. Yellowfin tuna , fish proteases and isolation methods of enzyme

2.1 Yellowfin tuna and tuna canning process

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 (a single species) 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 (Ramon *et al.*, 2004).

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

Yellowfin (*Thunnus albacares*) is one kind of large tuna (Figure 7). The major morphological characteristics include: 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; the common sizes lie between 35 and 180 cm fork length, that is between 0.8 and 111kg (Allen and Demer, 2003).

Yellowfin tunas are found worldwide in tropical and subtropical waters, from latitudes of approximately 40°N to 35°S. They are absent in the Mediterranean Sea. The yellowfin tuna is a highly migratory fish. In the Pacific Ocean, however, there is little evidence for long-range north-south or east-west migration. This suggests relatively little genetic exchange between the eastern, central, and western Pacific Ocean and perhaps the development of subspecies (Ramon *et al.,* 2004).

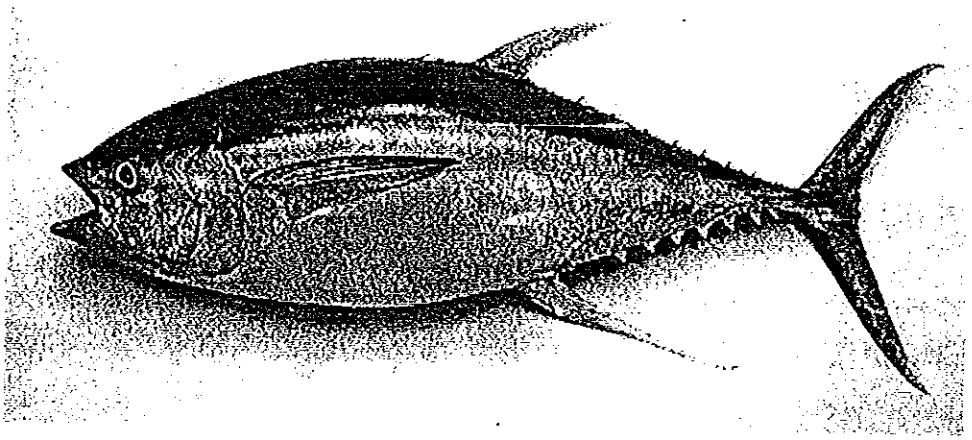


Figure 7 Yellowfin tuna
Source: Camera Hawaii, Inc (1995)

Tuna canning industry is a large industry in Thailand. Canned tuna in Thailand ranks first in the world since 1985 (Economic Agriculture Office, 1999). Figure 8 showed the flow-chart for the tuna canning and the waste from canning process.

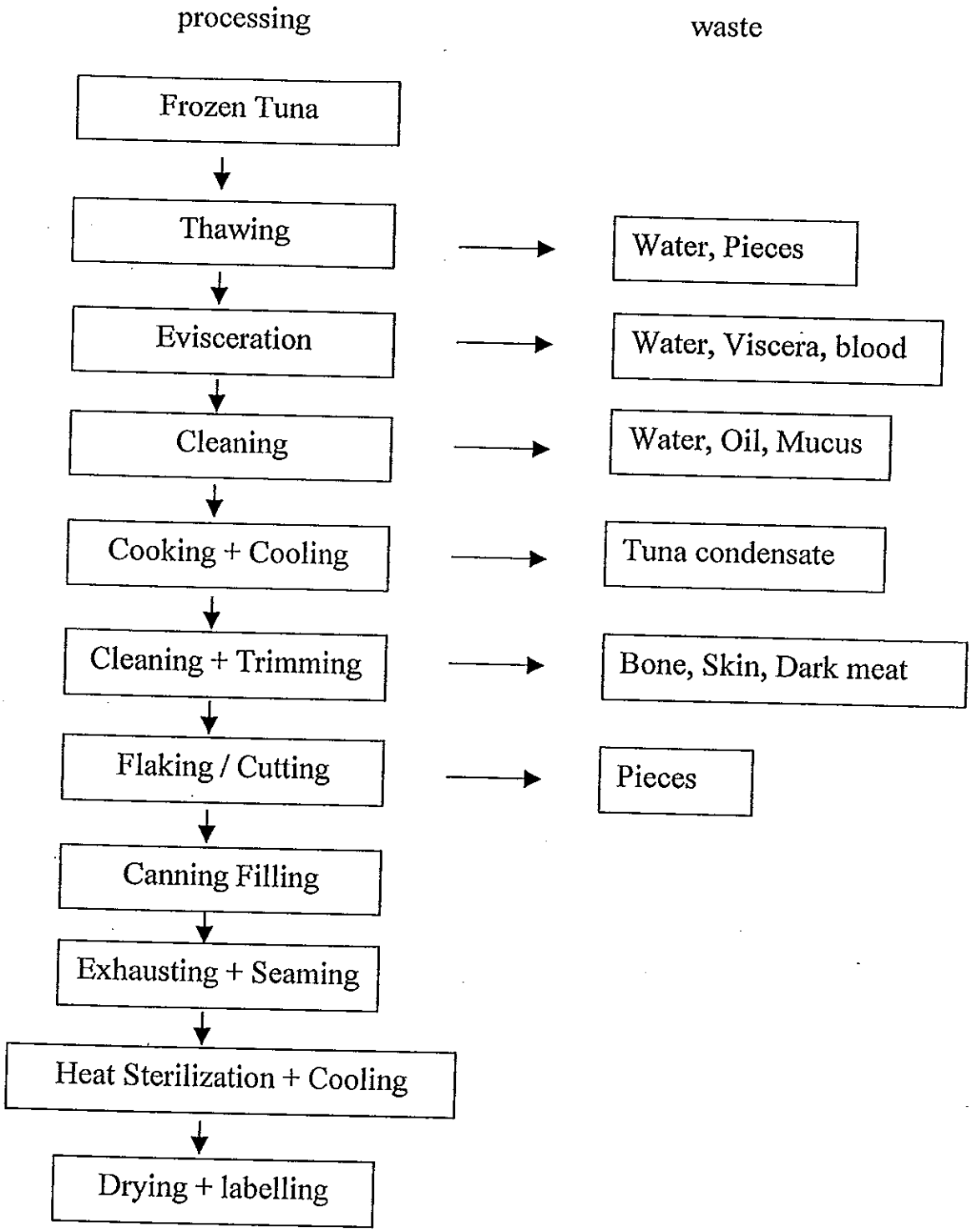


Figure 8 Typical flow-chart for the canning of tuna

Source: H-Kittikun (2003)

During tuna canning process, there are 25-30% solid waste (e.g. head, skin, viscera) and about 35% liquid waste (e.g. blood, tuna condensate, oil). More than 200,000 metric tons of tuna viscera and offal can be collected annually (Visessanguan *et al.*, 2003).

Since it is necessary to put by-products of seafood harvesting better economic use and the demand for alternate sources of enzymes, the waste from tuna canning process becomes the potential source for enzymes production.

2.2 Fish proteases and isolation methods

2.2.1 Types of commercially useful proteases

The term protease refers to all enzymes that hydrolyze peptide bonds. Other names include peptidase and peptide hydrolase. There are many applications of proteases in a lot of areas.

Proteases differ in their ability to hydrolyze various peptide bonds. Each type of protease has a specific kind of peptide bonds it breaks. Examples of proteases include : fungal protease, pepsin, trypsin, chymotrypsin, papain, bromelain, and subtilisin.

Proteases are classified into four groups according to the catalytic residue involved in the nucleophilic attack at the carbonyl carbon of the scissile bond (Table 2).

Table 2 Classification of commercial proteases and their fields of use

Class	I.U.B.No.	Use
Serine proteases (alkaline)	3.4.21	Pharmaceutical, Detergents, Food
Thiol proteases	3.4.22	Meat tenderizing, Beer haze prevention
Carboxyl proteases (acid)	3.4.23	Baking, Other food uses Milk-clotting in cheese manufacture
Metalloproteases (neutral)	3.4.24	Aspartame manufacture, Food, Brewing

Source : Frost *et al.*, (1987)

2.2.2 Fish proteases

The term "fish proteases" refers to hydrolytic enzymes from aquatic species that catalyze degradation of peptide bonds in protein molecules. There is a large pool of diversified species of fish adapted to a variety of habitat conditions. These organisms include about 7,000 fresh water species and 13,000 salt water species of fish. It is expected to find in these fish species the equivalent of any proteases found in other terrestrial animals and plants, since they all carry out virtually the same metabolic processes. Fish proteases may be classified using the same criteria used for proteases from other animal, plant or microbial sources, i.e. serine proteases (e.g. trypsin from catfish) (Yoshinaka, 1984), thiol proteases (e.g. cathepsin from tilapia) (Sherekar, 1988), acid proteases (e.g. pepsin from trout) (Twinning, 1983) and neutral proteases (e.g. collagenase from lobster) (Haard, 1994).

Genetic variations within species together with adaptation to different environmental conditions have resulted in that fish proteases have particular properties. Some of these properties include higher catalytic efficiency at low temperature, lower thermal stability and substantial catalytic activity at neutral to alkaline pH. For example, when compared to pepsins from animals living in temperate or warm habitat or those from endotherms, pepsins from cold water fish exhibit lower Arrhenius activation energy, temperature optima, thermal stability and a higher apparent Michaelis constant and pH optima (Lopez *et al.*, 1998).

A survey of proteolytic digestive enzymes in various species of fish has revealed that a serine protease is widely distributed in fish viscera. Trypsin and chymotrypsin belong to the family of serine

protease. They are important in the digestive system due to their high proteolytic activities.

The distribution and activity fo proteases in viscera of different species of tuna (skipjack tuna, yellowfin tuna and tonggol tuna) have been researched. The results proved that the protease extracted from yellowfin tuna viscera exhibited the maximum activity. The protease extracted from the tonggol tuna viscera showed the lowest activity. Comparison on the protease activity of the individual viscera organ (stomach, spleen, liver and pancreas) from all three tuna species showed that spleen was the best source for protease followed by liver, pancreas and stomach. The research also showed that the optimum pH for protease from skipjack tuna, yellowfin tuna and tonggol tuna were at pH 10.0, 10.0 and 9.5, respectively. The optimum temperature for activity of protease was at 50 °C. Protease extracted from spleen showed the best thermal stability (Prachumratana. 1998). Another previous research proved that spleen of yellowfin tuna was the best source for trypsin with the highest activity of 49.26 units/ml and the specific activity of 13.81 units/mg protein. The best source for chymotrypsin was pancreas of yellowfin tuna, chymotrypsin isolated from pancreas of yellowfin tuna had the highest activity of 4.13 units/ml and specific activity of 1.03 units/mg protein. The purified trypsin and chymotrypsin from yellowfin tuna viscera were characterized and the optimum pH was found at pH 8.0 and the optimum temperature was 50°C. They were stable in the pH range of 7.0-8.0. Their molecular weights were approximately 23 and 25 kDa, respectively (Jantaro, 2000).

2.2.3 Trypsin

Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains (Brown and Wold, 1973). It has been isolated from a number of sources including crayfish (Kim *et al.*, 1992), African lungfish (Reeck and Neurath, 1972), anchovy (Martinez *et al.*, 1988), moose (Stevenson and Voordouw, 1975), whale (Bricteux-Gregoire *et al.*, 1975), Atlantic cod (Gildberg, 1992), capelin (Hjelmeland and Raa, 1982), and so on.

Trypsin inactive precursor, trypsinogen is transformed into trypsin as the result of the cleavage of a single peptide bond (Lys_6-Ile_7) near the N-terminal of the zymogen. The activation process is catalyzed by a variety of enzymes including enterokinase, mold proteases and trypsin itself. The latter autocatalytic process is accelerated by calcium ions which bind to the N-terminal region of the zymogen and promote the specific bond cleavage (Walsh and Wilcox, 1970).

Trypsin is inhibited by organophosphorus compounds such as diisoprophyl fluorophosphate and natural "trypsin inhibitor" from pancreas. Soybean, lima bean, and egg white are sources of inhibitors. silver ion was also found to be a potent inhibitor (Chambers *et al.*, 1974).

Trypsin from marine animals resemble mammalian trypsins with respect to their molecular size (22.5-24 kDa), amino acid composition and sensitivity to inhibitors such as aprotinin (or trasylol), soybean trypsin inhibitor (SBTI) (Simpson, 2000). Their pH optima for the hydrolysis of various substrates have been reported 7.5 to 10.0, while their temperature optima for hydrolysis of those substrates ranged from 35-50°C (Jantaro, 2000; Vecchi and Coppes, 1996). Trypsin from marine animals tends to be more stable at alkaline pH, but are unstable at acid

pH, unlike mammalian trypsins that are most stable at acid pH (Simpson, 2000).

Trypsin from intestine and pancreas of animal is useful in physician side such as the post-mortem autolytic degradation of abdominal tissues by the proteases from intestine of anchovy (Heu *et al.*, 1995); tenderization; recovery of protein from bones; hydrolysis of blood proteins in meats commodity (Haard, 1992), etc.

2.2.4 Chymotrypsin

Chymotrypsin is an extracellular enzyme and activated from inactive proenzyme chymotrypsinogen. Proenzyme is transferred to small intestine and activated by that cleaved Arg_{15}-Ile_{16} to give chymotrypsin (Walsh and Wilcox, 1970)

Chymotrypsin has been isolated and characterized from marine species such as the anchovy (Heu *et al.*, 1995), Atlantic cod (Heu et al., 1995), capelin (Kalac, 1978), rainbow trout (Kristjansson and Nielson, 1992), carp (Cohen *et al.*, 1981), dogfish (Ramakrishna *et al.*, 1987), etc.

Chymotrypsin preferentially catalyzes the hydrolysis of peptide bonds involving L-isomers of tyrosine, phenylalanine, and tryptophan. It also readily acts upon amides and esters of susceptible amino acids. In addition to bonds involving aromatic amino acids, chymotrypsin catalyzes at a high rate the hydrolysis of bonds of leucyl, metyionyl, asparaginyl, and glutamyl residue (Berezin and Martinek, 1970).

In general, chymotrypsin is single-polypeptide molecules with molecular weight range between 25 and 28 kDa. They are most active within the pH range of 7.5 to 8.5, and most stable at around

pH 9.0 (Kristjansson and Nielson, 1992). Chymotrypsin from marine animals have a higher catalytic activity and hydrolyzed more peptide bonds in various protein substrates at subdenaturation temperature than mammalian chymotrypsins (Simpson, 2000)

Chymotrypsin can be inhibited by heavy metal, the natural trypsin inhibitors from potato, and organophosphorus compounds to various degrees. It was reported phenothiazine-N-carbonyl chloride also can be specific for chymotrypsin inhibition (Erlanger *et al.*, 1970).

Chymotrypsin is useful for food industry such as fish extract production, baked goods, egg and egg products and cheese curd formation in cheese products (Heu *et al.*, 1995; Haard, 1992).

2.2.5 Methods for isolation of enzymes from marine animal

Since enzymes from marine animal have potential value, a number of isolation methods have been developed. For example, two isozymes with chymotrypsinlike activities were isolated from the pyloric ceca of Atlantic cod by homogenizing the tissue in cold Tris-HCl buffer (pH 7.5), followed by centrifugation to exclude undissolved tissue, affinity chromatography on p-aminobenzamidine Sepharose 4B to remove trypsins and hydrophobic interaction chromatography (HIC) step with phenyl-Sepharose to obtain the chymotrypsins (Asgiersson and Bjarnasson, 1991).

Two chymotrypsinlike proteases were isolated from the pyloric ceca of rainbow trout by homogenizing the tissue in Tris-HCl buffer (pH 8.1) followed by ammonium sulfate precipitation, hydrophobic interaction chromatography on phenyl-Sepharose, and then ion-exchange (IEX) chromatography on EDAE-Sepharose (Kristjansson and Nielson, 1992).

Heu *et al.* (1997) purified and characterized Cathepsin L-like enzyme from the muscle of anchovy (*Engraulis japonica*). After the pre-treatment of raw material, the crude enzyme solution was subjected to 30-80% ammonium sulfate (AS) fractionation. The fraction was treated by a series of steps including dialysis, UF and chromatography (loading onto Sephadex G-75 column and CM-Sephadex C-50 column), the fractions with high catheptic activity were concentrated and stored at –40°C.

Simpson and Haard (1984a) purified and characterized trypsin from the Greenland cod (*Gadus ogac*). The method involved ammonium sulfate fractionation, affinity chromatography(SBTI-Sepharose) and electrophoresis.

Jantaro (2000) isolated trypsin and chymotrypsin from viscera of yellowfin tuna (*Thunnus albacares*) by the method involving ammonium sulfate precipitation, dialysis, gel filtration column chromatography, affinity column chromatography, anion-exchange column chromatography and gel electrophoresis.

Some researchers studied the application of membrane technology in this field. For example, Chen and Zall (1985) studied concentration and fractionation of proteases from clam viscera by UF using Amicon thin channel UF system (TCF-10). The membrane was operated at 2.46 Kgf /cm^2 at 2 °C with a recirculation rate of 200 ml h^{-1}.

Gildberg and Shi (1994) isolated tryptic enzymes from fish sauce which prepared by salt fermentation from mixed viscera of Atlantic cod (*Gadus morhua*). UF was carried out using an Alfa-Laval UF pilot unit (UFP 11) with a minimum recirculation volume of 6 liter. The unit was fitted with two Amicon polysulphone hollow fibre

cartridges (HF 26.5-43-PM10) giving a total membrane area of 5 m^2 and a nominal molecular weight cut-off of 10 kDa. The temperature of the retentate was maintained between 22 and 27 °C during filtration. The same researchers also recovered proteases from Atlantic cod (*Gadus morhua*) stomach and intestines by the UF unit with hollow fibre membrane (Amicon, H1P10-20, MWCO 10 kDa) (Gildberg, 1992).

In addition, Dewitt and Morrissey (2002b) studied the parameters for the recovery of proteases from surimi wash water. The centrifugal units (Pall filtron, Northborough, MA) with MWCO 30, 50 and 100 kDa were tested. These researchers also have been successful in recovery of catheptic proteases from surimi wash water at pilot scale by using cross flow UF unit (Maximate™-EXT, Pall filtron) with the MWCO 30 or 50 kDa polyethersulfone membrane which had been specifically modified to minimize protein binding to the surface and interstitial binding.

On the other hand, the research has not been reported to separate proteases from yellowfin tuna viscera by membrane. The present work emphasized on this field.

Objectives

1. To separate proteases from yellowfin tuna viscera by UF;
2. To study the effects of membrane molecular weight cut off (MWCO), transmembrane pressure (TMP), cross flow rate, operation temperature and prei-ncubation time on enzyme separation during UF.

Chapter 2

MATERIALS AND METHODS

Materials

1. Raw materials

Viscera of yellowfin tuna (*Thunnus albacares*) were kindly provided by Chotiwat Manufacturing Co., Ltd., Hat Yai, Songkhla. The viscera were kept in a freezing room (-20°C).

2. Chemicals

Analytical grade chemicals were used for enzyme extraction from yellowfin tuna viscera, assay of raw material, enzyme activities, protein concentration, gel electrophoresis.

3. Membranes

1. Regenerated cellulose membranes with MWCO 30 kDa and 100 kDa, Pellicon 2 module, Millipore Corporation.
2. Polyethersulfone membranes with MWCO 30 kDa and 100 kDa, Biomax module, Millipore Corporation.
3. Polyethersulfone UF membrane with MWCO 10 kDa, PBCC module, Millipore Corporation.

4. Instruments

1. Spectrophotometer, Model U-2000, Hitachi Koki Co., Ltd.
2. Water bath, Model W 350, Memmert Ltd.

3. pH meter, Model 420A, Orion Research, Inc.
4. Refrigerated centrifuge, Model Himac SCR-20B, Hitachi Koki Co., Ltd.
5. Peristaltic pump, Model CH2A, Amicon Ltd.
6. Plate and frame membrane filter holder, Model Pellicon 2 casstte filter stainless steel holder and assembly, Millipore Corporation.
7. Reciprocal displacement pump, model Procon XX814V230, Millipore Corporation.
8. UV / VIS Spectrophotometer, Model V-530, Jasco Ltd.
11. Electrophoresis Mini-Protein II Dual Slab Cell with power supply, Model 1000-500, Bio-RAD Laboratories.
12. Kjeltech system, Model 1002 Distilling Unit, Tecator Company.
13. Stirred ultrafiltratin cell, Model 8050, Amicon Ltd.

Analytical methods

1. **Soluble protein** was measured by the method of Lowry *et al.* (1951).
2. **Moisture** was measured by the method of A.O.A.C. (1990).
3. **Crude fat** was measured by the method of A.O.A.C. (1990).
4. **Ash** was measured by the method of A.O.A.C. (1990).
5. **Salt** was measured by the method of A.O.A.C. (1999).
6. **Total amino nitrogen** was measured by method of A.O.A.C. (1980).
7. **General protease activity assay** (modified from the method of Munilla-Moran and Saborido-Rey, 1996)

Casein suspension 0.5 ml of 10mg/ml (in 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3) and 0.1 ml of enzymatic extract were mixed and incubated for 1 hour under 50°C. The reaction was stopped by adding 1.0 ml of chilled trichloroacetic acid

(TCA) (10% in distilled water) to precipitate the non-digested protein. After 1 hour in the refrigerator, the suspension was centrifuged at 10,000 rpm for 10 min at room temperature (30°C). The clear supernatant was measured spectrophotometically at 280 nm against blanks in which casein solution and enzymatic extract were substituted by buffer. Control was made in the same way, but the enzymatic extract was added at the end of the incubation period and just before adding TCA. The absorbance was converted in μg of tyrosine by using standard curves in which the enzymatic extract was substituted by different concentrations of tyrosine. One unit of activity was defined as the amount of enzyme that liberated 1 μg of tyrosine per minute.

8. Trypsin activity assay

The activity of trypsin was measured by the modified method of Cano-lope *et al.* (1987) and Simpson *et al.* (1984b). The activity of trypsin was determined in a reaction mixture (total volume of 3 ml) consisting of 0.10 ml of enzyme solution, 0.30 ml of 10 mM N-toluenesulfonyl-L-arginine methyl ester (TAME) and 2.60 ml of 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3, then incubated at 50°C for 10 minutes. The initial rate of change in absorbance was measured at 247 nm. Trypsin activity and its specific activity were expressed in TAME units per ml and units per mg, respectively. One unit of trypsin activity was defined as 1 μmol substrate hydrolyzed per minute using the extinction coefficient of 540 $M^{-1}cm^{-1}$ at 247 nm.

$$\text{Enzyme activity (units/ml)} = \frac{A_{247nm}/\text{min} \times 1000 \times 3}{540 \times \text{volume of enzyme solution in assay}}$$

9. Chymotrypsin activity

The activity of chymotrypsin was determined in the reaction mixture consisting of 0.1 ml enzyme solution, 1.5 ml of 1.07 mM benzoyl-L-tyrosine ethyl ester (BTEE) in 50% methanol (v/v) and 1.4 ml of 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3, then incubated at 50 °C for 5 minutes. The initial rate of change in absorbance was measured at 256 nm. Activity and specific activity of chymotrypsin were expressed in BTEE units per ml and per mg, respectively. One unit of chymotrypsin activity was defined as 1 μmol substrate hydrolyzed per minute using the extinction coefficient of 964 $M^{-1}cm^{-1}$ at 256 nm (Ramakrishna *et al.*, 1987).

$$\text{Enzyme activity (units/ml)} = \frac{A_{256nm}/\text{min} \times 1000 \times 3}{964 \times \text{volume of enzyme solution in assay}}$$

10. Caluculation of parameters of enzyme

10.1 Specific activity

$$\text{U/mg} = \frac{\text{Activity of enzyme (U/ml)}}{\text{Soluble protein concentration (mg/ml)}}$$

10.2 Total activity

U = Enzyme solution volume (ml) × enzyme activity (U/ml)

10.3 % yield

$$\%\text{yield} = \frac{\text{Total activity of enzyme}}{\text{Total activity of initial enzyme}} \times 100$$

10.4 Purification factor (PF)

$$PF = \frac{\text{Enzyme specific activity after purification}}{\text{Enzyme specific activity before purification}}$$

11. Caluculation of parameters of UF process

11.1 Diafiltration wash volume (V_{wash})

$$V_{wash} = Jv\ A\ t$$

where V_{wash} is the volume of the buffer added into the tank; Jv is average flux; A is membrane area; t is DF time.

11.2 Diafiltration number

$$N_D = \frac{V_{wash}}{V_0}$$

where V_0 is initial feed volume.

11.3 Transmission

$$\text{Transmission} = \frac{\text{Solute concentration in permeate}}{\text{Solute concentration in bulk solution (or feed)}}$$

11.4 Transmembrane pressure (TMP)

$$\text{TMP} = \frac{P_F + P_R}{2} - P_P$$

where P_F is the feed inlet pressure; P_R is retentate outlet pressure; P_P is the permeate outlet pressure.

Methods

1. Preparation of crude enzyme extract

The body weight, whole viscera weight and individual organ weight of the samples of yellowfin tuna were weighed. The viscera were kept at –20°C. The spleen or mixed viscera (100g each) was minced and homogenized in 300 ml of 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% sodium azide. The mixture was centrifuged at 10,000 rpm for 30 minutes and the supernatant was used as the crude enzyme solution (Heu *et al.*, 1995). The crude enzyme solution was assayed for soluble protein concentration, general protease activity, trypsin activity and chymotrypsin activity.

2. Effect of membrane MWCO on enzyme separation

The polyethersulfone membranes with MWCO 30 kDa and 100 kDa (effective area 50 cm^2) were used with peristaltic pump. The extract from spleen was used as feed. Batch concentration mode was used for this UF process. In this mode, retentate was recirculated to the feed bulk, and permeate was removed (Figure 9). The processes were operated at room temperature (30 °C). Feed volume was 500 ml. The cross flow rate was controlled at 50 ml/min. The total operation time was 1 hour. The protein concentration, enzyme activities and enzyme yields in both retentate and permeate were analyzed.

The membrane that provided higher proteases activity and yield was selected for further experiments.

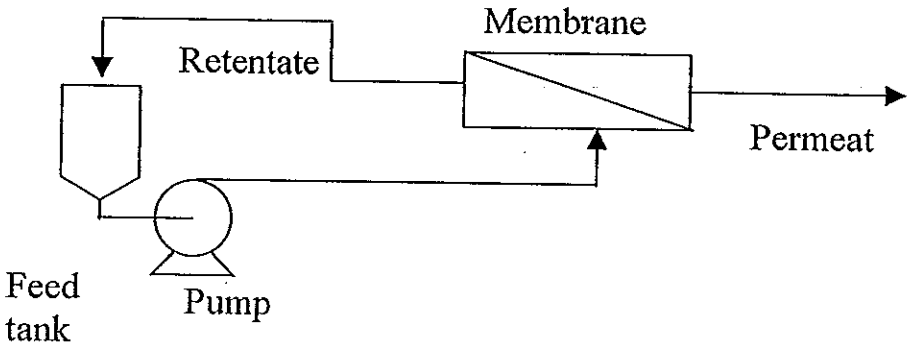


Figure 9 Batch concentration mode

3. Effect of operation temperature on enzyme separation

The method used for this study was the same as that for method 2 except temperature. In addition to room temperature (30 °C), 4 °C was also used. The temperature that provided higher proteases activity and yield was selected for further experiments.

4. Effect of pre-incubation time on enzyme separation

The method used for this study was as that in method 2. The extracts were incubated for different time (0.5, 1.0, 2.0, 3.0, 6.0 hours) at 50°C in water bath before UF. The permeate flux, protein concentration, proteases activity and yield were measured. Permeate average flux was measured by collecting permeate sample in the volumetric cylinder. The incubation time that provided higher proteases activity and yield was selected for further experiments.

5. Effect of cross flow rate and pressure on enzyme separation

The regenerated cellulose membrane with MWCO 30 and 100 kDa (effective area 0.5 m^2) were used with plate and frame membrane filter stainless steel holder and reciprocal displacement pump. The total recycle mode was used in the step (Figure 10). In this mode, both retentate and permeate were recirculated to the feed tank. The cross flow rate was controlled at 120 L/h, 240 L/h and 360 L/h for both two membranes. The transmembrane pressure (TMP) 0.5 bar, 1.5 bar, 2.5 bar, 3.5 bar and 4.5 bar were used for the membrane with MWCO 30 kDa. The TMP 1 bar, 1.5 bar, 2.5 bar, 3.5 bar and 4.5 bar were used for the membrane with MWCO 100 kDa. TMP was controlled by pump. The cross flow rate was controlled by flow rate meter and pump. Permeate flux was measured every 1 minute by collecting permeate sample in the volumetric cylinder over a period of time. The permeate was sampled for protein and proteases analysis.

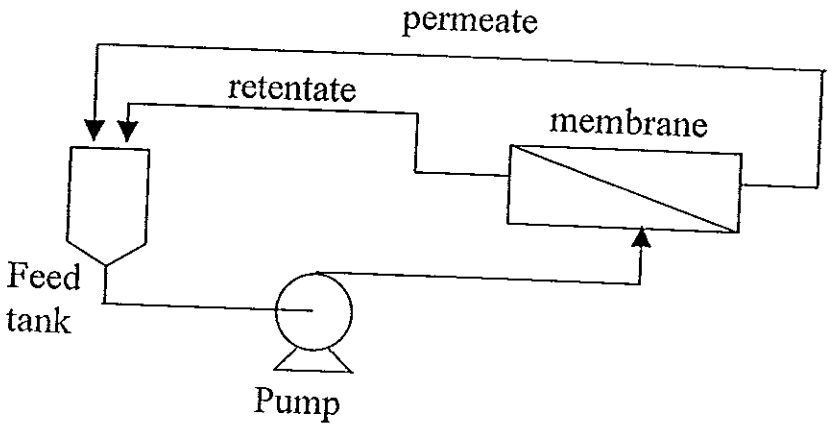


Figure 10 Total recycle mode

6. Continuous diafiltration (DF)

The regenerated cellulose membrane with MWCO 30 kDa (effective area 0.5 m^2) were used with plate and frame membrane filter holder and reciprocal displacement pump. In continuous DF process, the retentate was recirculated to the feed bulk and permeate was removed. The Tris-HCl buffer (20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% sodium azide) was added into feed tank continuously to keep feed volume constant (Figure 11). Both spleen and whole viscera extracts were used in the study. The cross flow rate was controlled at 360 L/h and TMP was controlled at 1.5 bar. The cross flow rate was controlled by flow rate meter and pump. The permeate flux was measured every 10 minutes by collecting permeate sample in the volumetric cylinder. The feed bulk and permeate were sampled at the same time. The DF time was 120 minutes. Soluble protein concentration, proteases acitvities in feed bulk and permeate samples were assayed.

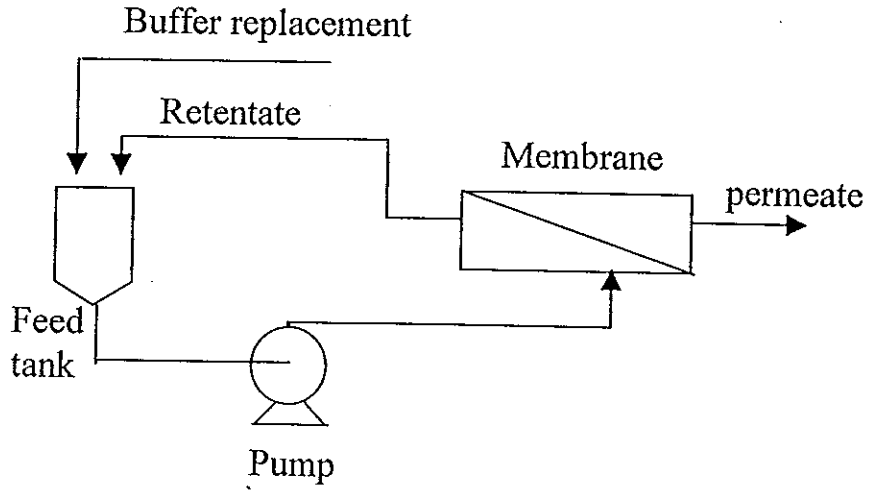


Figure 11 Diafiltration

7. Concentration of product

The purified enzyme solution after DF was concentrated by dead end system using stirred ultrafiltration cell by polyethersulfone membrane with MWCO 10 kDa at pressure 2.5 bar. In this system, the membrane was put on the bottom of the tank. Solution was filtered through membrane by pressure. A magnetic bar was stirred near membrane surface to decrease concentration polarization. The initial feed volume was 50 ml, the volume was decreased to 10 ml after concentration. The soluble protein concentration and proteases activities were assayed before and after concentration.

8. Gel electrophoresis

8.1 SDS-PAGE (Appendix B7 and Appendix B8)

The samples from extract (both spleen and whole viscera) before UF and after incubation, DF and concentrated product were assay by SDS-PAGE to detect the protein composition. One volume sample was diluted with four volume sample buffer. Samples were loaded and runned in Mini-Protein II Dual Slab Cell with 10% or 12% gel. Each well contained about 5 μg protein. The standard protein marker consisted of the proteins with molecular weight 225, 150, 100, 75 and 50 kDa.

8.2 Activity staining (Appendix B9)

The samples from extract before UF, product after DF and product after concentration were used for SDS-PAGE at first, then for activity staining to detect the position of proteases. The 10% gel was used for electrophoresis. After electrophoresis, the gel was soaked in the casein suspension, then incubated at 50°C in 20 mM Tris-HCl buffer (pH 8.0 containing 5 mM $CaCl_2$ and 0.02% sodium azide) (modified from the

method of Carcia-Carreno *et al.,* 1993). The gel was stained by Coomasie brilliant blue R-250 after incubation. The clear zone on blue background indicated the protease after destaining.

Chapter 3

RESULTS AND DISCUSSION

1. Raw materials analysis

1.1 Major parts of viscera

The means weight value of total body weight of yellowfin tuna tested was about 1,222 g. The mixed viscera weight was about 61.90 g giving the ratio of 5.07% of body weight. The spleen weight was 20.99 g giving the ratio of 1.72% of body weight. Other major parts of viscera, i.e. stomach, liver, pancreas, spleen and intestine, were also analyzed. The stomach weight was 19.29 g, which calculated as the ratio of 1.58% of body weight. Liver weight was 13.60 g, which was 1.11% of body weight. Pancreas weight was 1.83, which was 0.15% of body weight. Intestine weight was 3.82, which was 0.31% of body weight (Table 3). The weight and percentage of individual organs approximated to the results from other researcher (Jantaro, 2000). The spleen was almost as the ratio of 30% of mixed viscera. Therefore, the economic value will be significant if this waste can be utilized.

Some previous research revealed that the spleen is the best source for proteases in the tuna viscera (Trairatananukoon, 2001). In this work, the spleen was separated manually and used as the enzyme source at first. The mixed viscera were also used as enzyme source for diafiltration.

Table 3 Weight of total body, mixed viscera and individual organ of the viscera of yellowfin tuna (*Thunnus abacares*)

	Individual Organ (g)					
Total body (g)	Mixed viscera	Stomach	Liver	Pancreas	Spleen	Intestine
1,200	60.13	18.74	12.45	1.86	22.75	4.01
1,108	55.42	18.13	14.03	1.67	18.34	3.39
1,300	68.52	20.48	14.73	2.12	22.58	4.79
1,400	66.82	20.29	13.33	1.84	23.08	3.57
1,100	58.63	18.81	13.48	1.67	18.20	3.34
[1]1,222	61.90	19.29	13.60	1.83	20.99	3.82
	[2] 5.07	1.58	1.11	0.15	1.72	0.31

[1] means weight values

[2] percentage (%) of individual organ in mixed viscera

1.2 Chemical compositions of spleen and mixed viscera

Table 4 showed the major compositions of spleen and mixed viscera. Total proteins were 19.29% and 17.42% of spleen and mixed viscera, respectively. Crude fat in spleen was 13.14% which was much higher than that (5.82%) in mixed viscera. The contents of ash, salt and moisture were similar in both spleen and mixed viscera. The results were also similar to the results from other researcher (H-Kittikun, 2003).

Table 4 Major compositions of yellowfin tuna spleen and mixed viscera

	Mixed viscera	Spleen
Moisture[1](%)	77.79 ±0.35	74.48±0.36
Total protein[1](%)	17.42±0.31	19.29±1.03
Salt[1](%)	0.63±0.045	0.41±0.03
Crude fat[2](%)	5.82±0.31	13.14±1.02
Ash[2](%)	5.57±0.47	6.44±0.24

[1] calculated from wet weight

[2] calculated from dry weight

Mean ± standard deviation of three replications.

2. Effect of MWCO on enzyme separation

The assay of soluble protein provided evidence that the amounts of protein were similar in permeates or retentates separated by polyethersulfone membranes with MWCO either 30 kDa or 100 kDa.

The soluble protein content in retentate separated by MWCO 30 kDa membrane was 14.57 mg/ml while it was 13.79 mg/ml in the retentate separated by MWCO 100 kDa membrane (Table 5). This table also showed the volume concentration factor (VCF) which is defined as the ratio of initial feed to final retentate volume (Zeman, 1996b). The VCF did not have significant difference. It indicated that these two membranes had similar separation ability for proteins distributed in this extract.

Table 5 Soluble protein concentration in permeate and retentate from polyethersulfone membranes with MWCO 30 kDa and 100 kDa

Membrane		Soluble protein (mg/ml)	Volume (ml)	VCF[1]
Crude enzyme solution		13.95	500	
MWCO 30kDa	Retentate	14.57	471	1.04
	Permeate	7.86	26	
MWCO 100kDa	Retentate	13.79	419	1.19
	Permeate	10.76	79	

[1]VCF: volume concentration factor

In theory, the VCF from MWCO 100 kDa membrane should be much higher than that from MWCO 30 kDa. But a greater amount of protein fouled the membrane pore for larger pores size membranes (Youravong, 2001). It was probable that the fouling mechanism resulted in the lacking of difference for VCF. On the other hand, in this research, since the pump could not provide enough cross flow rate and extract contained a large amount of impurity. It is probably that the cake layer was formed rapidly and this cake layer can provide an additional filter layer instead of membrane itself (Vilker *et al.,* 1981). Therefore, the separation of enzymes by membrane itself was only achieved in initial several minutes. This would mean that the permeate flux was governed mainly by the membrane shear rate and not by details of the flow (Ding *et al.*, 2002)

The results also indicated that the membrane with MWCO 30 kDa provided higher yields of enzymes than the membrane with MWCO 100 kDa (Figure 12). Almost all proteases were held in the retentate separated by membrane with MWCO 30 kDa, especially trypsin and chymotrypsin. When the MWCO 100 kDa membrane was used, a part of enzymes was lost to permeate. Dewitt and Morrissey (2002b) also found that protease was distributed almost equally between permeate and retentate when using a lab scale centrifugal UF unit with a 100 kDa MWCO membrane.

The figures of enzyme purity (Figure 13) revealed that the purity of enzymes in the retentate separated by MWCO 30 kDa membrane was much higher than that in permeate. In retentate the specific activities were 3.24 U/mg, 0.82 U/mg and 0.62 U/mg and for general protease, trypsin and chymotrypsin, respectively while they were 0.11 U/mg, 0.01 U/mg and 0.01U/mg in permeate (Table-Appendix C1).

The retentate and permeate separated by MWCO 100 kDa membrane had similar purity. In the retentate, the specific activities were 3.34 U/mg, 0.82 U/mg and 0.63 U/mg for general protease, trypsin, and chymotrypsin while they were 2.85 U/mg, 0.84 U/mg and 0.59 u/mg for general protease, trypsin and chymotrypsin, respectively in the permeate. It meaned that MWCO 100 kDa membrane had poor enzyme separation ability for this feed.

The purities of enzymes in retentate separated by MWCO 100 kDa membrane were higher than those in renteate separated by MWCO 30 kDa (Table-Appendix C1). It was probably due to higher transmission of other proteins using MWCO 100 kDa membrane (Ghosh, 2000). It also suggested that proteases purity could be increased using MWCO 30 kDa membrane by removing impurity by diafiltration system.

Results of the enzyme transmissions from Table-Appendix C1 also confirmed this finding. The transmissions of enzyme by using MWCO 30 kDa membrane were 0.02, 0.01 and 0.01 and for general protease, trypsin and chymotrypsin respectively. The transmissions of these enzymes by using MWCO 100 kDa membrane were 0.66, 0.79 and 0.72 for general protease, trypsin and chymotrypsin, respectively.

From this study, it was found that the membrane with MWCO 30 kDa showed much better selectivity for enzyme recovery from the raw enzyme solution by holding the enzymes in the retentate.

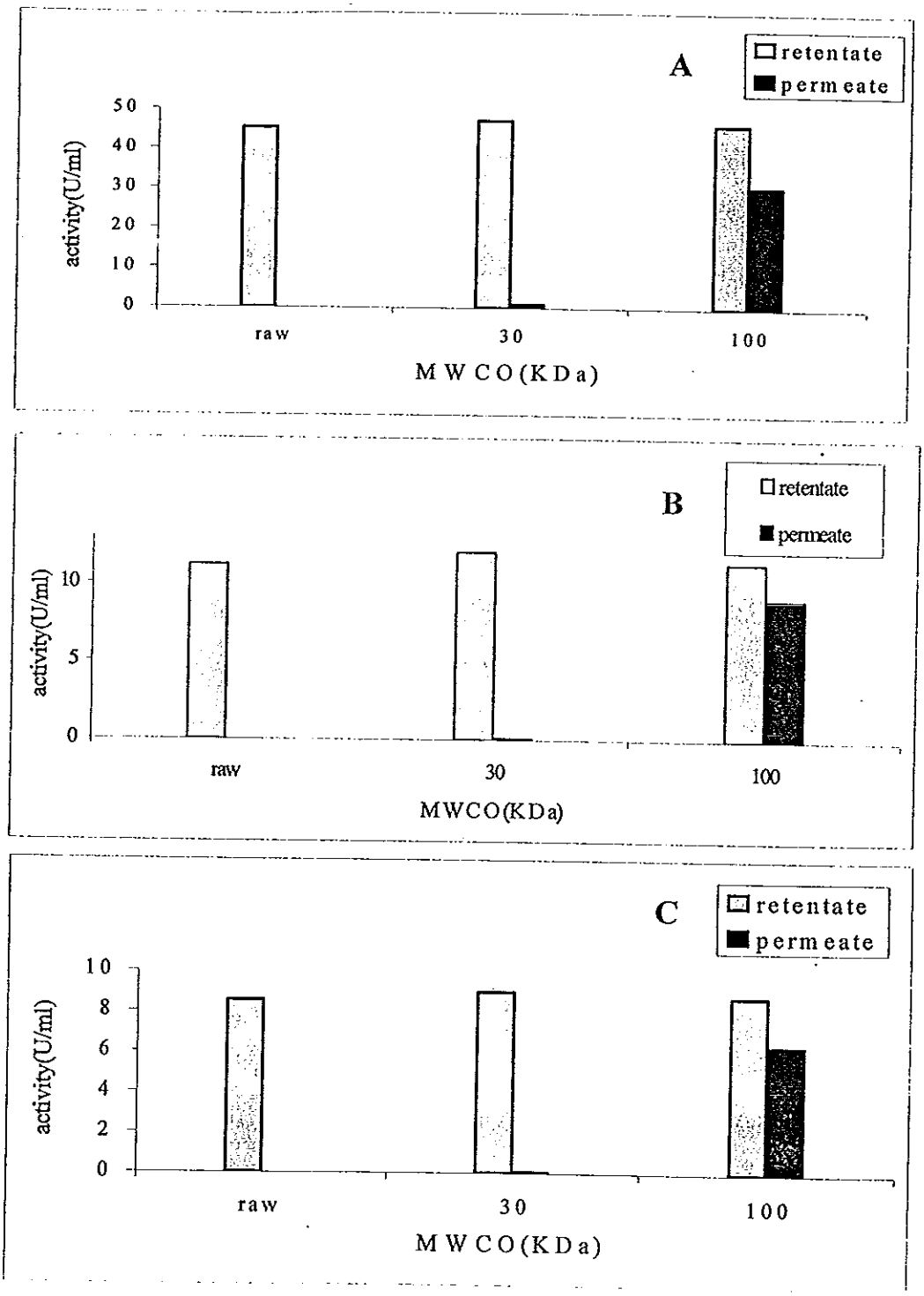


Figure 12 Effect of UF using polyethersulfone membrane with MWCO 30 and 100 kDa on enzyme activity of general protease (A), trypsin (B) and chymotrypsin (C) from yellowfin tuna spleen extract at room temperature(30°C)

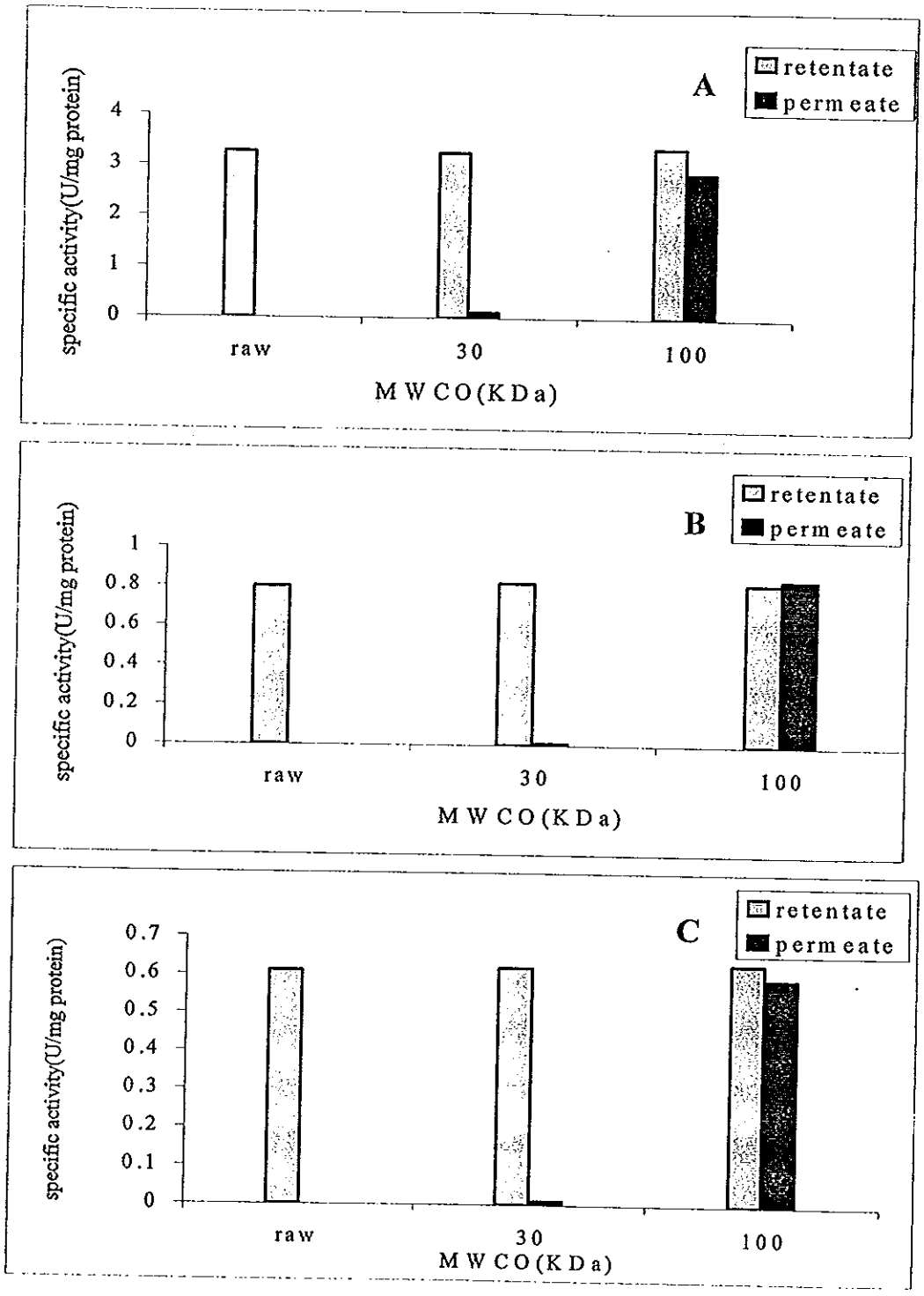


Figure 13 Effect of UF using polyethersulfone membrane with MWCO 30 and 100 kDa on enzyme specific activity of general protease (A), trypsin (B) and chymotrypsin (C) from yellowfin tuna spleenextract at room temperature (30°C)

3. Effect of operation temperature on enzyme separation

The UF process was operated at both room temperature (30°C) and 4°C by batch concentration mode using polyethersulfone membrane with MWCO 30 kDa. The results indicated that there was no significant difference for membrane selectivity during the UF operated at these two temperatures (Table 6). The room temperature (30°C) was selected for further works. Other research revealed that the optimal temperature for these was around 50°C (Jantaro, 2000). Higher temperature could result in loss of enzyme activity. It was due to the denaturation of enzyme. But these enzymes may have more flexible structure adapted to work at low temperature. Therefore, no different effect on enzyme activity at both room temperature (30°C) and 4°C. The enzyme activity was increased a little in retentate comparing to the crude enzyme solution. It was due to the volume reduction of feed after UF.

On the other hand, the volume concentration factor from the process at room temperature (30°C) is a little higher than that from the process at 4°C. It may be due to the composition of spleen. As mentioned above, the spleen contained more crude fat than other organs. It was possible that the fat formed a compact layer at low temperature and covered the membrane (Mohammadi *et al.*, 2003). This layer may prevent the passage of solute and solvent. The low temperature also increased viscosity of extract which decreased the permeate flux (equation 1).

Table 6 Effect of operation temperature on enzyme separation by UF using polyethersulfone membrane with MWCO 30 kDa

	Crude. enzyme. extract	30°C		4°C	
		Retentate	Permeate	Retentate	Permeate
VCF		1.06		1.04	
Protease					
Act.(U/ml)[1]	48.64	51.69	0.74	50.48	0.74
Sp. Act.(U/mg)[2]	4.06	4.22	0.13	4.14	0.13
Trypsin					
Act.(U/ml)	12.16	12.74	0.02	12.74	0.02
Sp. Act.(U/mg)	1.02	1.05	0.01	1.04	0.01
Chymotrypsin					
Act.(U/ml)	8.89	9.61	0.03	9.33	0.04
Sp. Act.(U/mg)	0.74	0.79	0.01	0.76	0.01
Soluble protein Concentration (mg/ml)	11.98	12.09	5.92	12.21	5.77

[1] volume concentration factor
[2] enzyme activity
[3] specific activity

4. Effect of pre-incubation time on enzyme separation

From previous study, it was found that polyethersulfone membrane with MWCO 30 kDa had favorable separation ability to separate proteases from crude extract. Therefore, this membrane was selected for this study. The yellowfin tuna spleen extract was incubated at 50 °C for 0~24 hours. The incubated extract was used as feed with batch concentration mode.

The results in Table 7 showed the effect of pre-incubation time on the soluble protein concentration in crude extract, retentate and permeate separated by UF. In the crude extract, the soluble protein concentration was almost unchanged. However, in the retentate separated from crude extract incubated for 3 hour, the soluble protein concentration showed lowest value (9.86 mg/ml) compared to the retentates separated from crude enzyme solution incubated for 0.5, 1.0, 6.0 and 24 hours.

The result of soluble protein concentration in permeate also confirmed the result mentioned above. The permeate separated from crude extract incubated for 3 hours contained the highest soluble protein concentration (9.02 mg/ml).

The change of soluble protein concentration was due to the protein hydrolysis by proteases presented in crude enzyme solution during incubation. The large molecular weight protein was hydrolyzed to small molecular peptide and passed the membrane to the permeate.

Table 7 Effect of pre-incubation time on average flux and protein separation during UF using polyethersulfone membrane with MWCO 30 kDa

		Soluble protein. concentration (mg/ml)	VCF	Average flux (10^{-3}ml/cm^2.min)
Crude.enzyme solution[1]		12.05		–
0.5 h	Incubated solution[2]	11.94		
	Retentate	11.43	1.04	9.2
	Permeate	8.57		
1.0 h	Incubated solution	11.26		
	Retentate	10.93	1.04	11.2
	Permeate	8.68		
3.0 h	Incubated solution	11.08		
	Retentate	9.86	1.04	11.8
	Permeate	9.02		
6.0 h	Incubated solution	11.33		
	Retentate	10.48	1.05	12.0
	Permeate	8.91		
24.0 h	Incubated solution	11.27		
	Retentate	10.87	1.05	12.2
	Permeate	8.29		

[1] crude enzyme solution before incubation

[2] incubated enzyme solution before UF

Table 7 also showed average flux of UF process when different incubated extracts were used as feed. After incubation, the average flux was increased. It was because that proteases themselves played an active role to produce aqueous hydrolysates which could be subjected to ultrafiltration (Gildberg, 1992). Since the protein molecule was hydrolyzed to small molecule hydrolysates, the viscosity of extract was decreased, so the average flux increased compared to the flux when crude enzyme solution without incubation was used as feed (Equation 1). It suggested that proteases maybe recovered from the ultrafiltration retentate, whereas the bulk of the protein, which could be digested to low molecular weight peptides and free amino acids, could be removed to permeate.

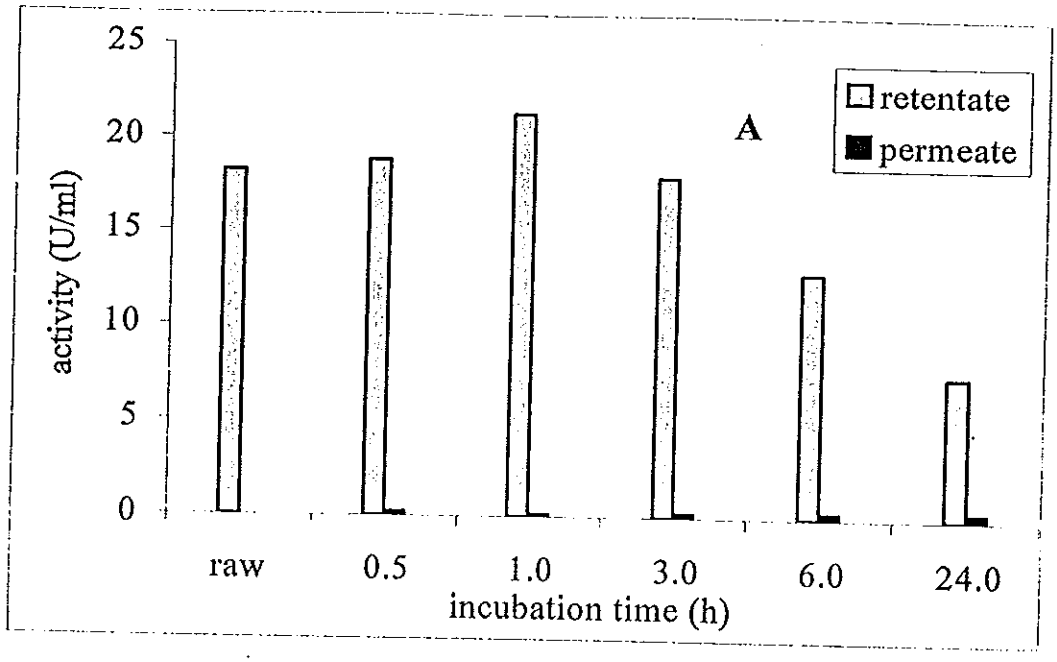
Figure 14, 15 and 16 indicated that different incubation time had effect on enzyme activity in the retentates separated by UF process. The highest enzyme activities in the retentate were 58.43 U/ml (Table-Appendix C2), 14.35 U/ml (Table-Appendix C3) and 15.73 U/ml (Table-Appendix C4) for general protease, trypsin and chymotrypsin respectively when the crude enzyme solution was incubated at 50°C for 1 hour. In this study, several factors may affect on the enzyme activity, such as volume concentration factor and loss of enzyme activity during the process. Since incubation time had not affected volume concentration factors significantly, so the loss of enzyme activity during process became the major reason affecting on recovery of enzyme activity. Loss in protease activity during ultrafiltration was due to protease denaturation and/or binding of protease onto the membrane (Dewitt and Morrissey, 2002a). As mentioned above, the temperature had no significant effect on enzyme activity in this study. It was assumed that losses to the membrane

fraction of enzyme activity was attributed to adsorption/precipitation of protease onto the membrane (Belter *et al.*, 1998). Visual observation of protein precipitated on centrifugal unit membrane surfaces helped to support this conclusion (Dewitt and Morrissey, 2000b).

On the other hand, the long incubation time may resulted in decay of enzyme activity. Benjakul *et al.* (1997) also found that some of the observed reduction in activity was a result of loss in protease stability over time at optimal incubation temperature.

The results in Table-Appendix C2 also showed that the general protease activity in retentate increased when the extract incubated for 6 or 24 hours was used. The reason for this was probably removal of low molecular weight inhibitory compounds form the retentate (Gildberg and Overbo, 1991).

These Figures also indicated that the pre-incubation time had similar effect on enzyme purity. As same as enzyme activity mentioned above, the highest specific activities in retentates were found when the crude extracts were incubated at 50°C for 1 hour bbefore UF. The highest activities were 5.35 U/mg (Table-Appendix C2), 1.46 U/mg (Table-Appendix C3) and 1.44 U/mg (Table-Appendix C4) for general protease, trypsin and chymotrypsin respectively. The reasons for purity change were similar to those for activity change.



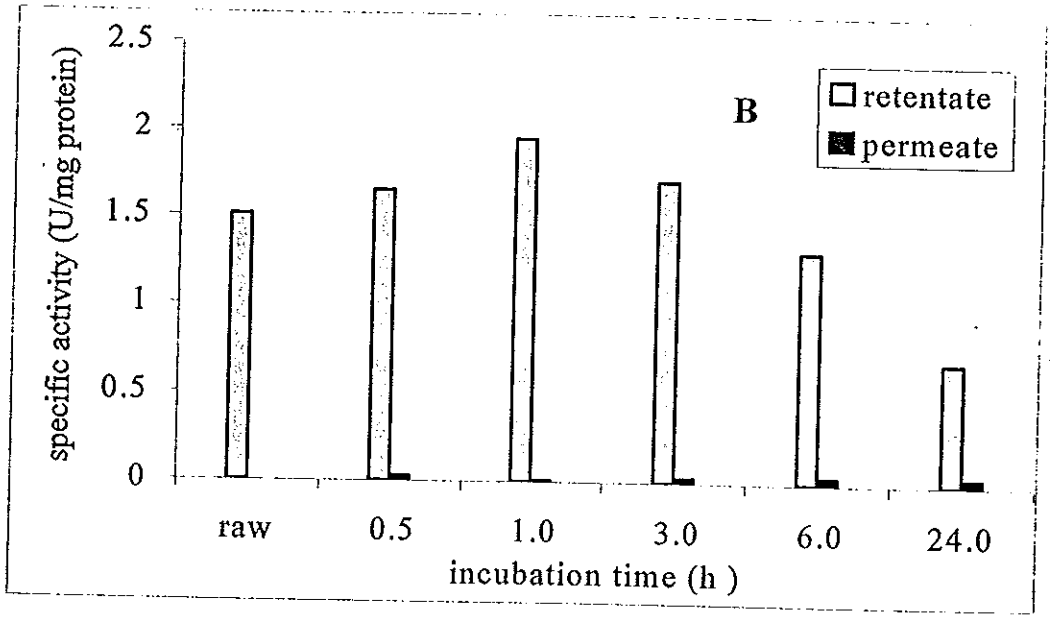


Figure 14 Effect of pre-incubation time on activity (A) and specific activity (B) of general protease from yellowfin tuna spleen extract separated by batch concentration UF mode using polyethersulfone membrane with MWCO 30 kDa

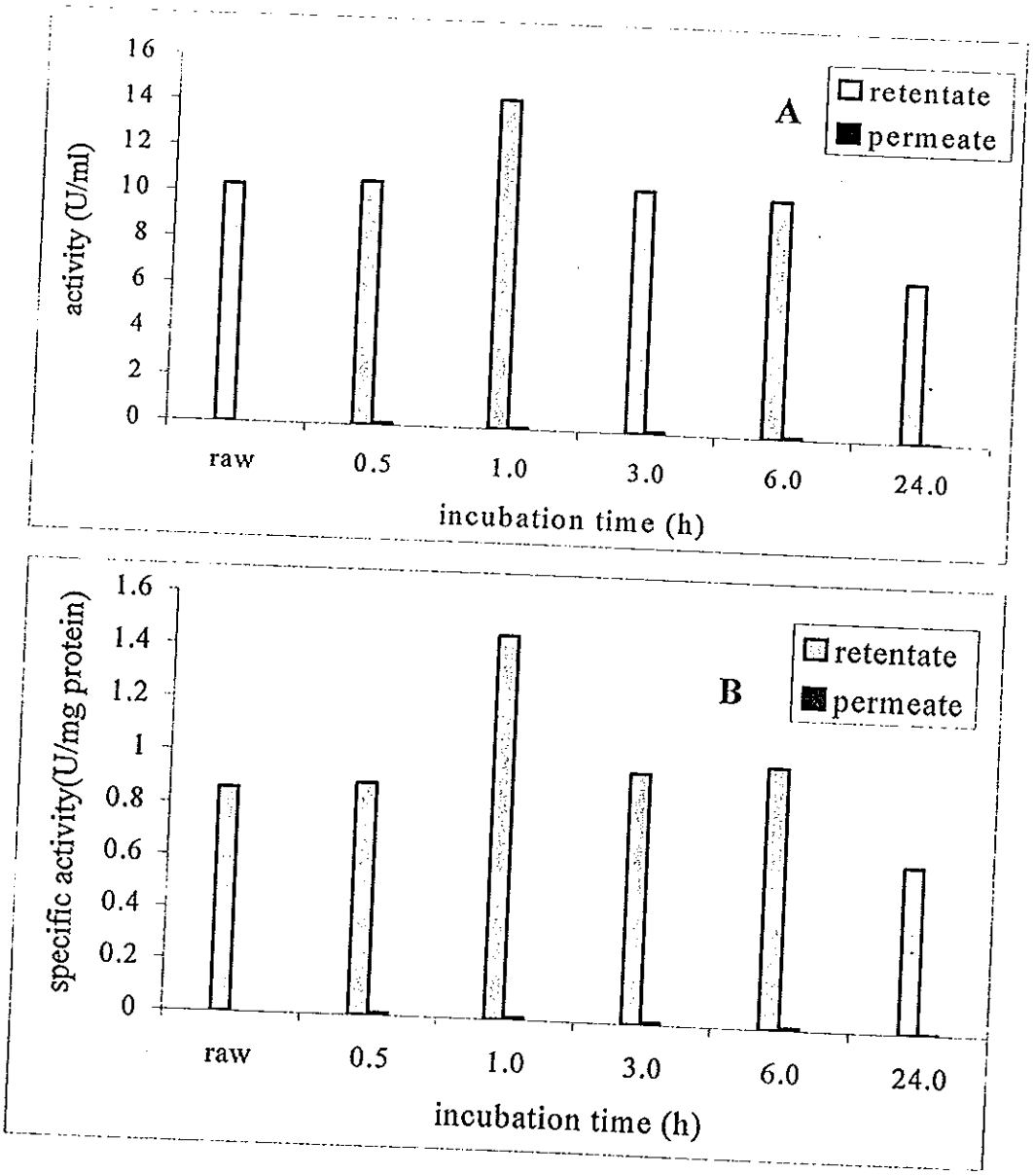


Figure 15 Effect of pre-incubation time on activity (A) and specific activity (B) of trypsin from yellowfin tuna spleen extract separated by batch concentration UF mode using polyethersulfone membrane with MWCO 30 kDa

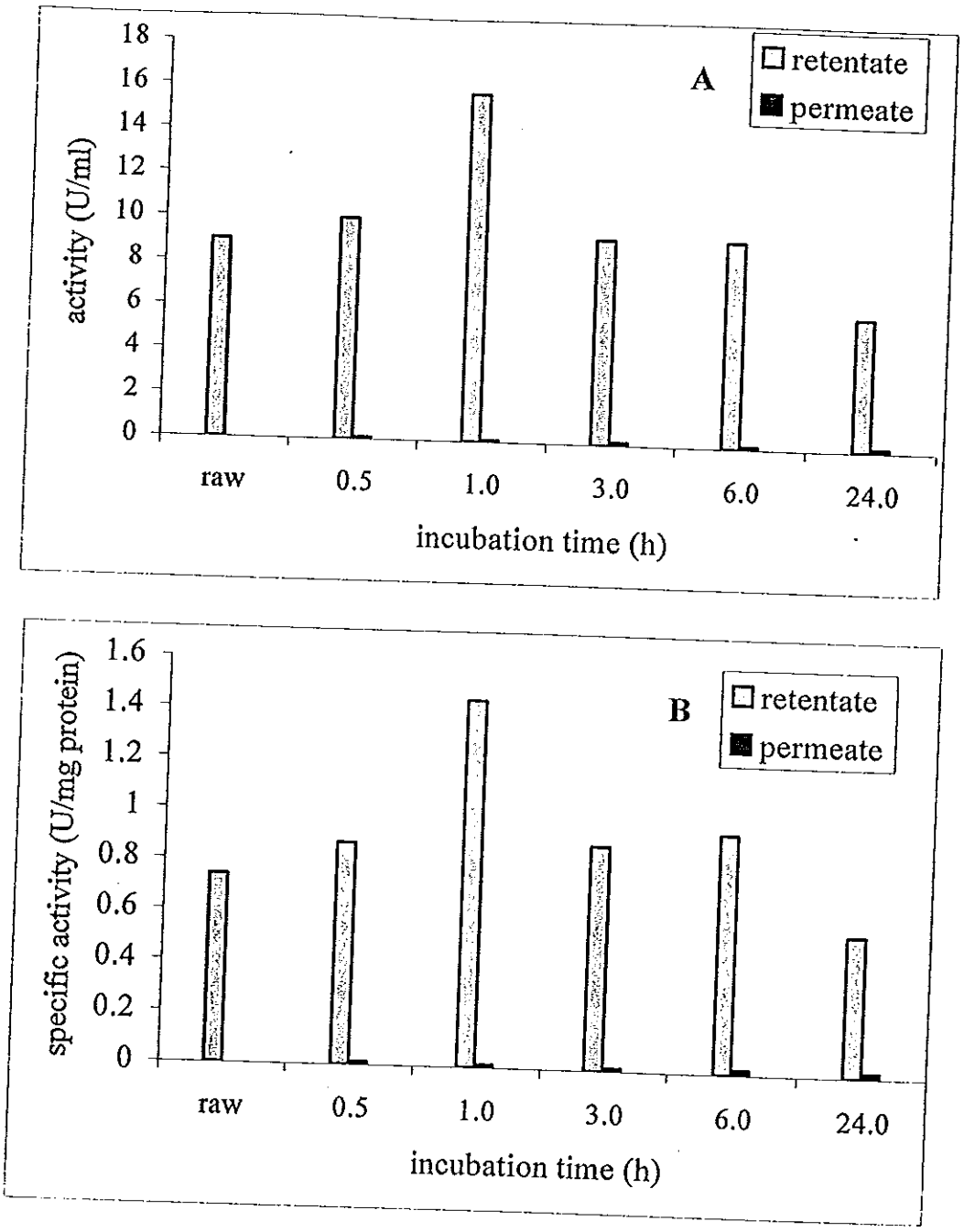


Figure 16 Effect of pre-incubation time on activity (A) and Specific activity (B) of chymotrypsin from yellowfin tuna spleen extract separated by batch concentration UF mode using polyethersulfone membrane with MWCO 30 kDa

5. Effect of cross flow rate and transmembrane pressure on enzyme separation by UF

5.1 Effect on proteases and transmission of protein

Regenerated cellulose membrane with MWCO 30 kDa and 100 kDa were used in this step with total recycle mode. Both retentate and permeate were recycled to the feed tank. The permeate flux and enzyme activities were measured.

The transmissions of protein and enzymes were studied. Table 8 and 9 indicated that TMP and cross-flow rate had no impactful effects on enzyme and protein transmission. It was probably due to that the low molecular weight solute was very easily transmitted through this membrane and, hence, the extent of concentration polarization of these solutes was expected to be negligible (Ghosh *et al.*, 2000). These two membranes had similar rejection ability to protein, but little enzymes could pass membrane with MWCO 30 kDa while about half amount of enzymes passed membrane with MWCO 100 kDa. When MWCO 30 kDa membrane was used, the transmissions of enzymes (protease, trypsin and chymotrypsin) were very low, but the transmission of protein was approximately 0.80. It indicated that enzymes did not pass this membrane but most other proteins did. Therefore, the separation of enzymes from other protein could be achieved by using this membrane. This conclusion could be confirmed by other researchers. Dewitt and Morrissey (2002b) found that 90% protease had been recovered by using pilot UF unit (Maximate^{TM} EXT, Pall Filtron, Northborough, MA) with MWCO 30 kDa membrane.

On the other hand, when MWCO 30 kDa membrane was used, the high TMP and high cross flow rate also caused the increase of enzyme

loss, but no effect on protein transmission. For instance, when TMP was 2.5 bar and cross flow rate was 240 L/h, the protease transmission was 0.03, but the protease transmission was increased to 0.15 if the TMP was increased to 3.5 bar and this transmission was increased to 0.07 when cross flow rate was increased to 360 L/h. No distinct change was found during using MWCO 100 kDa membrane. For example, when TMP was 2.5 bar and cross flow rate was 240 L/h, the protease transmission was 0.39, the protease transmission was 0.30 if the TMP was increased to 3.5 bar and this transmission was 0.39 when cross flow rate was increased to 360 L/h.

It was also found that protein transmission by using MWCO 100 kDa membrane was lower than using MWCO 30 kDa. The reason was probable that a greater amount of protein fouled the membrane pore for larger pore size membranes (Youravong, 2001).

Transmission change during using MWCO 30 kDa membrane with higher TMP or cross flow rate was due to the fact that the higher cross flow rate decreased the effect of concentration polarization and higher TMP enhanced the passage of solutes and flux. The reason was probably that the molecular weight of these enzymes were similar to the MWCO of this membrane, so the transmissions of these enzymes were enhanced. It was reported that protease loss may result from using a membrane with a molecular weight cut off similar to that of the desired component (Benjakul *et al.,* 1997). On the other hand, general proteases may include some proteases with smaller molecular weight, so more activity was detected in permeate if the passage was enhanced. For UF using the membrane with MWCO 100 kDa, since the larger membrane pore size

Table 8 Transmissions of protein, protease, trypsin and chymotrypsin during UF using regenerated cellulose membrane with MWCO 30 kDa at 30°C

Cross flow rate (L/h)	TMP (bar)	Transmission			
		Protein	Protease	Trypsin	Chymotrypsin
120	0.5	0.81	0.01	0.01	0.01
	1.5	0.80	0.01	0.02	0.01
	2.5	0.86	0.03	0.01	0.01
	3.5	0.82	0.01	0.01	0.01
	4.5	0.82	0.02	0.03	0.01
240	0.5	0.82	0.02	0.02	0.01
	1.5	0.83	0.02	0.01	0.01
	2.5	0.81	0.03	0.01	0.01
	3.5	0.81	0.15	0.06	0.02
	4.5	0.82	0.17	0.05	0.01
360	0.5	0.83	0.01	0.01	0.01
	1.5	0.85	0.02	0.02	0.01
	2.5	0.77	0.07	0.01	0.01
	3.5	0.85	0.18	0.04	0.02
	4.5	0.85	0.14	0.06	0.02

Table 9 Transmissions of protein,protease, trypsin and chymotrypsin during UF using regenerated cellulose membrane with MWCO 100 kDa at 30°C

Cross flow rate (L/h)	TMP (bar)	Transmission			
		Protein	Protease	Trypsin	Chymotrypsin
120	1.0	0.77	0.41	0.71	0.62
	1.5	0.77	0.43	0.71	0.61
	2.5	0.76	0.33	0.65	0.53
	3.5	0.76	0.36	0.66	0.53
	4.5	0.74	0.31	0.65	0.51
240	1.0	0.75	0.49	0.74	0.65
	1.5	0.73	0.48	0.76	0.66
	2.5	0.80	0.39	0.69	0.55
	3.5	0.80	0.30	0.64	0.56
	4.5	0.79	0.31	0.65	0.55
360	1.0	0.88	0.47	0.74	0.66
	1.5	0.91	0.47	0.75	0.64
	2.5	0.84	0.39	0.69	0.67
	3.5	0.82	0.40	0.70	0.67
	4.5	0.82	0.38	0.68	0.66

caused more serious blocking in the membrane, so no impactful transmission changes of these enzyme was detected.

5.2 Permeate flux profile

It was found similar curves of permeate flux vs. time with different cross flow rate and TMP were obtained by using MWCO 30 kDa membrane (Figure 17A, 18A and 19A). and MWCO 100 kDa membrane (Figure 17B, 18B and 19B.). In the initial several minutes, the fluxes decreased sharply, it was due to concentration polarization. Then, the fluxes decreased slightly, it was due to protein adsorption and particle deposition on the surface or interior wall of membrane. After about 20-30 minutes, the flux kept almost constant for a long period. This stage was due to particle deposition, consolidation of fouling materials on the interior space of membrane or formation of cake layer (Marshall *et al.*, 1993).

Steady state flux vs. TMP in Figure 20 indicated that higher cross flow rate resulted in higher permeate flux. The increase in cross flow rate caused reduction in concentration polarization and this led to an increase in the permeate flux (Ghosh et al., 2002). From Figure 20, no limiting flux was found during increasing cross flow rate. The experimental trends seemed to indicate that the permeate flux could be increased further by increasing the cross flow rate. So, cross flow rate should be kept as higher as possible (depend on the work range of pump) during the UF.

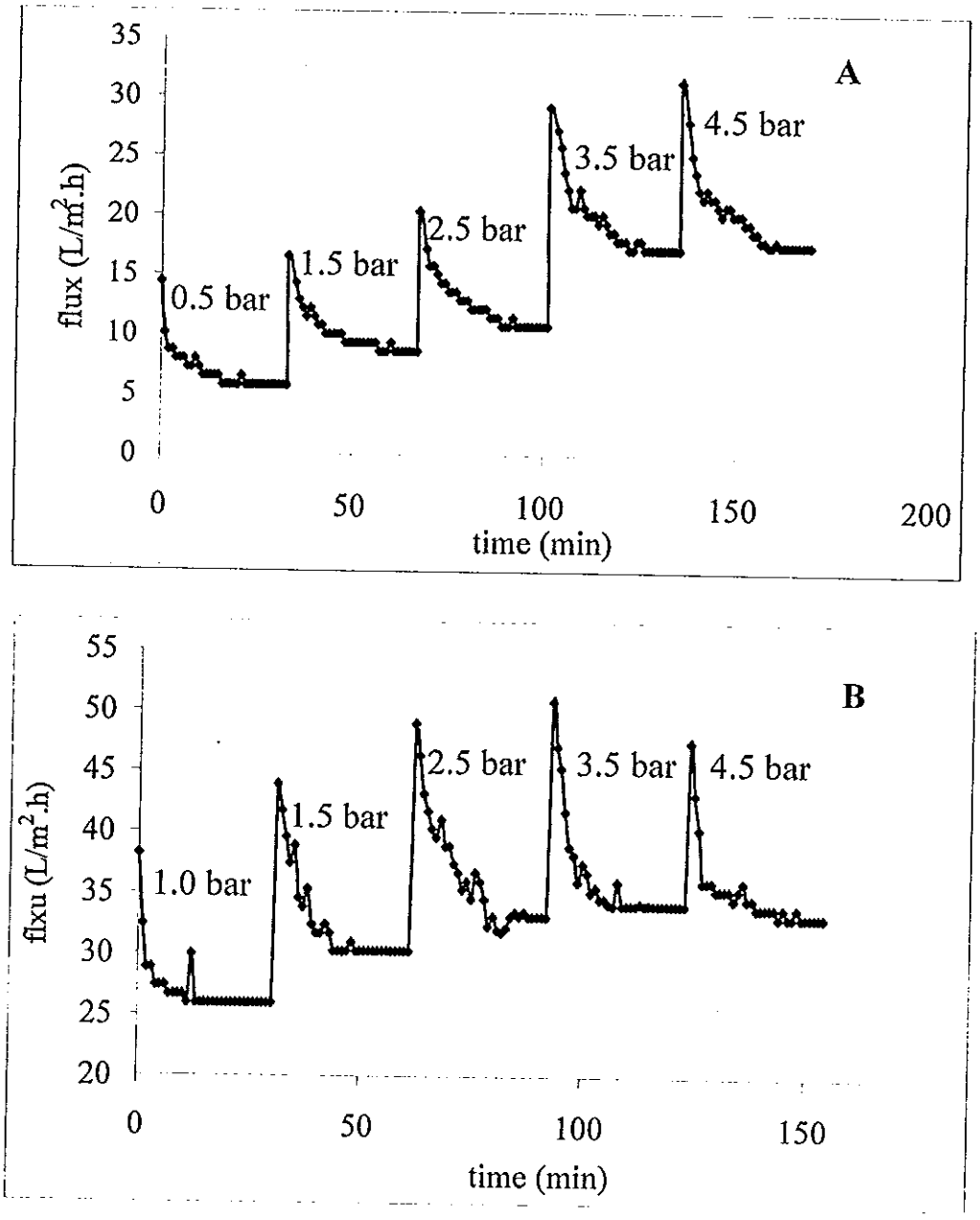


Figure 17 Flux vs. time during UF using MWCO 30 kDa (A) and 100 kDa (B) regenerated cellulose membranes with cross flow rate 120 L/h at room temperature (30°C)

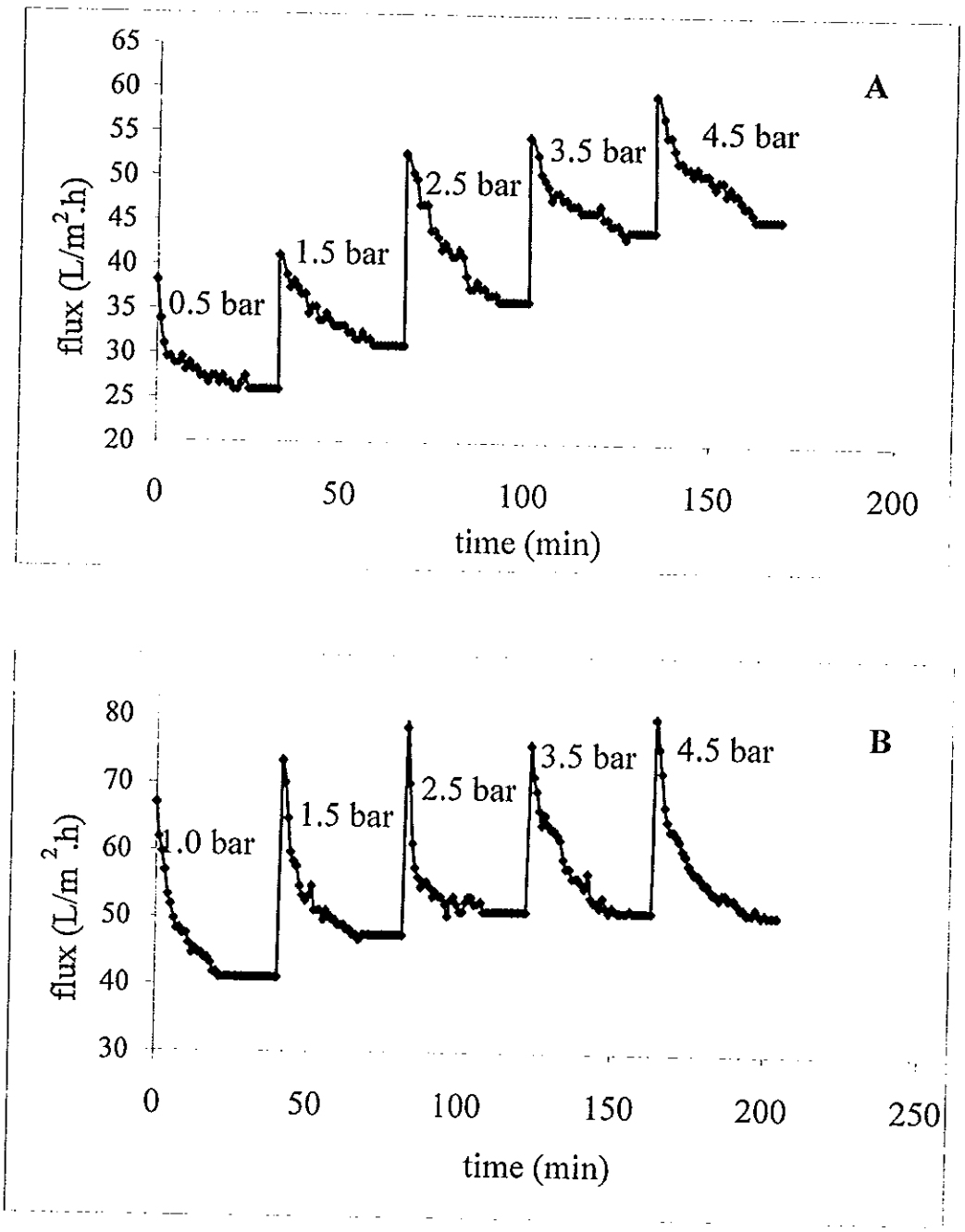


Figure 18 Flux vs. time during UF using MWCO 30 kDa (A) and 100 kDa (B) regenerated cellulose membranes with cross flow rate 240 L/h at room temperature (30°C)

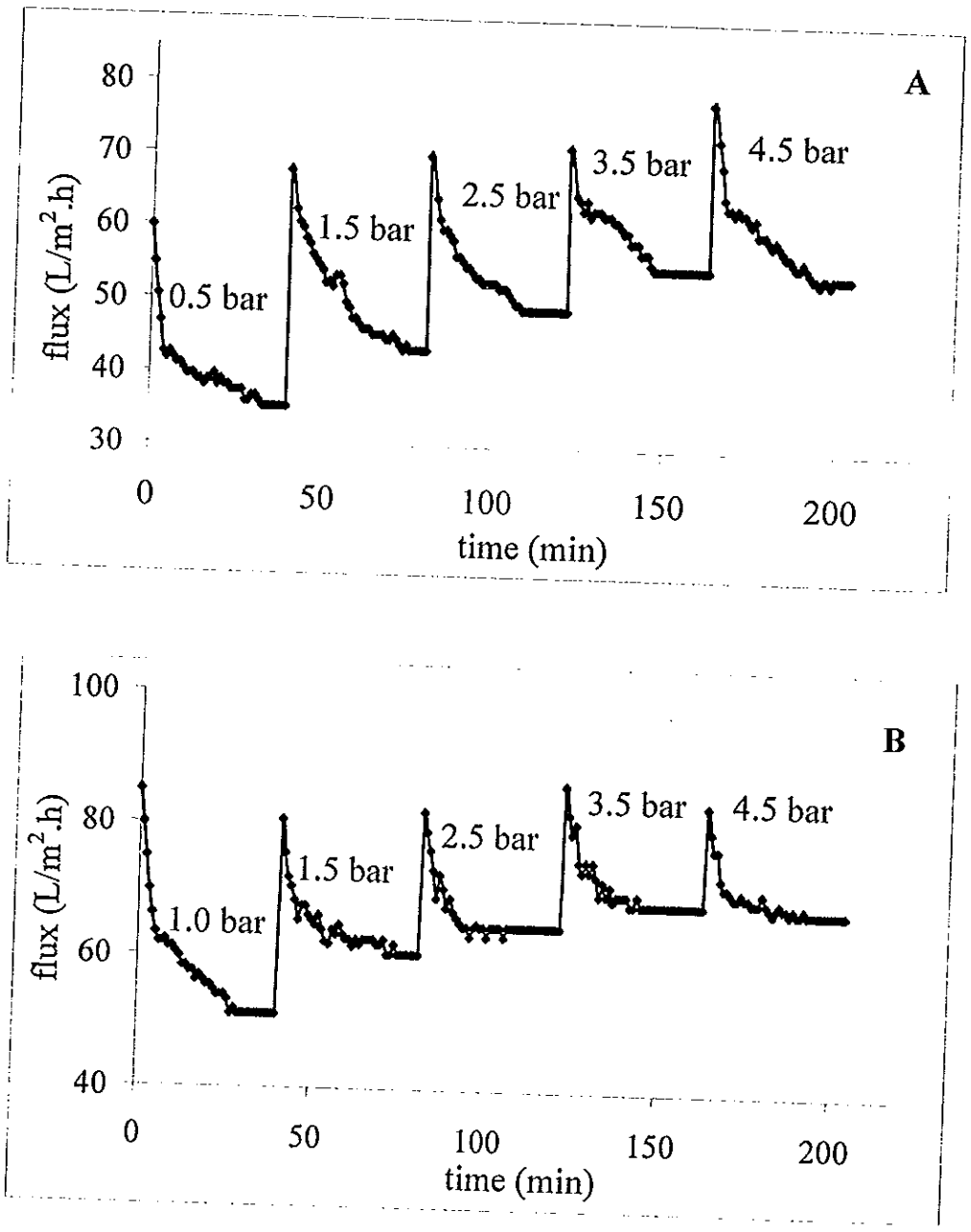


Figure 19 Flux vs. time during UF using with MWCO 30 kDa (A) and 100 kDa (B) regenerated cellulose membranes with cross flow rate 360 L/h at room temperature (30°C)

Figure 20 also indicated that increasing TMP enhanced the steady state permeate flux. For MWCO 30 kDa membrane, the highest permeate flux (54.72 L/m^2.h) was found at TMP 3.5 bar and cross flow rate 360 L/h (Table-Appendix C5). Higher TMP (i.e. 4.5 bar) didn't increase the permeate flux. It meaned that limiting flux occurred at TMP 3.5 bar. It is because that TMP can enhance the permeate flux, but fouling and concentration were also more severe at high TMP (Ghosh *et al.*, 2002). The similar phenomena were found during using MWCO 100 kDa membrane. The highest permeate was 68.4 L/m^2.h with cross flow rate 360 L/h and TMP 2.5 bar (Table-Appendix C5). It is probable that the fouling in the pore resulted in the steady state flux at lower TMP using MWCO 100 KDa membrane comparing to the MWCO 30 KDa membrane.

On the other hand, it was found that increasing cross flow rate had more effect on enhancing flux than increasing TMP. For example, during using MWCO 30 kDa membrane, the steady state flux was increased from 5.76 to 17.64 L/m^2.h if TMP was increased from 0.5 to 4.5 bar and cross flow rate was kept at 120 L/h. but the steady state flux was increased from 5.76 to 25.92 L/m^2.h if cross flow rate was increased from 120 to 240 L/h and TMP was kept at 0.5 bar. The similar phenomena were found during using MWCO 100 kDa membrane. The reason was probable that the concentration polarization was the major reason for flux decline in this work.

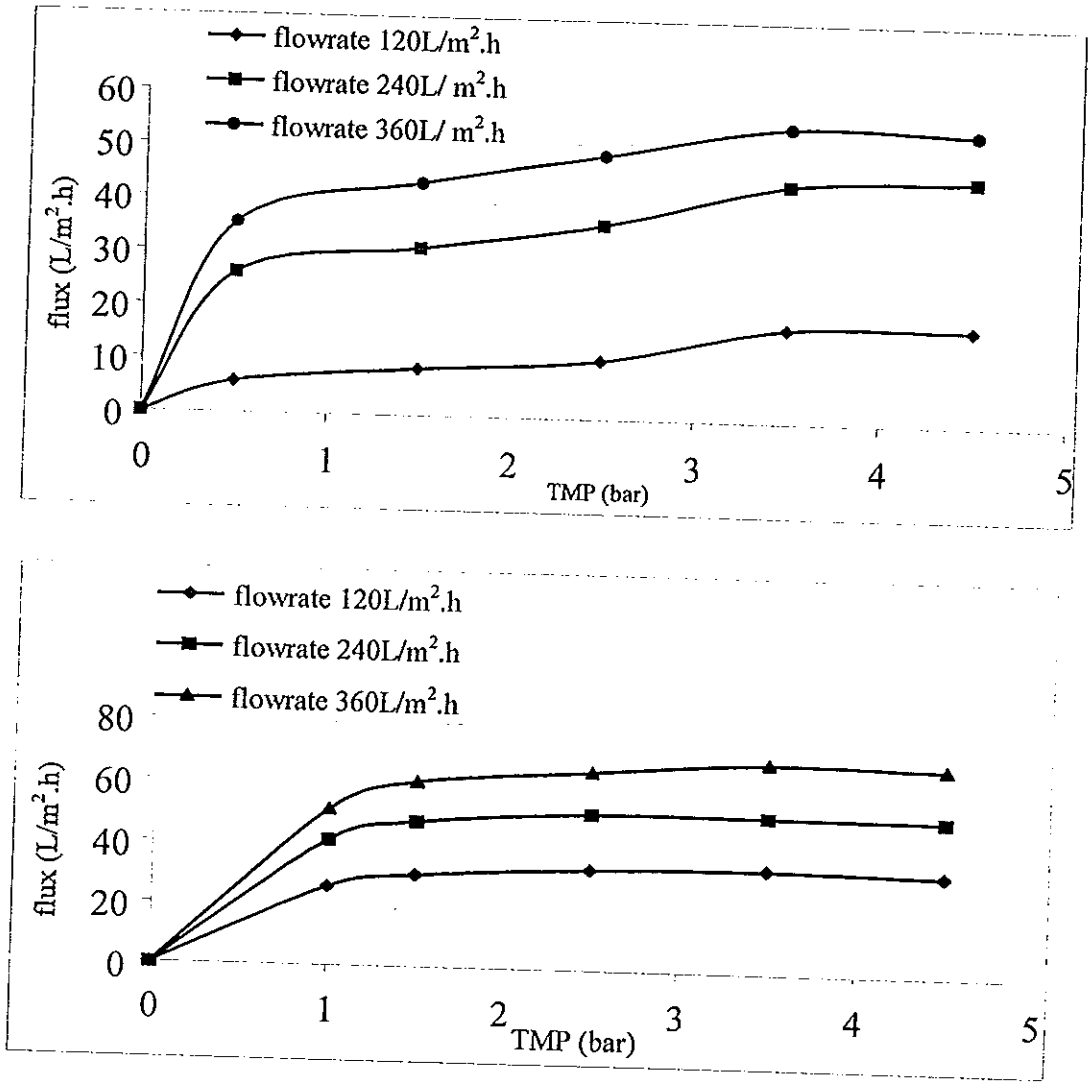


Figure 20 Flux vs. TMP with different cross flow rate using regenerated cellulose membrane with MWCO 30 kDa (A) and 100 kDa (B)

6. Continuous diafiltration

The extract from both spleen and mixed viscera were used as feed. In order to maximize permeate and minimize enzyme loss, regenerated cellulose membrane (Pellicon 2) with MWCO 30 kDa was used at TMP 1.5 bar and cross flow rate 360 L/h. For continuous diafiltration, the retentate was sent back to the feed tank, but the permeate was not recycled. The volume of liquid in the feed tank was kept constant by replacing the volume lost in the permeate with buffer continuously (Ghosh *et al.*, 2002). The results indicated that continuous diafiltration was a method with high efficiency to increase enzyme purity.

6.1 Tuna spleen extract

For extract from tuna spleen, the soluble protein concentration was decreased from 11.34 mg/ml to 0.61 mg/ml after DF for 60 minutes while the activities of enzymes was kept almost constant (Figure 22, 23). The steady state flux (56.45 $L/m^2.h$) was achieved after DF 100 minutes (Figure 21 and Table-Appendix C6). The steady state flux of DF was higher than that in total recycle mode (43.23 $L/m^2.h$). It was because that adding buffer diluted the feed. Since the dilution helped to remove the fouling problem during DF, but precipitation of protein on membranes was more evident during the total recycle mode, so the permeate flux was higher during continuous diafiltration than that during total recycle mode (Dewitt and Morrissey, 2002b).

Figure 21 shows the permeate flux at different time intervals. The flux decreased from about 70 $L/m^2.h$ to 56 $L/m^2.h$. This was probably due to fouling and not due to concentration polarization, since the feed concentration decreased with time due to loss of solute along with the permeate (Higuchi *et al.*, 1991).

The enzyme purity was increased dramatically (Figure 24). The purification factors were 18.01, 18.42 and 16.37 for general protease, trypsin and chymotrypsin, respectively (Table-Appendix C7). 60 minutes was found as the minimum DF time for improvement of enzyme purity. Diafiltration for longer than 60 minutes had no distinct effect on improvement of enzyme purity. It was probably due to the gel layer formed on the membrane surface and this layer prevented the passage of protein (Youravong, 2001).

The DF wash volume at 60 minutes (Table-Appendix C6) was 30.86 L and number of DF was 6.17. In a real operation, the DF wash volume and number of DF may help decide when to terminate the diafiltration operation. This will primarily depend on the solute concentration recovered (Ghosh, *et al.*, 2002).

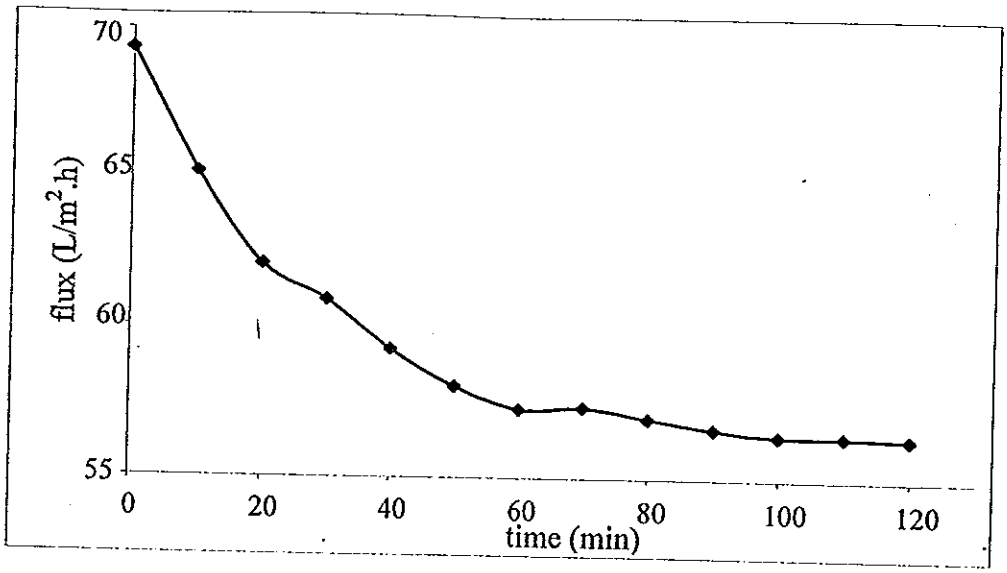


Figure 21 Flux vs. time during continuous DF for extract of tuna spleen by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

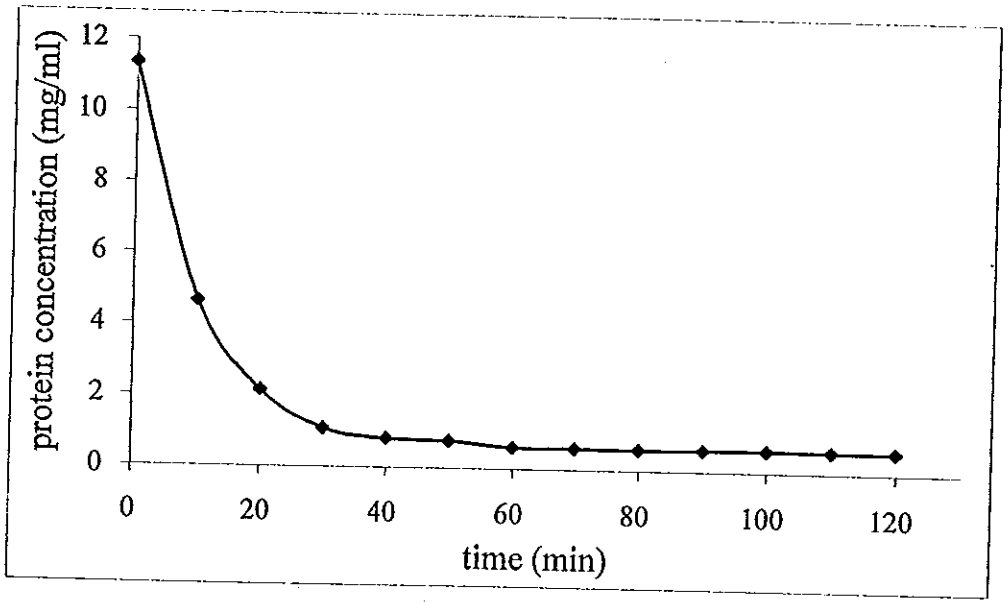


Figure 22 Soluble protein concentration vs. time during continuous DF for extract of tuna spleen by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

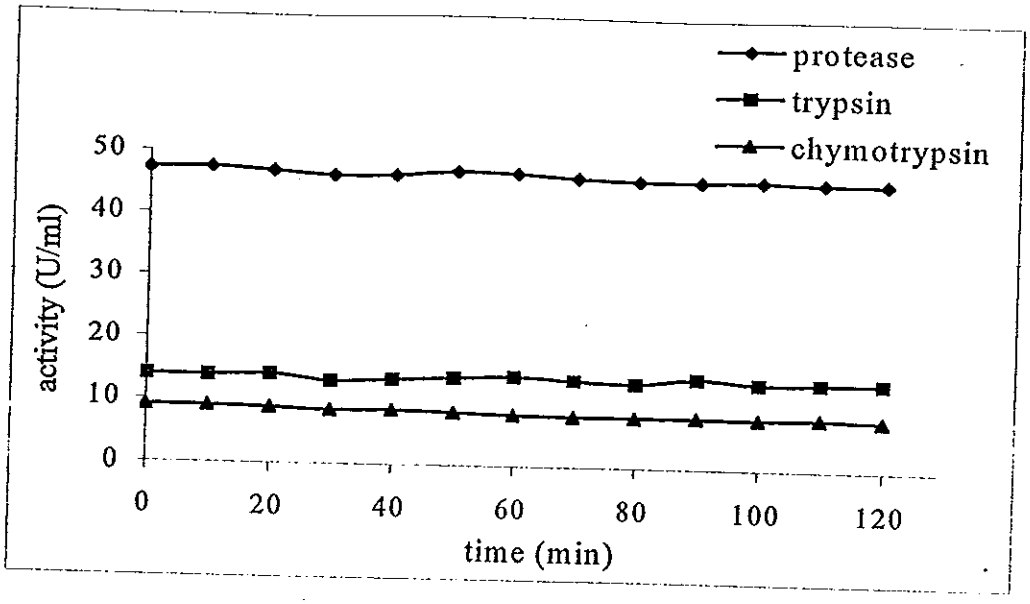


Figure 23 Enzyme activity vs. time during continuous DF for extract of spleen by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

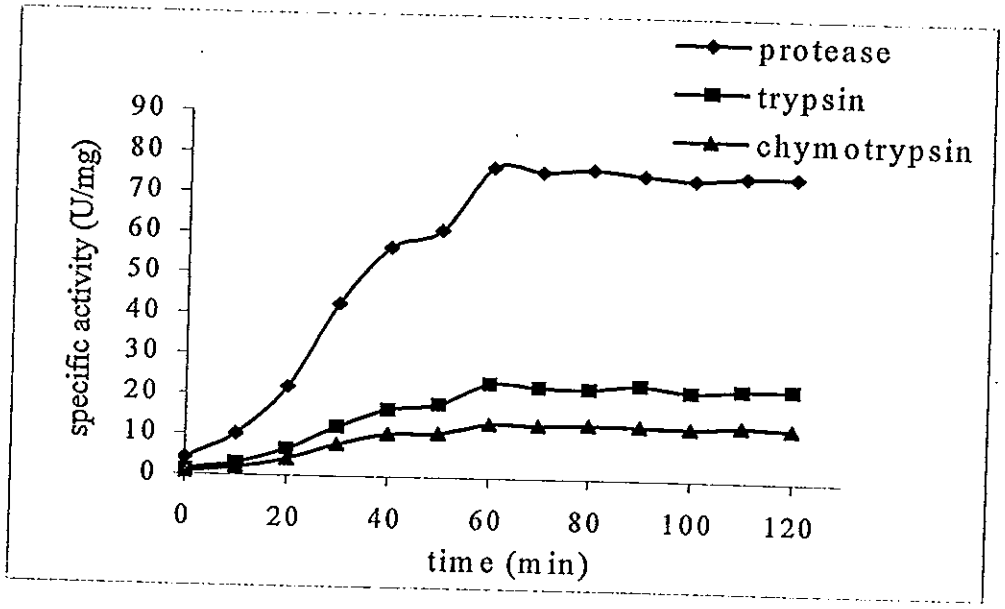


Figure 24 Enzyme specific activity vs. time during continuous DF for extract of spleen by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

6.2 Extract of mixed viscera

For extract from mixed viscera, the same operation conditions (TMP 1.5 bar and cross flow rate 360 L/h) were used. The soluble protein concentration was decreased from 16.17 mg/ml to about 3.02 mg/ml after DF for 90 minutes while the activities of enzymes was kept almost constant (Figure 25, 26). The steady state flux (43.71 $L/m^2.h$) was achieved after DF 80 minutes (Figure 27).

The enzyme purity was increased too (Figure 28). The purification factors were 5.02, 5.09 and 5.48 for general protease, trypsin and chymotrypsin, respectively (Table-Appendix C9). 90 minutes was found as the minimum DF time for improvement of enzyme purity. Increasing operation time also had no distinct effect on improvement of enzyme purity. The purification factors were less than those in the DF for extract of spleen. It was probably due to a lot of impurity presented in the extract of mixed viscera. The DF wash volume at 90 minutes was 28.21 L and number of DF was 5.64.

From the results, it was found that diafiltration wash volume for spleen extract was higher that for mixed viscera extract. It is probably because the spleen extract contained less impurity than mixed viscera extract. Thus, less membrane fouling resulted in higher permeate. So the diafiltration wash volume for spleen extract was higher.

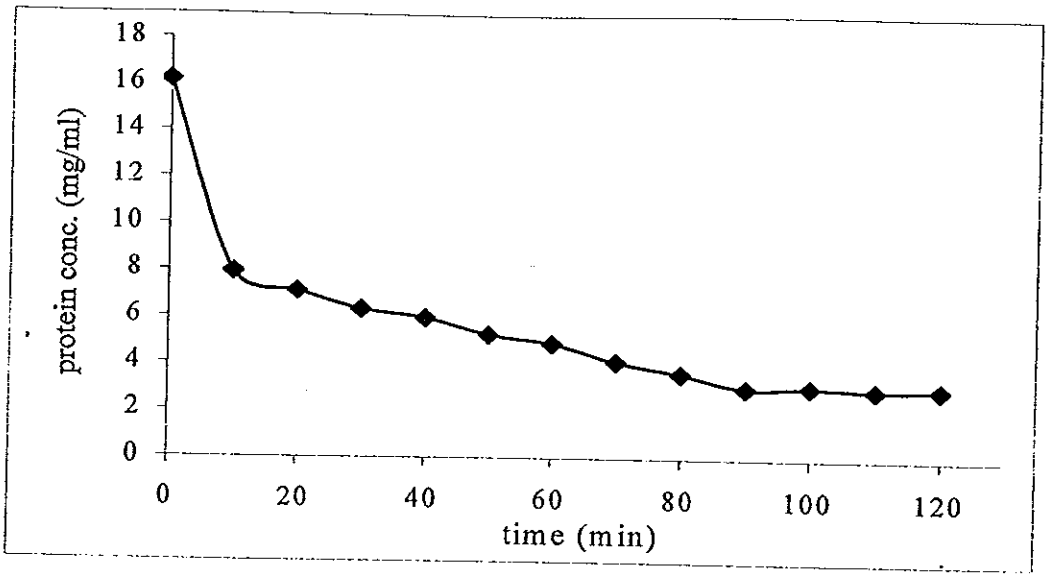


Figure 25 Soluble protein concentration vs. time during continuous DF for extract of mixed viscera by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

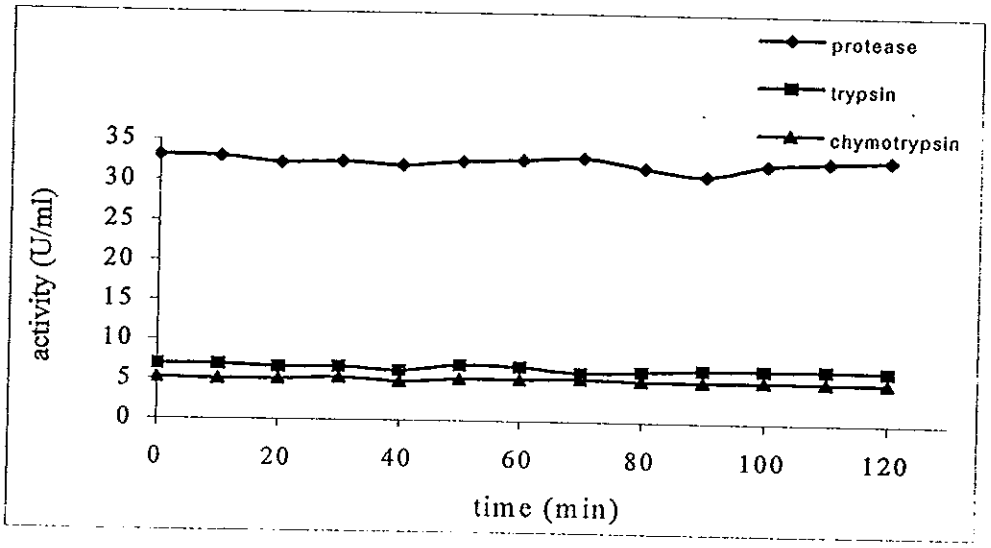


Figure 26 Enzyme activity vs. time during continuous DF for extract of mixed viscera by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

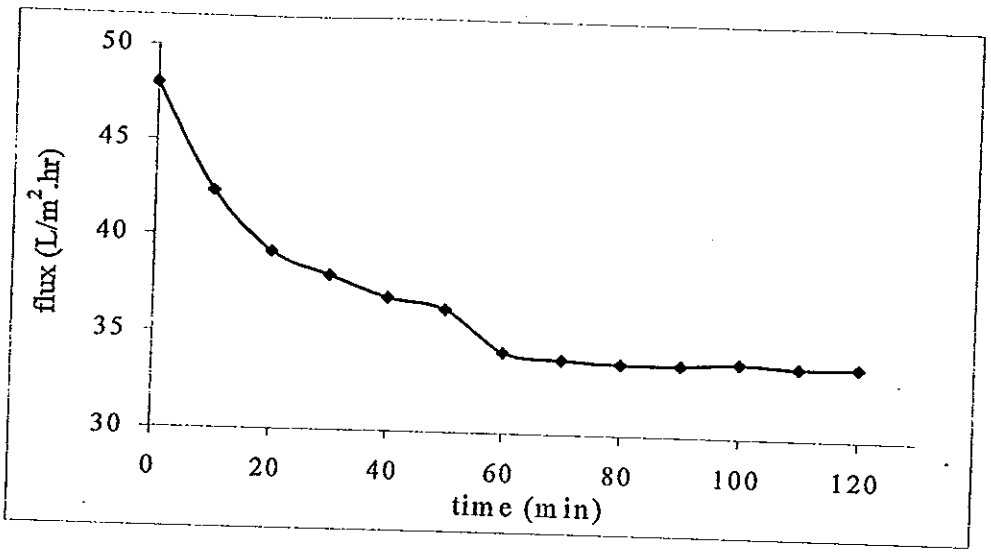


Figure 27 Flux vs. time during continuous DF for extract of mixed viscera by regenerated membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

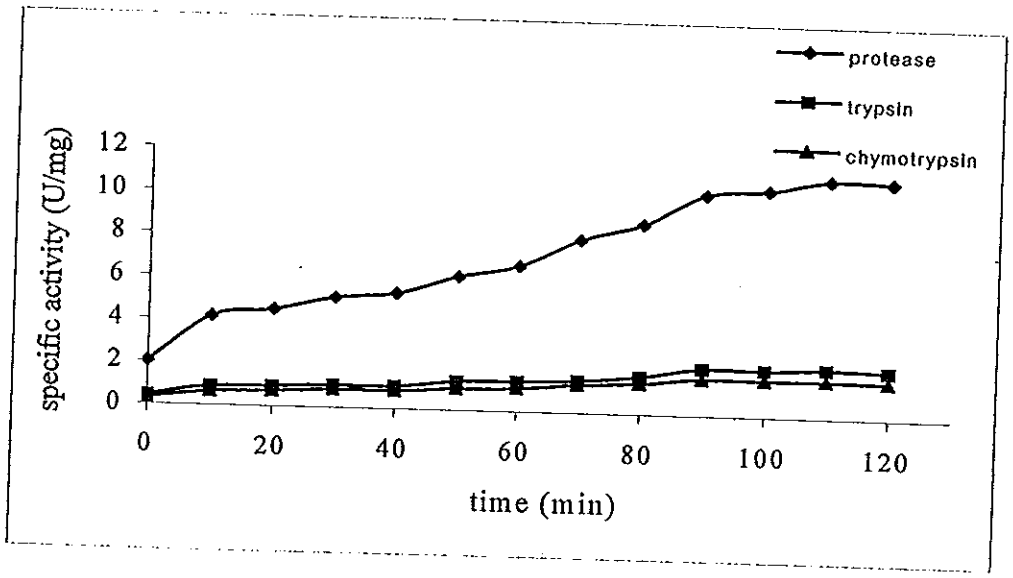


Figure 28 Enzyme specific activity vs. time during continuous DF for extract of mixed viscera by regenerated cellulose membrane with MWCO 30 kDa using cross flow rate 360 L/h and TMP 1.5 bar at 30°C

7. Concentration of product

The purified enzyme solution from continuous DF was concentrated 5 times (from 50 ml to 10 ml) by dead-end system using polyethersulfone membrane with MWCO 10 kDa. As that expected, the enzyme activity and soluble protein concentration were increased after concentration while specific activity was kept almost constant. For extract of spleen, the soluble protein concentration was changed from 0.68 mg/ml to 3.27 mg/ml. The general protease activity was increased from 39.48 U/ml to 194.2 U/ml. The trypsin activity was increased from 13.45 U/ml to 67.13 U/ml, and chymotrypsin activity was increased from 7.62 U/ml to 38.24 U/ml (Table 10).

For extract of mixed viscera, the soluble protein concentration was changed from 3.09 mg/ml to 15.38 mg/ml. The general protease activity was increased from 32.89 U/ml to 162.97 U/ml, trypsin activity was increased from 6.48 U/ml to 31.48 U/ml, and chymotrypsin activity was increased from 4.96 U/ml to 23.85 U/ml (Table 10).

For all of these enzymes, the specific activity did not change much.

The enzyme activity was changed a little in the feed before concentration comparing to that after diafiltration. It probably was due to enzyme denaturation during storage.

The results indicated that the purified solution could be concentrated easily by membrane filtration. On the other hand, the purified solution from diafiltration also can be concentrated by system as same as that for diafiltration. It can be achieved by changing continuous diafiltration system to batch concentration mode.

Table 10 Soluble protein concentration, enzyme activity and specific activity of enzyme solution before and after concentration using purified extracts from DF

		Before concentration	After concentration
Spleen extract			
	Volume (ml)	50	10
	Soluble protein (mg/ml)	0.68	3.27
Protease	Act.[1] (U/ml)	39.48	194.20
	Sp.Act[2] (U/mg)	58.06	59.39
Trypsin	Act. (U/ml)	13.45	67.13
	Sp.Act.(U/mg)	19.78	20.53
Chymotrypsin	Act. (U/ml)	7.62	38.24
	Sp.Act.(U/mg)	11.21	11.69
Mixed viscera extract			
	Volume (ml)	50	10
	Soluble protein (mg/ml)	3.09	15.38
Protease	Act. (U/ml)	32.89	162.97
	Sp.Act.(U/mg)	10.64	10.60
Trypsin	Act. (U/ml)	6.48	31.48
	Sp.Act.(U/mg)	2.10	2.05
Chymotrypsin.	Act. (U/ml)	4.96	23.85
	Sp.Act.(U/mg)	1.61	1.55

[1] activity

[2] specific activity

8. Gel electrophoresis

From diafiltration, the enzyme mixture was obtained. Thus, more evidence was needed to prove the change of protein in the extract and the presence of proteases. Electrophoresis method is the effective tool in protein isolation, purification and characterization. On the other hand, a new development in electrophoresis, i.e. substrate electrophoresis or activity staining, has been utilized in the assessment of the composition, molecular mass and classes of proteases present in crude extracts from fish digestive tracts (Carcia-Carreno *et al.*, 1993). Both SDS-PAGE and activity staining were used to detect the protein composition and presence of proteases in the extract during process in this study.

Figure 29 showed the zymogram of proteins in the crude extract before and after incubation and purified extract after UF. For both extract of spleen and extract of mixed viscera, some large molecular weight proteins were removed after incubation. As mentioned above, it was due to that the large molecular weight protein was hydrolyzed by proteases in the extract. Comparing the extract before and after UF, it was clear that most proteins were removed. On the bottom of gel, some small molecular weight proteins also presented. It was probably due to protein autolysis during storage.

Figure 30 showed the protein in the extract of spleen during the DF. It showed that most protein were removed in the initial 40 minutes. The results confirmed the results mentioned before (Figure 22).

Figure 31 showed the protein in the extract of mixed viscera during the DF. It was similar to the DF of extract of spleen. Most proteins were removed in the initial 60 minutes. The results also confirmed the results in the study about DF of extract of mixed viscera (Figure 25)

It was found that molecular weight of major protein (position A) in the solution after DF was about 23.70 kDa. It approximately in the range of the molecular weight of trypsin (23 kDa) and chymotrypsin (25 kDa) (Jantaro, 2000).

Figure 32 showed the activity of protease in crude extract, extract after DF and concentrated enzyme extract by activity staining. The results showed that all of these extract contained protease activity. The molecular weight of protease at position A was about 25.43 kDa. it confirmed that trypsin and chymotrypsin showed the activity at this position.

On the other hand, it is difficult to separate trypsin and chymotrypsin completely since the molecular weights of these are very close.

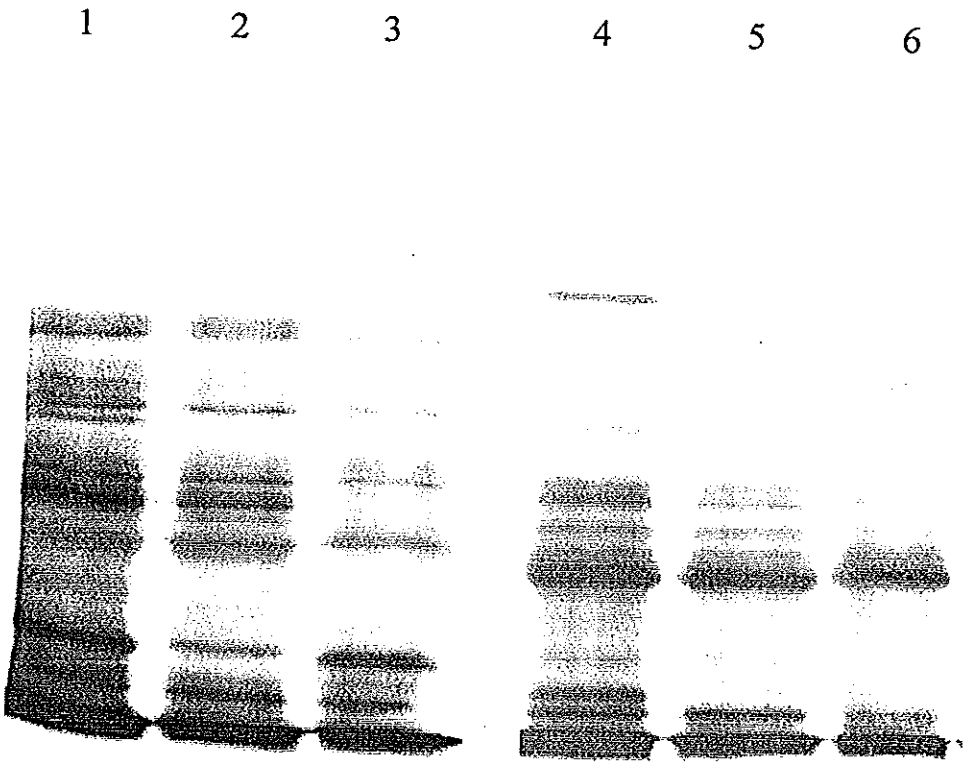


Figure 29 The extract of mixed viscera and spleen of yellowfin tuna before incubation, after incubation and after DF using 12% gel Lane 1, protein in the extract of mixed viscera before incubation at 50°C; 2, protein in the extract of mixed viscera after incubation at 50°C for 1 hour; 3, protein in the extract of mixed viscera after DF; 4, protein in extract of spleen before incubation at 50°C; 5, protein in the extract of spleen after incubation at 50°C for 1 hour; 6, protein in the extract of spleen after DF

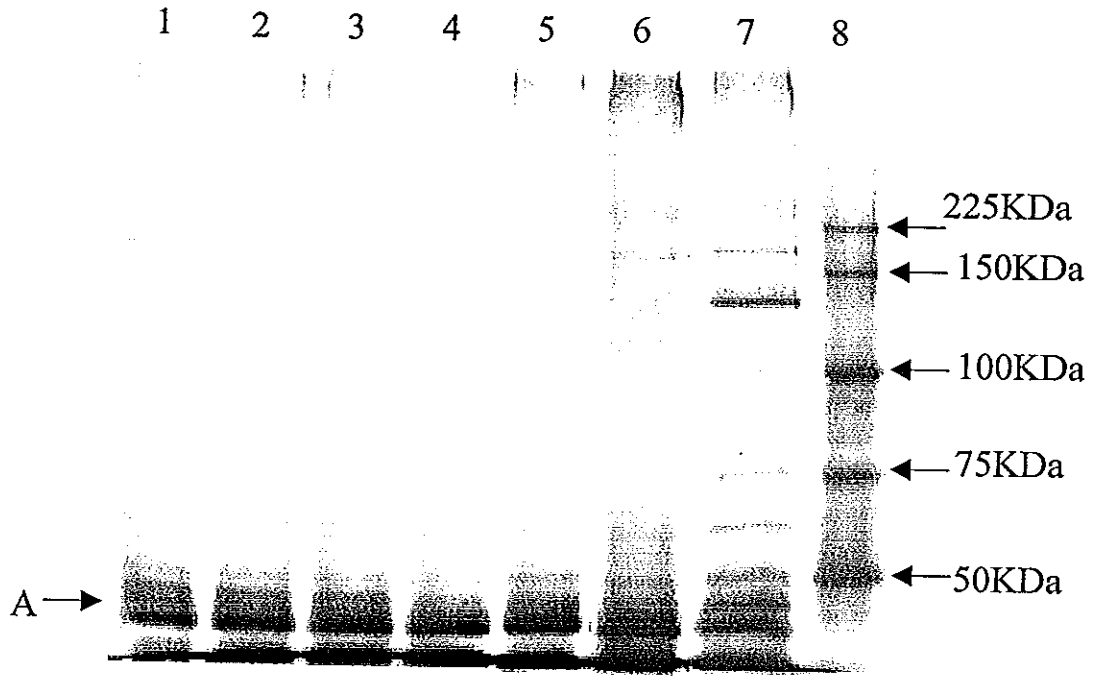


Figure 30 The extract of spleen during DF using 10% gel. Lane 1, extract after DF for 120 minutes; 2, extract after DF for 100 minutes; 3, extract after DF for 80 minutes; 4, extract after DF for 60 minutes; 5, extract after DF for 40 minutes; 6, extract after DF for 20 minutes; 7, extract before DF; 8, protein standard marker; A, the position containing protein with molecular weight around 23.70 kDa

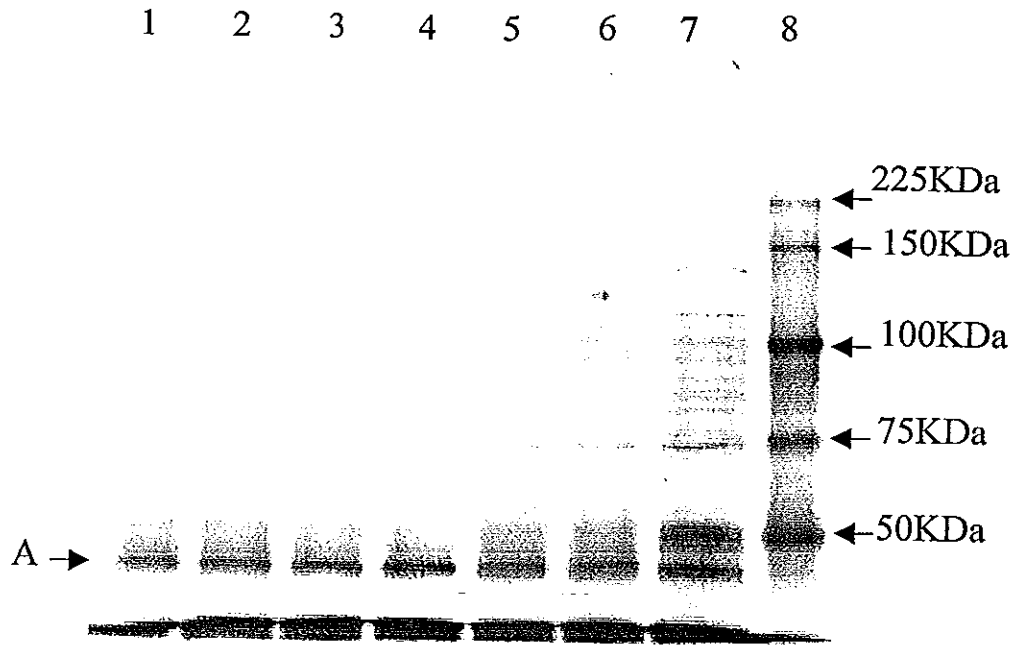


Figure 31 protein composition in the extract of mixed viscera during DF using 10% gel. Lane 1, extract after DF for 120 minutes; 2, extract after DF for 100 minutes; 3, extract after DF for 80 minutes; 4, extract after DF for 60 minutes; 5, extract after DF for 40 minutes 6, extract after DF for 20 minutes; 7, extract before DF; 8, protein standard marker; A, the position containing protein with molecular weight around 23.70 kDa

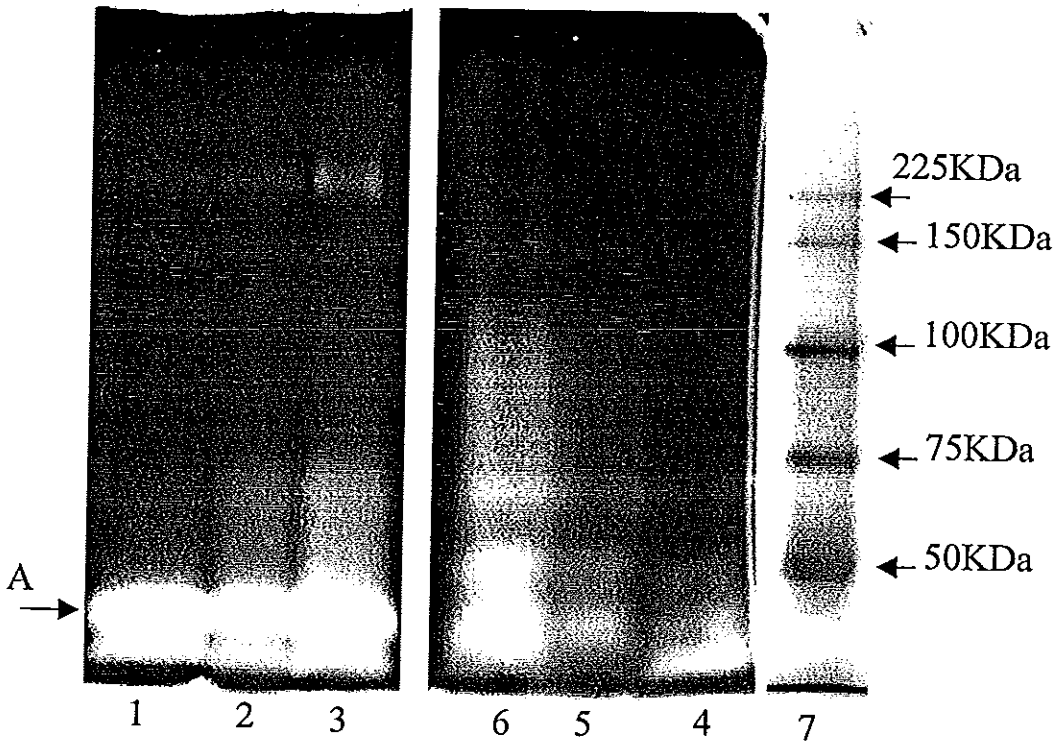


Figure 32 Activity staining of SDS-PAGE gel using 10% gel. Lane 1, protease in the extract of mixed viscera before DF; 2, protease in the extract of mixed viscera after DF; 3, protease in the extract of mixed viscera after concentration; 4, protease in the extract of spleen before DF; 5, protease in the extract of spleen after DF; 6, protease in the extract of spleen after concentration; 7, protein standard marker; A, position containing proteases with molecular weight around 25.43 kDa

Chapter 4

CONCLUSION

This work investigated the major factors affecting on enzyme recovery by UF, i.e. membrane molecular weight cut off, operation temperature, transmembrane pressure (TMP) and cross flow rate, as well as the methods which may improve the enzyme recovery, i.e. pre-incubation time and continuous diafiltration.

At low TMP and cross flow rate, the membranes with MWCO 30 kDa and 100 kDa had similar rejection for protein. Most protein passed these two membranes. But the transmissions of general protease, trypsin and chymotrypsin using the membrane with MWCO 100 kDa were much higher than that using membrane with MWCO 30 kDa. Recovery and higher yield of enzymes could be achieved by using membrane with MWCO 30 kDa.

No distinct difference was found during UF at room temperature (30°C) and 4°C. The room temperature (30°C) was selected for this research because of easiness of operation.

Pre-incubation induced the protein hydrolysis. As a result, the large molecular protein was hydrolyzed to small molecular protein and the permeate flux was enhanced. Long-time incubation hydrolyzed more protein but it also caused denaturation of enzymes. For this work, 1 hour was found as the optimal incubation time that provide the highest enzyme activity and purity.

Both TMP and cross flow rate had no distinct effect on protein transmission. Transmission of protein was about 0.80 for membrane with

MWCO 30 kDa and 100 kDa. At different TMP and cross flow rate, the membrane with MWCO 30 kDa had much better separation ability for enzymes than the membrane with MWCO 100 kDa. TMP 1.5 bar and cross flow rate 360 L/h was the best operation condition for both enhancing flux and minimizing enzyme loss in this research.

Continuous DF was a strong method to improve enzyme purity. For extract of spleen, the purification factors were 18.01, 18.42 and 16.37 for general protease, trypsin and chymotrypsin respectively by continuous DF for 60 minutes. And for extract of mixed viscera, the purification factors were 5.02, 5.09 and 5.48 for general protease, trypsin and chymotrypsin respectively by continuous DF for 90 minutes.

The concentration was achieved by membrane filtration with dead end system. The enzyme activity was increased about 5 times while the feed volume was decreased to 1/5. The specific activity of enzyme was almost kept constantly.

Gel electrophoresis provided evidences for enzyme purification and presence of proteases. The gel showed that the large molecular protein had been removed after pre-incubation and most protein had been removed after DF. Both silver staining and activity staining provided the evidences that the protein with molecular weight around 23.70 kDa or 25.43 kDa presented in the extract after purification and this protein showed protease activity. The molecular weight of this protein was very close to the molecular weight of trypsin and chymotrypsin. It was confirmed that trypsin and chymotrypsin had been kept in the extract after purification while most other proteins had been removed .

From the results, it was concluded that ultrafiltration was an efficient method to separate proteases from extract of yellowfin tuna

viscera. This method can be used for a large volume feed, e.g. several liter, comparing to other method, such as chromatography. The operation was very simple. A high purification factor was achieved by only one step, i.e. continuous diafiltration. Ultrafiltration has strong potential for application in tuna canning industry to recover valuable bioactive compound from waste.

SUGGESTIONS

1. Some pretreatment, e.g. microfiltration, should be used to remove impurity in the extract before UF. It may improve UF performance and product color.
2. Crystallization can be used to get pure enzyme crystal after purification and concentration.
3. Freeze drying can be used to produce powder from the purified and concentrated enzyme extract. And it is valuable to study the applications of these purified and concentrated enzyme using both liquid extract or powder after freeze drying.
4. Except proteases recovered, the protein and protein hydrolysates in permeate may be used for various purposes including food, animal feed and microbial growth substrates.

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APPENDICES

Appendix A

Buffer preparation

Tris-HCl buffer was prepared by the method of Prachumratana (1998)

×10 Stock solutions

A: 0.2 M solution of tris (hydroxymethyl) aminomethane (24.23 g in 1000 ml)

B: 0.2 M HCl

50 ml of A + *X* ml of B

X	pH
40.3	7.5
38.5	7.6
36.6	7.7
34.5	7.8
32.0	7.9
29.2	8.0
26.2	8.1
22.9	8.2
19.9	8.3
17.2	8.4
14.7	8.5

Appendix B

Analytical methods

1. **Quantitatin of soluble protein was conducted by the method of Lowry *et al.*, (1951)**

Reagents

1. 2% Na_2CO_3 in 0.1 N NaOH solution
2. 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate solution
3. Alkaline copper solution was prepared by mixing 50 ml of solution1 and 1 mL of solution 2 immediately before using.
4. Folin-ciocateus reagent was diluted with distilled water in the ration of 1:1 rapidly before using.

Procedures

1. A 0.5 mL of appropriated dilution of sample was placed into the tube.
2. A 0.3 mL of alkaline copper was added and incubated at room temperature (30°C) for 10 minutes
3. Added 0.3 mL of Folin-ciocatues reagent, vortexed immediately and incubated at room temperature (30°C) for 30 minutes.
4. Measured the absorbance at 750 nm

standard curve of protein

1. Bovine serum albumin (BSA) was prepared in various concentrations of 0.01, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25 and 0.30 mg/ml.
2. graph was plotted for standard curve of BSA concentration and optical density at 750 nm (Figure-Appendix B1).

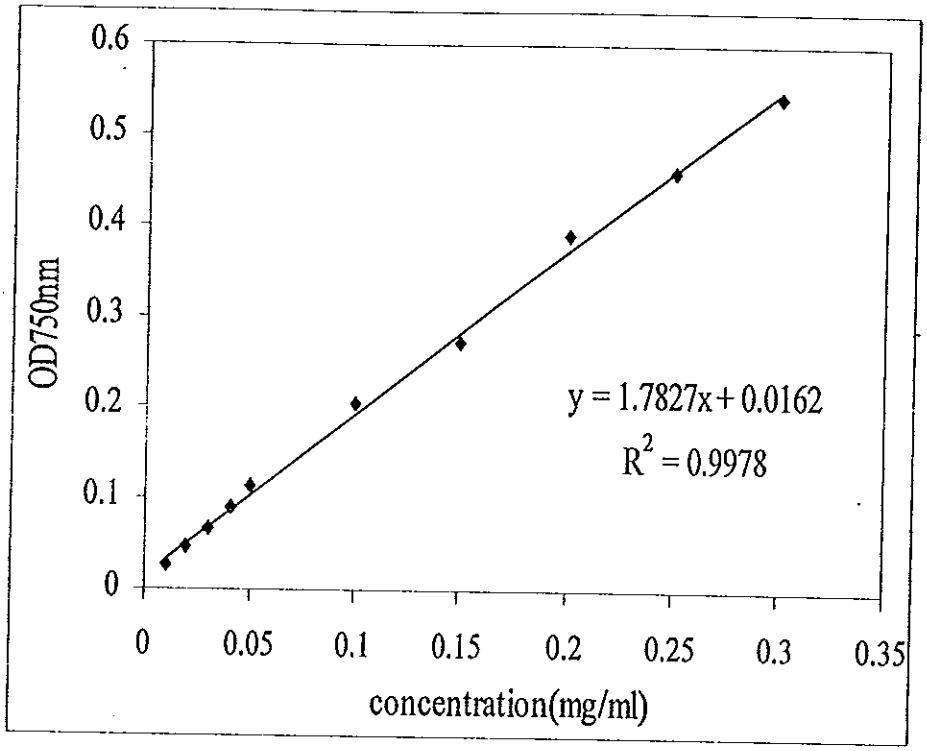

Figure-Appendix B1 Standard curve of BSA at the absorbance of 750 nm

2. Total protein and total nitrogen was measured by the Kjeldahl method (modified from A.O.A.C, 1990)

Apparatus

1. Kjeldahl flasks (800ml capacity: used for both digestion and distillation)
2. Digestion heaters
3. Digestion unit; consists of electric heaters, large lead tube, and plastic fume stack with suction fan capable of exhausting toxic fumes to outside air.
4. Distillation unit
5. Volumetric flask (250 ml)
6. Pipette (5 and 10 ml)
7. Burette (25 ml)
8. Digestion machine

Reagents

1. H_2SO_4 solution
2. Catalyst consists of CuSO4 ANDK2SO4 in the ration of 1:10
3. 40% NaOH
4. 2% H_3BO_3 solution
5. Mixed indicator
   - 5.1 Methyl red (0.125 g) and methylene blue (0.082 g) were weighed and dissolved in 100 ml of 95% ethanol.
   - 5.2 Bromocrasol green (0.1 g) was dissolved in distilled water and adjusted to 100 ml
   - 5.3 Mixed solution on 5.1 and 5.2 in the ratio of 5:1

Procedures

1. Digestion step

1.1 The 0.2-0.5 g or 5-10 ml of sample was placed into digestion flask. Blank control was included by using distilled water instead sample.

1.2 Added 1-2 g of catalyst

1.3 Poured a 5-10 ml of H_2SO_4 solution into flask, and fitted with a gas-trapped apparatus

1.4 Conducted digestion over heating by starting to 200 °C for 1 hour and heated up to 350 °C for 4 hours.

1.5 Reaction mixture gave clearly blue or green-blue supernatant and was cooled down for the next step of distillation.

2. Distillation step

2.1 Added 60-100 ml portion of distilled water into Kjeldahl flask.

2.2 Fitted with rubber stopper in Kjeldahl machine and opened cooling water pump in the flow rate of 3-4 liters per minute.

2.3 Pumped 40% NaOH into Kjeldahl flask until the color of sample solution was black.

2.4 A 10 ml of 2% H_3BO_3 solution containing 2-3 drops of mixed indicator in 250 ml flask trapped about 100-150 ml outlet of condenser.

2.5 Cleaned distillation unit by repeating distillation with distilled water.

2.6 The solution was titrated with 0.02-0.1 N HCl or H_2SO_4 solution.

Calculation

$$\text{Total nitrogen (\%)} = \frac{[(a - b) \times N \times 14]}{W}$$

where, a = volume of HCl or H_2SO_4 solution I sample titration (ml)

b = volume of HCl or H_2SO_4 solution in blank titration (ml)

N = concentration of HCl or H_2SO_4 solution (N)

W = weight or volume of sample

Factor = 6.25

Total protein (%) = total nitrogen (%)× 6.25

3. Moisture (A.O.A.C., 1990)

Procedures

1. Moisture can was heated in hot air oven at 105 °C for 3 hours and moved to cooled down in desiccator, then weighed.
2. Repeated as 1. until have no significant change of its weight.
3. A 1-3 g of sample was added into moisture can and incubated in hot air oven at 105 °C for 5-6 hours, then placed into desiccator and weighed. Repeated step 3 until have no significant change of its weight.

Calculation

$$\%M = \frac{W1 - W2}{W1} \times 100$$

where, M = moisture (%)

W1 = weight of sample before incubation

W2 = weight of sample after incubation

4. Crude fat (A.O.A.C., 1990)

Procedures

1. Incubated at 250 ml spherical flask in hot air oven at 105 °C for 3 hours and cooled down in desiccator, then weighed.
2. A 1-2 g of sample was putted on filtered membrane, then wrapped it tightly and placed into Soxhlet bag which was covered by cotton wool and moved to Soxhlet.
3. Poured petroleum ether into spherical flask about 150 ml and placed on heating mantle, then started cooling water controller and Soxhlet apparatus.
4. Crude fat was extracted for 14 hours and left in flask slightly, placed to incubate in hot air oven at 105 °C for 3 hours and cooled down in desiccator.
5. Weighed and reincubated at 105 °C for 30 minutes until have no significant change of its weight.

Calculation

$$\% \text{ crude fat} = \frac{\text{weight of curde fat after incubation}}{\text{weight of sample}} \times 100$$

5. Ash (A.O.A.C., 1990)

Procedures

1. Heated the crucible in muffle furnace at 600 °C for 3 hours and left until temperature down in room temperature (30°C), then putted into desiccator and weighed
2. Repeated the heating for 30 minutes following as stated on 1. until its difference of weight less than 1-3 g.
3. A 2 g of sample was added into the crucible and heated in muffle furnace at 600 °C for 3 hours and repeated the method of 1. and 2.

Calculation

$$\%\text{Ash} = \frac{\text{weight of ash after heating}}{\text{weight of ash before heating}} \times 100$$

6. Salt (Chlorine as Sodium Chloride) in Seafood (modified from A.O.A.C., 1999)

Reagents

1. Silver nitrate standard solution: weigh out about 8.5 g of the silver nitrate dissolve it in water, and make up to 500 ml in a volumetric flask. Shake well.
2. The ferric indicator solution: solution consists of a cold, saturated solution of $FeNH_4(SO_4)_2.12H_2O$ in water (about 40%) to which a few drops of 6 N nitric acid has been added.
3. 0.1 N NH_4SCN

Procedures

1. Use suitable size sample, depending on NaCl content.

2. Add known volume 0.1 N $AgNO_3$ solution, more than enough to precipitate all Cl^- as AgCl, and then add 20 ml HNO_3.
3. Boil gently on hot plate or sand bath until all solids except AgCl dissolve (usually 15 min).
4. Cool, add 50 ml H_2O and 5 ml indicator, and titrate with 0.1 N NH_4SCN solution until solution becomes permanent light brown.

Calculation

1. Subtract ml 0.1 N NH_4SCN used from ml 0.1 N $AgNO_3$ added and calculate difference as NaCl.
2. With 10 g sample each ml 0.1 N $AgNO_3$= 0.058% NaCl.

7. Sodium dodecyl sulfate – polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970; Mini-PROTEIN II Electrophoresis Cell Instruction Manual of BIO-RAD Laboratories company)

Stock solutions and buffers

1. Acrylamide / bis (30%T, 2.67%C): weigh 87.6 g acrylamide and 2.4 g N'N'-bis- methylene-acrylamide, make to 300 ml with deionized water. Filter and store at 4 °C in the dark (30 days maximum)
2. 10%(w/v) SDS: dissolve 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with deionized water.
3. 1.5 M Tris-HCl, pH 8.8

   27.23 g Tris base

   80 ml deionized water

   Adjust to pH 8.8 with 6N HCl. Bring total volume to 150 ml with deionized water and store at 4 °C.
4. 0.5 M Tris-HCl, pH6.8

6 g tris base

60 ml deionized water

Adjust to pH 6.8 with 6 N HCl. Bring total volume to 100 ml with deionized water and store at 4°C.

5. sample buffer

3.55 ml deionized water

1.25 ml 0.5 M tris-HCl, pH 6.8

2.5 ml glycerol

2.0 ml 10% (w/v) SDS

0.2 ml 0.5%(w/v) bromophenol blue

Store at room temperature (30°C). Add 50 µl β- mercaptoethanol to 950 µl sample buffer prior to use. Dilute the sample at lease 1:2 with sample buffer and heat at 95 °C for 4 minutes.

6. 10× electrode (running) buffer, pH8.3 (makes 1 L)

30.3 g tris base

144.0 g glycine

10.0 g SDS

Dissolve and bring total volume up to 1,000 ml with deionized water. Do not adjust pH with acid or base. Store at 4 °C. If precipitation occurs. Warm to room temperature (30°C) before use.

7. 10% APS (fresh daily)

100 mg ammonium persulfate

Dissolved in 1 ml of deionized water

Gel formulation (10 ml)

1. Prepare the monomer solution by mixing all reagents except the TEMED and 10% APS. Degas the mixture for 15 minutes.

Percent Gel SDS	DDI H_2O (ml)	Acrylamide/bis(ml) 30%	Gel buffer * (ml)	10%(w/v) (ml)
4%	6.1	1.3	2.5	0.1
10%	4.1	3.3	2.5	0.1
12%	3.4	4.0	2.5	0.1

* resolving (running) Gel buffer: 1.5 M Tris – HCl, pH 8.8
stacking gel buffer: 0.5M tris-HCl, pH 6.8

2. Immediately prior to pouring the gel, add:
   For 10 ml monomer solution:
   Resolving gel: 50 µl 10% APS and 5 µl TEMED
   Stacking gel: 50 µl 10% APS and 10 µl TEMED

Electrophoresis Procedures

1. The glass plate sandwiches was assembled.
2. Prepared resolving (running) gel monomer solution as formulation
3. Place a comb completely into the assembled gel sandwich. With a marker pen, place a mark on the glass plate 1 cm below the teeth of the comb. This will be the level to which the separating gel is poured. Remove the comb.
4. Add APS and TEMED to the resolving gel monomer solution and pour the solution to the mark, using a glass pipet and bulb.

5. Immediately overlay the monomer solution with water.
6. Allow the gel to polymerize for 45 minutes to 1 hour. Rinse off the overlay solution completely with distilled water.
7. Prepare the stacking gel monomer solution. Combine all reagents except APS and TEMED.
8. Dry the area above the resolving gel with filter paper before pouring the stacking gel.
9. Place a comb in the gel sandwich and tilt it so that the teeth are at a slight (~ 10^{o}) angle. This will prevent air from being trapped under the comb teeth while the monomer solutions are poured.
10. Add APS and TEMED to the stacking gel monomer solution and pour the solution down the spacer nearest the upturned side of the comb in the sandwich and add monomer to fill completely. The comb is properly seated when the T portion of the comb rests on top of the spacers.
11. Allow the gel to polymerize 30-45 minutes. Remove the comb by pulling it straight up slowly and gently.
12. Rinse the wells completely with distilled water. The gels are now ready to be attached to the inner cooling core, the sample loaded and gels run.
13. Assembly the upper buffer chamber and inner cooling core. Lower the inner cooling core into the lower buffer chamber. Add approximately 115 ml of buffer to the upper buffer chamber. Fill until the buffer reaches a level halfway between the short and long plates. Do not overfill the upper buffer chamber.
14. Pour the remainder of the buffer into the lower buffer chamber so that at least the bottom 1 cm of the gel is covered. Remove

any air bubbles from the bottom of the gel so that good electrical contact is achieved. This can be done by swirling the lower buffer with pipet until the bubbles clear.

15. Samples were mixed with sample buffer. Load the samples into the wells with a Hamilton syringe or a pipette using gel loading tips. Samples should be loaded slowly to allow them to settle evenly on the bottle of the well. Be careful not to puncture the bottom of the well with the syringe needle or pipette tip.
16. Place the lid on the top of the lower buffer chamber to fully enclose the cell. The correct orientation is made by matching the colors of the plugs on the lid with the jacks on the inner cooling core. Attach the electrical leads to a suitable power supply with the proper polarity.
17. Apply power for electrophoresis. The recommended power condition for optimal resolution with minimal thermal band distortion is 200 volts, constant voltage setting. The usual run time is approximately 45 minutes.
18. After electrophoresis is complete, turn off the power supply and disconnect the electrical leads.
19. Remove the cell lid and carefully pull the inner cooling core out of the lower chamber. Pour off the upper buffer.
20. Lay the inner cooling core on its side and remove the clamp assembly by pushing down on both sides of the cooling core latch and up on the clamps until clamp assembly is released.
21. Remove the gels.

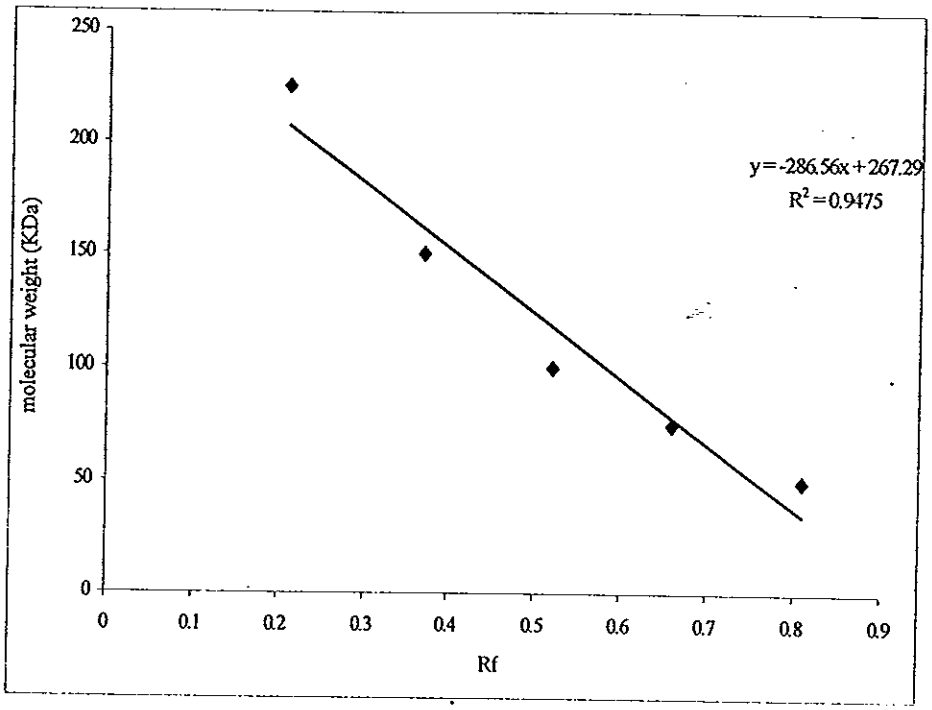

Figure-Appendix B7 Calibration curve of molecular weight protein by SDS - PAGE. The standard consisted of protein Molecular weight 225kDa, 150 kDa, 100 kDa, 75 kDa, 50 kDa

8. Silver Staining procedure (Yaobo, 2002)

Stock solution

1. Solution A : 50% methanol and 10% acetic acid
2. Solution B: 0.8 g $AgNO_3$ was dissolved in 4 ml deionized water
3. Solution C: 0.36% NaOH 21 ml; 14.8 M (30%) ammonium
4. Solution D: add solution B into solution C, bring the volume to 100 ml with deionized water (prepare before using).
5. Solution E:0.5 ml 1% citric acid; 50 µl 38% formaldehyde bring the volume to 100 ml with deionized water (prepare before using)
6. Solution F: 1% acetic acid

procedure

1. Soak the gels in solution A for at lease 1 hour, shake slightly and renew solution A 2 -3 times.
2. Wash gel with water 30 minutes, change water 3 -4 times
3. Staining with solution D for 15 minutes with slight shaking.
4. Wash gel with water for 2 minutes.
5. Wash gel with solution E. the protein lane can appear in 10 minutes.
6. Gel was soaked in solution F when gel background become slight yellow.
7. Wash gel with water for at lease 1 hour, change water 2 –3 times.
8. Dry gel or keep gel in water.

9. Activity staining procedure (Carcia-Carreno *et al.,* 1993)

1. After electrophoresis, gels were immersed in 100 ml of 2% casein in 0.02 M Tris-HCl buffer (pH 8.0) for 1 hour at 4°C, in order to allow the substrate to diffuse into the gel, at reduced enzyme activity.
2. Then the temperature was raised to 50 °C and the gels were incubated for 90 minutes for the digestion of the protein substrate by the active fractions.
3. After substrate hydrolysis and incubation, gels were washed with distilled water and immediately fixed and stained in a one-step process by immersing them in a filtered staining solution containing 40% ethanol, 10% acetic acid, and 0.1% Coomassie brilliant blue R-250.
4. The staining period was carried out for 2 hours. After staining, a couple of hours of washing with 40% ethanol-10% acetic acid solution was enough to destain and improve the contrast of the clear zones.
5. Clear zones on blue background indicated protease activity.

10. Tyrosine standard curve

Casein suspension 0.5 ml of 10 mg/ml (in 20 mM Tris-HCl buffer, pH 8.0 containing 5 mM $CaCl_2$ and 0.02% NaN_3) and 0.1 ml of tyrosine solution were mixed and incubated for 1 hour under 50°C. then 1.0 ml of chilled trichloroacetic acid (TCA) (10% in distilled water) was added to precipitate the protein. After 1 hour in the refrigerator, the suspension was centrifuged at 10,000 rpm for 10 min at room temperature (30°C). The clear supernatant was measured spectrophotometically at 280 nm against blanks in which casein solution and tyrosine solution were substituted by buffer.

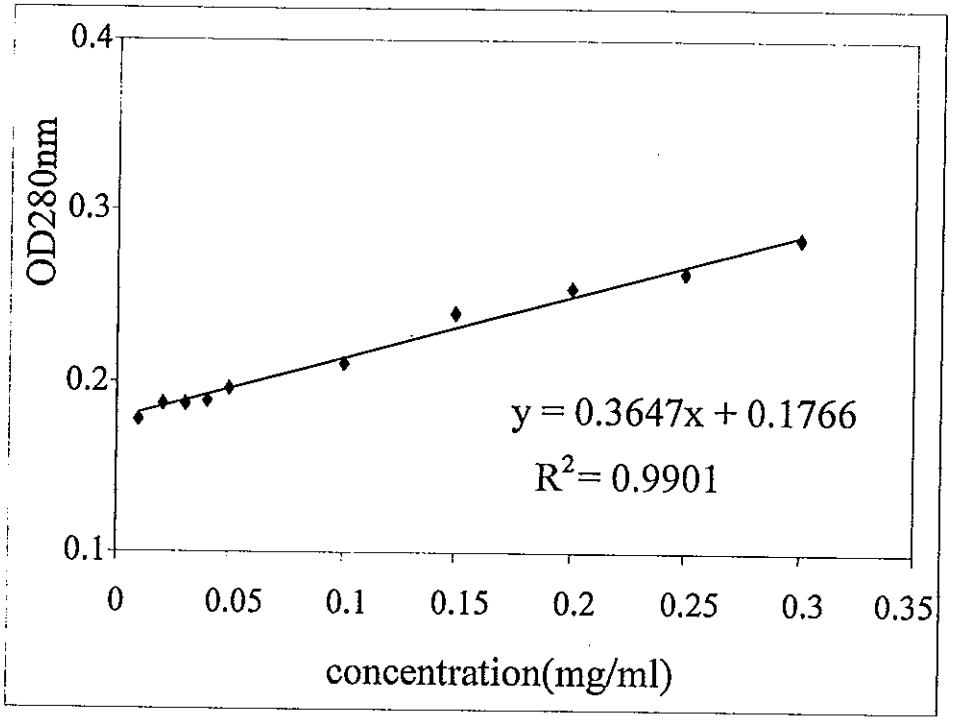

Figure-Appendix B10 Tyrosine standard curve at OD 280 nm using the standard consisted tyrosine concentration of 0.01, 0.02, 0.03, 0.04, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30 mg/ml

Appendix C

Experimental data

Table-Appendix C1 Effect of membrane MWCO on enzyme activity, purity, transmission and yield during UF using polyethersulfone membrane

membrane		Act.[1] (U/ml)	Sp. Act.[2] (U/mg)	Trans.[3]	Vol.[4] (ml)	Yield (%)
Protease						
Crude enzyme extract		45.41	3.26		500	
30 kDa	Retentate	47.13	3.24	0.02	471	98
	Permeate	0.85	0.11		26	
100 kDa	Retentate	46.17	3.34	0.66	419	85
	Permeate	30.06	2.85		79	
Trypsin						
Crude enzyme extract		11.21	0.80		500	
30 kDa	Retentate	12.01	0.82	0.01	471	100
	Permeate	0.09	0.01		26	
100 kDa	Retentate	11.36	0.82	0.79	419	85
	Permeate	9.03	0.84		79	

Table-Appendix C1 (continue)

membrane		Act. (U/ml)	Sp. Act. (U/mg)	Trans.	Vol. (ml)	Yield (%)
Chymotrypsin						
Crude enzyme extract		8.53	0.61		500	
30 kDa	Retentate	8.96	0.62	0.01	471	99
	Permeate	0.05	0.01		26	
100 kDa	Retentate	8.74	0.63		419	
	Permeate	6.33	0.59	0.72	79	86

[1] enzyme activity

[2] enzyme specific activity

[3] enzyme transmission

[4] volume

Table-Appendix C2 Effect of pre-incubation time on general protease activity and specific activity

		Activity (U/ml)	Specific activity (U/mg)
Crude enzyme extract		49.96	4.41
0.5 h	Incubated enzyme extract	51.95	4.35
	Retentate	51.60	4.51
	Permeate	0.60	0.08
1.0 h	Incubated enzyme extract	52.09	4.63
	Retentate	58.43	5.35
	Permeate	0.38	0.03
3.0 h	Incubated enzyme extract	33.29	3.00
	Retentate	35.40	3.59
	Permeate	0.90	0.11
6.0 h	Incubated enzyme extract	37.43	3.30
	Retentate	49.19	4.69
	Permeate	0.66	0.08
24 h	Incubated enzyme extract	20.14	1.78
	Retentate	20.54	1.89
	Permeate	0.54	0.07

Table-Appendix C3 Effect of pre-incubation time on trypsin activity and specific activity

		Activity (U/ml)	Specific activity (U/mg)
Crude enzyme extract		10.31	0.86
0.5 h	Incubated enzyme extract	10.27	0.86
	Retentate	10.58	0.89
	Permeate	0.11	0.01
1.0 h	Incubated enzyme extract	10.19	0.90
	Retentate	14.35	1.46
	Permeate	0.06	0.01
3.0 h	Incubated enzyme extract	8.47	0.76
	Retentate	8.54	0.86
	Permeate	0.11	0.01
6.0 h	Incubated enzyme extract	7.89	0.70
	Retentate	8.33	0.79
	Permeate	0.09	0.01
24 h	Incubated enzyme extract	5.13	0.46
	Retnetate	6.92	0.64
	Permeate	0.02	< 0.01

Table-Appendix C4 Effect of pre-incubation time on chymotrypsin activity and specific activity

		Activity (U/ml)	Specific activity (U/mg)
Crude enzyme extract		8.97	0.74
0.5 h	Incubated enzyme extract	9.04	0.76
	Retentate	9.97	0.87
	Permeate	0.08	0.01
1.0 h	Incubated enzyme extract	8.93	0.79
	Retentate	15.73	1.44
	Permeate	0.07	0.01
3.0 h	Incubated enzyme extract	7.12	0.64
	Retentate	7.31	0.74
	Permeate	0.14	0.02
6.0 h	Incubated enzyme extract	6.68	0.59
	Retentate	6.97	0.66
	Permeate	0.13	0.01
24 h	Incubated enzyme extract	4.29	0.38
	Retentate	5.99	0.55
	Permeate	0.17	0.02

Table-Appendix C5 Steady state flux during UF using regenerated cellulose membrane at different cross flow rates and TMPs

Cross flow rate L/h	TMP (bar)	steady state flux ($L/m^2.h$) 30 kDa[1]	100 kDa[2]
120	0.5/1.0[3]	5.76	25.92
	1.5	8.64	30.24
	2.5	10.80	33.12
	3.5	17.28	34.06
	4.5	17.64	33.12
240	0.5/1.0	25.92	41.08
	1.5	30.96	47.52
	2.5	36.00	51.12
	3.5	43.92	51.12
	4.5	45.36	50.76
360	0.5/1.0	35.28	51.12
	1.5	43.20	60.48
	2.5	48.96	64.80
	3.5	54.72	68.40
	4.5	54.00	67.68

[1] using membrane with MWCO 30 kDa

[2] using membrane with MWCO 100 kDa

[3] 0.5 bar for membrane with MWCO 30 kDa; 1.0 bar for membrane with MWCO 100 kDa

Table-Appendix C6 The permeate flux, soluble protein concentration during DF for extract of spleen

Time (min)	Permeate flux ($L/m^2.h$)	Soluble protein concentration (mg/ml)	Diafiltration wash volume
0	69.32	11.34	0
10	65.17	4.64	5.60
20	62.0	2.15	10.92
30	60.89	1.09	16.09
40	59.25	0.82	21.11
50	58.01	0.77	26.02
60	57.26	0.61	30.86
70	57.33	0.61	35.68
80	56.98	0.60	40.47
90	56.64	0.61	46.72
100	56.45	0.62	49.95
110	56.45	0.61	54.68
120	56.39	0.61	59.40

Table-Appendix C7 Enzyme activity and specific activity during DF for extract of spleen

Time	protease		trypsin		chymotrypsin	
(min)	Act[1] (U/ml)	Sp.Act[2] (U/mg)	Act (U/ml)	Sp.Act. (U/mg)	Act. (U/ml)	Sp.Act. (U/mg)
0	47.19	4.16	13.94	1.23	8.97	0.79
10	47.43	10.22	13.87	2.99	9.02	1.94
20	46.97	21.28	14.09	6.55	8.83	4.11
30	46.33	42.50	13.12	12.04	8.49	7.79
40	46.41	56.60	13.49	16.45	8.62	10.51
50	47.06	61.11	13.86	18.00	8.31	10.79
60	46.87	76.83	14.17	23.22	8.06	13.21
70	46.29	75.88	13.69	22.44	7.95	13.03
80	46.01	76.68	13.31	22.18	7.98	13.30
90	45.97	75.68	13.31	22.18	8.01	13.13
100	46.12	74.38	13.52	21.81	7.96	12.84
110	45.89	75.23	13.67	22.41	8.17	13.39
120	45.94	75.31	13.82	22.66	7.89	12.93
PF[3]		18.01		18.42		16.37

[1] enzyme activity

[2] specific activity

[3] purification factor

Table-Appendix C8 The permeate flux, soluble protein concentration during DF for extract of mixed viscera

Time (min)	Permeate flux ($L/m^2.h$)	Soluble protein concentration (mg/ml)	Diafiltration wash volume
0	47.98	16.17	0
11	42.33	7.93	3.76
20	39.17	7.12	7.19
30	37.98	6.33	10.47
40	36.89	5.96	13.62
50	36.32	5.24	16.71
60	34.19	4.87	19.64
70	33.85	4.11	22.51
80	33.71	3.62	25.37
90	33.71	3.02	28.21
100	33.85	3.08	31.06
110	33.71	2.97	33.89
120	33.76	3.01	36.72

Table-Appendix C9 Enzyme activity and specific activity during DF for extract of mixed viscera

Time (min)	prototease		trypsin		chymotrypsin	
	Act[1] (U/ml)	Sp.Act[2] (U/mg)	Act (U/ml)	Sp.Act. (U/mg)	Act. (U/ml)	Sp.Act. (U/mg)
0	33.08	2.04	6.94	0.43	5.21	0.31
10	32.95	4.16	6.97	0.88	5.09	0.64
20	32.17	4.52	6.65	0.93	5.12	0.72
30	32.36	5.11	6.71	1.06	5.34	0.84
40	31.96	5.36	6.28	1.05	4.92	0.83
50	32.44	6.20	7.03	1.34	5.27	1.01
60	32.69	6.67	6.84	1.40	5.28	1.08
70	33.01	8.03	6.17	1.50	5.38	1.31
80	31.84	8.80	6.36	1.76	5.19	1.43
90	30.95	10.24	6.62	2.19	5.14	1.70
100	32.33	10.49	6.69	2.17	5.24	1.70
110	32.71	11.01	6.77	2.28	5.16	1.74
120	33.03	10.97	6.65	2.20	5.12	1.70
PF[3]		5.02		5.09		5.48

[1] enzyme activity

[2] enzyme specific activity

[3] purification factor

Publication

Zhenyu L., Yonravong, W. and H-Kittikun, A. 2004. Separation of proteases from yellowfin tuna viscera by ultrafiltration. Regional Symposium Membrane Science and Technology (MST 2004). 22nd ~24th April 2004. University Technology Malaysia (UTM). Johor. Malaysia.

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