Purification and Characterization of Trypsin and Chymotrypsin from Viscera of Yellowfin Tuna (*Thunnus albacares*) and Enzyme Application

Saowarath Jantaro

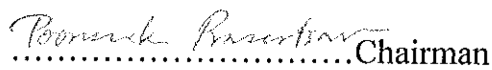
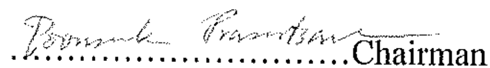
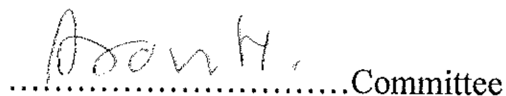
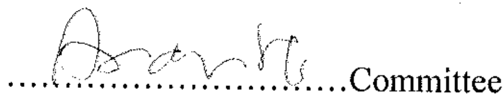
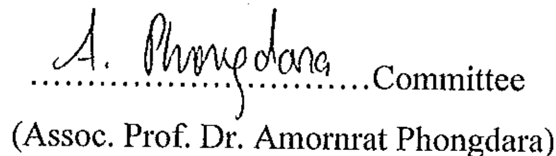
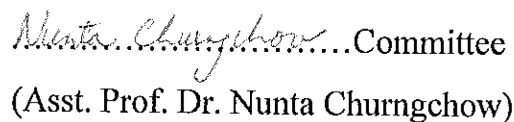
Master of Science Thesis in Biotechnology

Prince of Songkla University

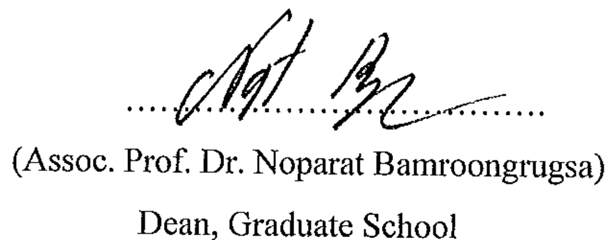
2000

เลขหมู่ QL638.S36 S37 2000 C.2
Order Key 28809
Bib Key 177576
1 ต.ค. 2543

Thesis Title	Purification and Characterization of Trypsin and Chymotrypsin from Viscera of Yellowfin Tuna (*Thunnus albacares*) and Enzyme Application
Author	Ms. Saowarath Jantaro
Major Program	Biotechnology

Advisory Committee	Examining Committee
 ....Chairman	 ....Chairman
(Assoc. Prof. Dr. Poonsuk Prasertsan)	(Assoc. Prof. Dr. Poonsuk Prasertsan)
 ....Committee	 ....Committee
(Asst. Prof. Dr. Aran H-Kittikun)	(Asst. Prof. Dr. Aran H-Kittikun)
	 ....Committee
	(Assoc. Prof. Dr. Amornrat Phongdara)
	 ....Committee
	(Asst. Prof. Dr. Nunta Churngchow)

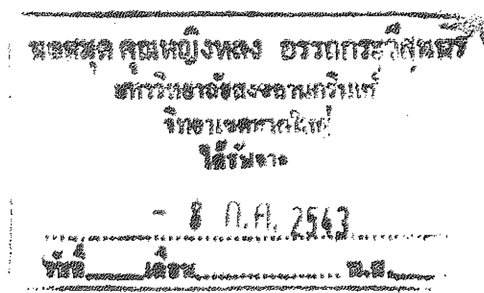
The Graduate School, Prince of Songkla University, has approved this thesis as partial fulfillment of the requirement for the Master of Science degree in Biotechnology.


....

(Assoc. Prof. Dr. Noparat Bamroongrugsa)

Dean, Graduate School

Thesis Title	Purification and Characterization of Trypsin and Chymotrypsin from Viscera of Yellowfin Tuna (*Thunnus albacares*) and Enzyme Application
Author	Miss Saowarath Jantaro
Major Program	Biotechnology
Academic Year	1999



Abstract

The activities of trypsin and chymotrypsin from the enzymes extracted from the whole viscera and the viscera organ (liver, pancreas, stomach, spleen, intestine) of yellowfin tuna (*Thunuus albacares*) were compared. Spleen was found to be the best source for trypsin with the highest activity of 49.26 units/ml and the specific activity of 13.80 units/mg protein, followed by the mixed viscera, pancreas, liver, intestine and stomach, respectively. The best source for chymotrypsin was pancreas, giving the highest activity and specific activity of 4.13 units/ml and 1.03 units/mg protein, respectively followed by the spleen, stomach, mixed viscera, liver and intestine, respectively. The enzymes extracted from their best sources were purified to separate trypsin and chymotrypsin by affinity chromatography on benzamidine-Sepharose column. The purity of the purified trypsin and chymotrypsin were increased 15.26 and 13.89 fold, respectively.

The purified enzymes were characterized and the maximal activity was found to be at pH 8.0 and the optimum temperature was 50 °C. They were stable in the pH range of 7.0 - 8.0 and stable up to 50°C for 60 minutes with residual activities of 56 % and 72 % for trypsin and

chymotrypsin, respectively. Trypsin and chymotrypsin activities were slightly activated by benzamidine and inhibited by 1-10 phenantroline, K^+, Ag^+ and 4-aminobenzoic acid. For EDTA and Hg^{++}, the inhibition effect was more pronounced on chymotrypsin than on trypsin. Their K_m values were 0.49 and 0.27 mg/ml, respectively as well as V_{max} values of 111.11 and 50 μmol/ml/min, respectively. The molecular weights of trypsin and chymotrypsin were approximately 23 and 25 kDa, respectively.

Crude enzyme extracted from viscera of yellowfin tuna and commercial enzymes were used for the production of fish extract from hydrolysis of tuna condensate. The product obtained was light to dark brown, clear and no fishy smell. Chemical compositions of fish extract using the crude enzyme were no significant difference from those using the other two sources of enzymes. However, when compared to the standard specification, it gave higher contents of moisture, total nitrogen, calcium and phosphorus and lower contents of total solid, ash, salt, crude fat, total amino nitrogen and total volatile nitrogen. After further improved by evaporation and desalting, the fish extract was similar to the commercial fish extract (Songkla Canning (Thailand) Co., Ltd.). The results indicated that the enzymes from tuna viscera could replace the commercial enzyme for the production of fish extract.

ชื่อวิทยานิพนธ์	การทำให้บริสุทธิ์และคุณลักษณะของเอนไซม์ทริปซินและไคโมทริปซินจากเครื่องในปลาทูน่าพันธุ์ครีบเหลือง (*Thunnus albacares*) และการประยุกต์ใช้
ผู้เขียน	นางสาวเสาวรัตน์ จันทะโร
สาขาวิชา	เทคโนโลยีชีวภาพ
ปีการศึกษา	2542

บทคัดย่อ

เมื่อเปรียบเทียบค่าแอคทิวิตี้ของทริปซินและไคโมทริปซินของเอนไซม์ที่สกัดจากเครื่องในรวมและเครื่องในแต่ละส่วน (ตับ, ตับอ่อน, กระเพาะ, ม้าม, ลำไส้) ของปลาทูน่าพันธุ์ครีบเหลือง (*Thunnus albacares*) พบว่าม้ามเป็นแหล่งเอนไซม์ที่ดีที่สุดสำหรับทริปซิน โดยให้ค่าแอคทิวิตี้สูงสุดเท่ากับ 49.26 ยูนิตต่อมิลลิลิตร และแอคทิวิตี้จำเพาะเท่ากับ 13.80 ยูนิตต่อมิลลิกรัมโปรตีน รองลงมาได้แก่ เครื่องในรวม, ตับอ่อน, ตับ, ลำไส้ และกระเพาะ ตามลำดับ แหล่งที่ดีที่สุดสำหรับไคโมทริปซินได้จากตับอ่อน โดยแอคทิวิตี้และแอคทิวิตี้จำเพาะสูงสุดเท่ากับ 4.13 ยูนิตต่อมิลลิลิตร และ 1.03 ยูนิตต่อมิลลิกรัมโปรตีน ตามลำดับ รองลงมาคือ ม้าม, กระเพาะ, เครื่องในรวม, ตับ และ ลำไส้ ตามลำดับ เอนไซม์ที่ได้จากแหล่งที่ดีที่สุดของแต่ละชนิด ถูกทำให้บริสุทธิ์เพื่อแยกทริปซินและไคโมทริปซินโดยผ่านโครมาโตกราฟีแบบจำเพาะเจาะจงในคอลัมน์ที่บรรจุ benzamidine-Sepharose ได้เอนไซม์ทริปซินและไคโมทริปซินมีความบริสุทธิ์ 15.26 และ 13.89 เท่า ตามลำดับ

เมื่อศึกษาคุณลักษณะของเอนไซม์ที่ผ่านการทำให้บริสุทธิ์ พบว่าเอนไซม์ทั้งสองชนิดให้ค่าแอคทิวิตี้สูงสุดที่พีเอช 8 และอุณหภูมิที่เหมาะสมคือ 50 องศาเซลเซียส เอนไซม์มีความคงตัวที่พีเอช 7.0 - 8.0 และสูญเสียแอคทิวิตี้ที่อุณหภูมิ 50 องศาเซลเซียส หลังการบ่มเอนไซม์เป็นเวลา 60 นาที โดยทริปซินมีแอคทิวิตี้เหลืออยู่ 56 เปอร์เซ็นต์ และ 72 เปอร์เซ็นต์สำหรับไคโมทริปซิน แอคทิวิตี้ของทริปซินและ

ไคโมทริปซินถูกกระตุ้นเล็กน้อยโดย benzamidine และถูกยับยั้งโดย 1-10 phenantroline, K^+, Ag^+ และ 4-aminobenzoic acid ส่วน EDTA และ Hg^{++} มีผลยับยั้งต่อไคโมทริปซินมากกว่าทริปซิน เอนไซม์ทริปซินและไคโมทริปซินที่ได้มีค่า K_m เท่ากับ 0.49 และ 0.27 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ และ V_{max} เท่ากับ 111.11 และ 50 ไมโครโมลต่อมิลลิลิตรต่อนาที ตามลำดับ น้ำหนักโมเลกุลของทริปซินและไคโมทริปซินมีขนาดประมาณ 23 และ 25 กิโลดาลตัน ตามลำดับ

มีการใช้เอนไซม์ที่สกัดจากเครื่องในย่อยน้ำนึ่งปลาทูน่าเพื่อผลิตสารสกัดจากปลา (fish extract) เปรียบเทียบกับการใช้เอนไซม์ทางการค้า พบว่าผลิตภัณฑ์ที่ได้มีสีน้ำตาลอ่อน-คล้ำ มีกลิ่นหอม ไม่มีกลิ่นคาวปลา องค์ประกอบทางเคมีของผลิตภัณฑ์ที่ใช้เอนไซม์สกัดจากเครื่องในปลาทูน่าไม่แตกต่างอย่างมีนัยสำคัญจากองค์ประกอบทางเคมีของผลิตภัณฑ์เมื่อใช้เอนไซม์ทางการค้า แต่เมื่อเปรียบเทียบกับมาตรฐานของผลิตภัณฑ์พบว่า จะมีค่าความชื้น, ปริมาณไนโตรเจนทั้งหมด, แคลเซียมและฟอสฟอรัสสูงกว่า และให้สารต่อไปนี้ในปริมาณที่ต่ำกว่า ได้แก่ ปริมาณของแข็งทั้งหมด, เถ้า, เกลือ, ไขมัน, ปริมาณกรดอะมิโนไนโตรเจน และสารไนโตรเจนที่ระเหยได้ หลังการปรับปรุงผลิตภัณฑ์โดยการนำไปประเหยและกำจัดเกลือออก พบว่าสารสกัดจากปลาที่ได้คล้ายคลึงกับผลิตภัณฑ์ทางการค้า (บริษัทสงขลาแคนนิ่ง (ประเทศไทย) จำกัด) จากผลการทดลองนี้จะเห็นได้ว่าสามารถใช้เอนไซม์ที่สกัดจากเครื่องในปลาทูน่าแทนการใช้เอนไซม์ทางการค้าในการผลิตสารสกัดจากปลา

Acknowledgement

I would like to express my deepest sincere and gratitude appreciation to my advisor, Assoc. Prof. Dr. Poonsuk Prasertsan of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University, for her suggestions, comment and her kind assistance of reading, correcting and also her invaluable guidance, understanding and encouragement throughout my master degree study.

I also would like to express my deepest appreciation to my co-advisor, Asst. Prof. Dr. Aran H-Kittikul of the Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University for his valuable advice and kindness suggestion and I am also very grateful to my thesis examining committee : Assoc. Prof. Dr. Amornrat Phongdara and Asst. Prof. Dr. Nunta Churngchow of the Department of Biochemistry, Faculty of Science, Prince of Songkla University for their helpful suggestions and dedicating valuable time for thesis examination.

Special thanks for my friends and staff who gave me their help heartily and shared a hard time with me during my study.

I would like to direct my appreciation to National Science and Technology Development Agency for their scholarship provided from June 1997 to May 1999 and also thanks go to Graduate School for every helpful supporting this thesis.

Finally, I am indebted and thankful to my family for their encouragement, willpower and heartiness support throughout my life.

Saowarath Jantaro

Contents

	Page
Abstract	(3)
Acknowledgement	(7)
Contents	(8)
List of Tables	(13)
List of Figures	(15)
List of Abbreviations	(18)
Chapter	
1. Introduction	1
Literature review	3
1. Yellowfin tuna (*Thunnus albacares*)	3
2. Proteolytic enzyme	6
2.1. Serine proteases	6
2.1.1. Trypsin-like proteases	6
2.1.2. Alkaline proteases	9
2.2. Acid proteases	9
2.3. Thiol proteases	9
2.4. Metalloproteases	10
3. Properties of trypsin and chymotrypsin	10
3.1. Molecular weight	10
3.2. Amino acid composition	11
3.3. Substrate specificity	15
3.4. Kinetic parameters	17

Contents (continue)

	Page
3.5. Denaturation constants	20
3.6. Inhibition constants	20
4. Factors affecting on property and activity of the enzyme	21
4.1. Temperature	21
4.2. pH	24
4.3. Substrates	25
4.4. Salt	25
4.5. Pressure	27
4.6. Chelating agent and metal ion	28
4.7. Inhibitors	29
5. Enzyme application	29
5.1. Fish sauce and fish silage	33
5.2. Protein hydrolysates	34
5.3. Ripening by enzyme addition	35
5.4. Carotenoid extraction	36
5.5. Fish extract production	37
Objectives	40
2. Materials and Methods	41
Materials	41
1. Raw materials	41
2. Chemicals	41
Instruments	41
Analytical methods	42

Contents (continue)

	Page
Methods	45
1. Purification and characterization of trypsin and chymotrypsin	45
1.1. Extraction of enzyme solution	45
1.2. Enzyme purification	45
1.2.1. Ammonium sulfate precipitation	45
1.2.2. Dialysis	46
1.2.3. Column chromatography	46
1.2.3.1. Gel filtration column chromatography	46
1.2.3.2. Affinity column chromatography	46
1.2.3.3. Anion-exchange column chromatography	47
1.2.4. Gel electrophoresis	47
1.2.4.1. Non-denatured polyacrylamide gel electrophoresis (Non-PAGE)	47
1.2.4.2. Sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	48
1.3. Characterization of the purified trypsin and chymotrypsin	48
1.3.1. Effect of pH on enzyme activity and stability	48
1.3.1.1. Effect of pH on enzyme activity	48
1.3.1.2. Effect of pH on enzyme stability	49
1.3.2. Effect of temperature on enzyme activity and stability	49

Contents (continue)

	Page
1.3.2.1. Effect of temperature on enzyme activity	49
1.3.2.2. Effect of temperature on enzyme stability	49
1.3.3. Effect of ions and chemicals on enzyme activity	50
1.3.4. Determination of kinetic parameters	50
2. Enzyme application	50
3. Results and Discussion	52
1. Purification and characterization of trypsin and chymotrypsin from yellowfin tuna	52
1.1. Extraction of trypsin and chymotrypsin from yellowfin tuna	52
1.2. Enzyme purification	55
1.2.1. Ammonium sulfate precipitation	55
1.2.2. Dialysis	57
1.2.3. Column chromatography	57
1.2.3.1. Gel filtration column chromatography	57
1.2.3.2. Affinity column chromatography	61
1.2.3.3.Anion-exchange column chromatography	64
1.2.4. Gel electrophoresis	68
1.2.4.1. Non-denatured polyacrylamide gel electrophoresis (Non-PAGE)	68
1.2.4.2. Sodiumdodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)	68

Contents (continue)

	Page
1.3. Characterization of the purified trypsin and chymotrypsin	72
1.3.1. Effect of pH on enzyme activity and stability	72
1.3.1.1. Effect of pH on enzyme activity	72
1.3.1.2. Effect of pH on enzyme stability	74
1.3.2. Effect of temperature on enzyme activity and stability	76
1.3.2.1. Effect of temperature on enzyme activity	76
1.3.2.2. Effect of temperature on enzyme stability	78
1.3.3. Effect of ions and chemicals on enzyme activity	80
1.3.4. Determination of kinetic parameters	84
2. Enzyme application	87
4. Conclusion	98
Suggestion	100
References	101
Appendices	109
Appendix A Buffer preparation	109
Appendix B Column chromatography preparation and $(NH_4)_2SO_4$ fractionation	112
Appendix C Media preparation	115
Appendix D Analytical methods	122
Vitae	147

List of Tables

Table		Page
1	Molecular weights of trypsin and chymotrypsin from some marine fish	12
2	Amino acid composition of anchovy trypsin and chymotrypsin compared with anchovy trypsin A and B and bovine trypsin and chymotrypsin	13
3	The N-terminal sequence of Atlantic cod trypsin (enzyme I) compared to trypsin from other species	14
4	N-terminal sequence of Atlantic cod chymotrypsin compared with bovine α-chymotrypsin	15
5	Kinetic parameters of some proteolytic enzymes from marine fish	18
6	Optimum temperature and pH on trypsin and chymotrypsin activities from marine fish	23
7	Kinetic constants for some synthetic substrates of bovine trypsin	26
8	Some inhibitors of bovine trypsin	30
9	Some processes facilitated by proteolytic enzymes	31
10	Some uses of proteolytic enzymes in the food industry	32
11	The composition of tuna condensate	38
12	The chemical composition of fish extract	39
13	Weight of total body, mixed viscera and individual organ of the viscera of yellowfin tuna (*Thunnus albacares*)	53

List of Tables (continue)

Table		Page
14	Comparison on activity of trypsin and chymotrypsin from each organ of yellowfin tuna (*Thunnus albacares*)	54
15	Ammonium sulfate precipitation at various salt saturation of trypsin and chymotrypsin from spleen and pancreas of yellowfin tuna (*Thunnus albacares*)	56
16	Summary of trypsin and chymotrypsin purification steps	58
17	Effect of ions and chemicals to trypsin and chymotrypsin activities from yellowfin tuna (*Thunnus albacares*)	81
18	Comparison on the quality of fish extract produced by crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) and the commercial enzymes to the standard specification of fish extract	89
19	Chemical composition of fish extract using crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) before and after evaporation and desalting step compared to those using the commercial product	96

Appendix

B2	Final concentration of ammonium sulfate : percentage saturation	113
D13	The composition of non-PAGE	141
D16	Most probable number (MPN) of *E. coli* per 100 ml	145

List of Figures

Figure		Page
1	Yellowfin tuna (*Thunnus albacares*)	3
2	Estimated distribution of yellowfin tuna	5
3	Formation of various forms of chymotrypsin on activation of chymotrypsinogen A by trypsin	8
4	Cleavage sites of oxidized β-chain of bovine insulin by anchovy trypsin and chymotrypsin	16
5	Gel filtration on Sephadex G-100 of trypsin and chymotrypsin from spleen of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	59
6	Gel filtration on Sephadex G-100 of trypsin and chymotrypsin from pancreas of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	60
7	Affinity column chromatography on Benzamidine-Sepharose of trypsin and chymotrypsin from spleen of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	62
8	Affinity column chromatography on Benzamidine-Sepharose of trypsin and chymotrypsin from pancreas of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	63
9	Anion exchange column chromatography on DEAE-Sephadex A-50 of trypsin from yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	65

List of Figures (continue)

Figure		Page
10	Anion exchange column chromatography on DEAE-Sephadex A-50 of chymotrypsin from yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm	66
11	Nondenatured polyacrylamide gel electrophoresis of protein fractions obtained during purification of trypsin and chymotrypsin from yellowfin tuna (*Thunnus albacares*)	69
12	SDS-polyacrylamide gel electrophoresis of protein fractions obtained during purification of trypsin from yellowfin tuna (*Thunnus albacares*)	70
13	SDS-polyacrylamide gel electrophoresis of protein fractions obtained during purification of chymotrypsin from yellowfin tuna (*Thunnus albacares*)	71
14	Effect of pH on the trypsin and chymotrypsin activities of yellowfin tuna (*Thunnus albacares*)	73
15	pH stability of trypsin and chymotrypsin from yellowfin tuna (*Thunnus albacares*)	75
16	Effect of temperature on the activities of trypsin and chymotrypsin at pH 8.0	77
17	Thermal stability of trypsin and chymotrypsin at various temperature	79
18	Lineweaver-Burk plot of trypsin activity in various TAME concentrations at pH 8.0, 50 °C	85

List of Figures (continue)

Figure		Page
19	Lineweaver-Burk plot of chymotrypsin activity in various BTEE concentrations at pH 8.0, 50 °C	86
20	Fish extract before evaporation step using crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) in comparison with the commercial enzyme trypsin and chymotrypsin	88
21	Fish extract using crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) in comparison with the commercial product	97

Appendix

B2	Nomogram for obtaining amounts of ammonium sulfate to be added	114
D1	Standard curve of BSA at the absorbance of 750 nm	123
D12	Calibration curve of molecular weight protein by SDS-PAGE	140

List of Abbreviations

ATEE	=	Acetyl-L-tyrosine ethyl ester
BAPNA	=	N-α-benzoyl-arginine-*p*-nitroanilide
BSA	=	Bovine serum albumin
BTEE	=	Benzoyl-L-tyrosine ethyl ester
BzArg-NH-Np	=	N-benzoyl-L-arginine-*p*-nitroanilide
BzTyrOEt	=	N-benzoyl-L-tyrosine ethyl ester
BzTyr-NH-Np	=	N-benzoyl-L-tyrosine-*p*-nitroanilide
CM	=	Carboxymethyl
DEAE	=	Diethylaminoethyl
EDTA	=	Ethylenediamine tetraacetic acid
EGTA	=	Ethylene glycol bis (*β*-aminoethyl ether) N,N,N′,N′,-tetraacetic acid
E 600	=	Ethyl-*p*-nitrophenyl phosphate
hr	=	hours
kcal	=	Kilocalories
kDa	=	Kilodaltons
K_m	=	Michaelis-Menten constant
l	=	Litre
min	=	Minutes
mg	=	Milligram
ml	=	Millilitre
mM	=	Millimolar
μM	=	Micromolar
MW	=	Molecular weight
°C	=	Degree celsius
O.D.	=	Optical density
PAGE	=	Polyacrylamide gel electrophoresis

List of Abbreviations (continue)

PMSF	=	Phenylmethyl sulfonylfluoride
rpm	=	Revolutions per minute
SAAPPNA	=	N-succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide
SDS	=	Sodiumdodecyl sulfate
SBTI	=	Soybean trypsin inhibitor
[S]	=	Substrate concentration
TAME	=	N-toluenesulfonyl-L-arginine methyl ester
TEMED	=	N,N,N′,N′,- tetramethyl ethylenediamine
TosArgOMe	=	*p*-tosyl-L-arginine methyl ester
TLCK	=	N-toluenesulfonyl-L-lysine chloromethyl ketone
TPCK	=	N-toluenesulfonyl-L-phenylalanine chloromethyl ketone
U	=	Units
V_{max}	=	Maximum velocity
w/v	=	Weight by volume
ZPCK	=	N-carbobenzoxy-L-phenylalanine chloromethyl ketone

Chapter 1

Introduction

Commercial enzymes widely used in various industries in Thailand are imported and very expensive. This results in high cost production and the limitation of using enzymes in industries. The most industrial enzymes imported are carbohydrases and proteases. About 60% of all industrial enzymes are protease (Haard, 1992). The sources of protease are plants, animals or microorganisms. Each source has its own specific characteristics.

Canned tuna in Thailand ranks first in the world since 1985. The quantity of canned tuna exported in 1998 and 1999 were 20,000 tons and 24,000 tons, respectively (Economic Agriculture Office, 1999). During tuna canning process, there were about 25-30% solid wastes such as fish head, viscera, bones, etc. and about 35% liquid wastes such as blood, tuna condensate (Prasertsan *et al.*, 1988). Fish viscera could be used as an enzymes source.

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 characterized thoroughly according to their physicochemical and enzymatic properties from the intestine of crayfish (Kim *et al.*, 1994), anchovy (Martinez *et al.*, 1988), poikilotherm *Gadus morhua* (Asgiersson *et al.*, 1989) and dogfish (Ramakrishna *et al.*, 1987). Chymotrypsin from the pancreas of carp

was similar to mammalian enzymes in both physical properties and kinetic properties (Heu *et al.*, 1995).

Prachumratana (1998) studied on the types and properties of enzymes from viscera of 3 species of tuna ; skipjack (*Katsuwonus pelamis*), yellowfin tuna (*Thunnus albacares*) and albacore (*Thunnus alalunga*). Enzymes extracted from viscera of these tunas showed the highest activities of protease and lipase with the values of 72.17 and 1.26 units/ml, respectively. Comparison among each viscera organ indicated that the spleen of yellowfin tuna gave the highest protease activity of 53.38 units/ml with the optimum temperature for its activity at 50 °C.

This work is the continuation of the previous study and will emphasize on the extraction, purification and characterization of trypsin and chymotrypsin from the viscera of yellowfin tuna (*Thunnus albacares*).

Literature review

1. Yellowfin tuna (*Thunnus albacares*)

Tunas are hard bone and epipelagic fish that can be classified to the family of Scrombroidae, the genus of Thunnidae (Saila and Norton, 1974). They are warm-blooded and keep their bodies at higher temperatures than the surrounding water. A higher body temperature allows increases in muscle power and may account for a tuna's ability to swim at speeds of over 50km/hr to catch smaller fish (King, 1995). Tropical tuna species include yellowfin, skipjack (*Katsuwonus pelamis*) and bigeye (*Thunnus obesus*) ; temperate species are albacore (*T. alalunga*) and bluefins (*T. thynnus* and *T. maccoyii*) (Saila and Norton, 1974). The tropical tunas tend to grow faster and live shorter than the temperate species with a total life span of 5 years or less (Joseph, 1973 cited by Saila and Norton, 1974). The size of yellowfin tuna could be up to 100 kilograms or more (Saila and Norton, 1974) with the average length of 50-150 centimeters. They are dark blue head, yellow-blue on abdomen surface and cover with dots (Figure 1) (Hmoejan, 1985).

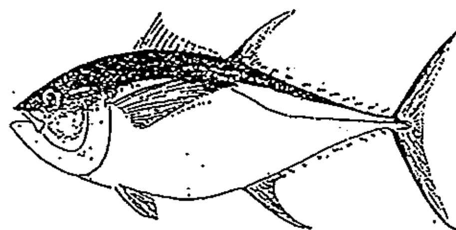


Figure 1 Yellowfin tuna (*Thunnus albacares*)
Source : Hmoejan (1985)

The International Commission for the Conservation of Atlantic Tunas (ICCAT) reports the movement of Atlantic tuna such as the movements of Atlantic bluefin between the eastern coast of the United States and Canada to the Bay of Biscay and Norwegian waters. The southern bluefin apparently migrates from spawning areas around Australia to the Atlantic, Pacific and Indian oceans. The yellowfin and bigeye tuna make somewhat less extensive migrations than the other major tuna species (Joseph, 1973 cited by Saila and Norton, 1974) that have a depth range from the surface to at least 150 meters. The fishing areas of yellowfin tuna were at the western of Pacific and Indean oceans as the mention in Saila and Norton (1974) that the yellowfin tuna were the first Indean and Pacific oceans tuna species exploited heavily and with higher concentrations in the western area and were not commercially exploited in the Atlantic ocean as shown the estimated distribution of yellowfin tuna in Figure 2. However, it is believed to be near its upper limit for rational exploitation at this time, so then the efforts of the Inter-American Tropical Tuna Commission (IATTC) have been directed primarily to yellowfin tuna conservation. Skipjack are not regarded as commonly occuring below 70 meters (Blackburn, 1965 cited by Saila and Norton, 1974).

There are about forty nations involved in tuna fishing include Japan, the United States of America, Taiwan, the Republic of Korea, France and Spain account for over 80% of the catch of the tuna market (Joseph, 1973 cited by Saila and Norton, 1974). The yellowfin tuna was the first tuna species exploited heavily that had large size and less movements. Over 90% of the recorded world consumption of tuna takes

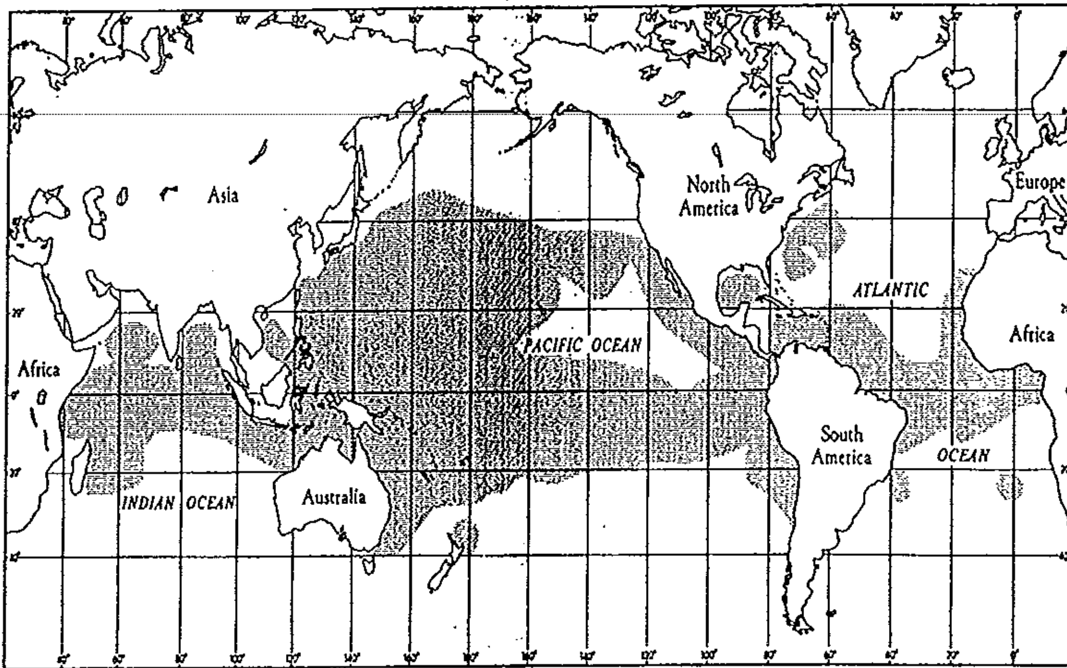


Figure 2 Estimated distribution of yellowfin tuna (shady area).

Source : Saila and Norton (1974)

place in the United States of America and Japan which catch the major share of the world tuna loadings (Saila and Norton, 1974).

2. Proteolytic enzyme

Proteases may be classified base on their similarities to well-characterized proteases, as trypsin-like, chymotrypsin-like, etc., their pH activity profiles as acid, neutral or alkaline proteases, substrate specificity and mechanism of catalysis (Haard and Simpson, 1994).

Protease based on mode of catalysis may also be classified into 4 groups as serine proteases, acid or aspartate proteases, thiol or cysteine protease and metalloproteases.

2.1. Serine proteases

The serine proteases comprise a large group of enzyme which is distinguished by the reaction of a serine residue in the active site (Walsh and Wilcox, 1970). Serine proteases can be subclassified into 4 groups as trypsin-like proteases, alkaline proteases, Myxobacter α-lytic proteases and Staphylococcal proteases. The last two groups were not be used in industries.

2.1.1. Trypsin-like proteases

Trypsin and chymotrypsin have specificity to digest substrates such as β-insulin at the position of Arg-Gly (22-23) and Lys-Ala (29-30) on the optimum pH at 8.0. They can be inhibited by diisopropyl fluorophosphate (DFP), soybean trypsin inhibitor and tosyl-L-lysine chloromethyl ketone (TLCK) (Ward, 1985). They also exhibit strong esterolytic activity toward esters analogous to the specific peptide substrate (Walsh and Wilcox, 1970). Trypsin and chymotrypsin from

fish are similar to those found in mammals (Heu *et al.*, 1995). They have been characterized thoroughly to their physicochemical and enzymatic properties from viscera of dogfish (Ramakrishna *et al.*, 1987), anchovy (*Engraulis japonica*) (Heu *et al.*, 1995), cod (*Gadus morhua*) (Asgiersson *et al.*, 1989), herring (*Clupea harengus*) and squid (*Illex illecebrosus*) (Simpson and Haard, 1984), salmon (*Salmo salar*) (Outzen *et al.*, 1996), fiddler crab (Eisen *et al.*, 1973). Shrimp (*Penaeus indicus*) (Doke and Ninjoor, 1987), etc.

The proteolytic enzyme trypsin and its inactive precursor, trypsinogen which 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).

Chymotrypsin is an extracellular enzyme and produced in chymotrypsinogen, inactive proenzyme, from pancreas. Proenzyme is transferred to small intestine and activated by that cleaved Arg_{15}-Ile_{16} to give chymotrypsin (Walsh and Wilcox, 1970). The activation process is shown in Figure 3 (Dixon and Webb, 1979). Activation of both trypsinogen and chymotrypsinogen appeared to be enhanced following freezing and thawing at -20°C. Possibly freeze-thaw action may disrupt cells/granules resulting in release of zymogen form which is then activated (Mullally *et al.*,1995).

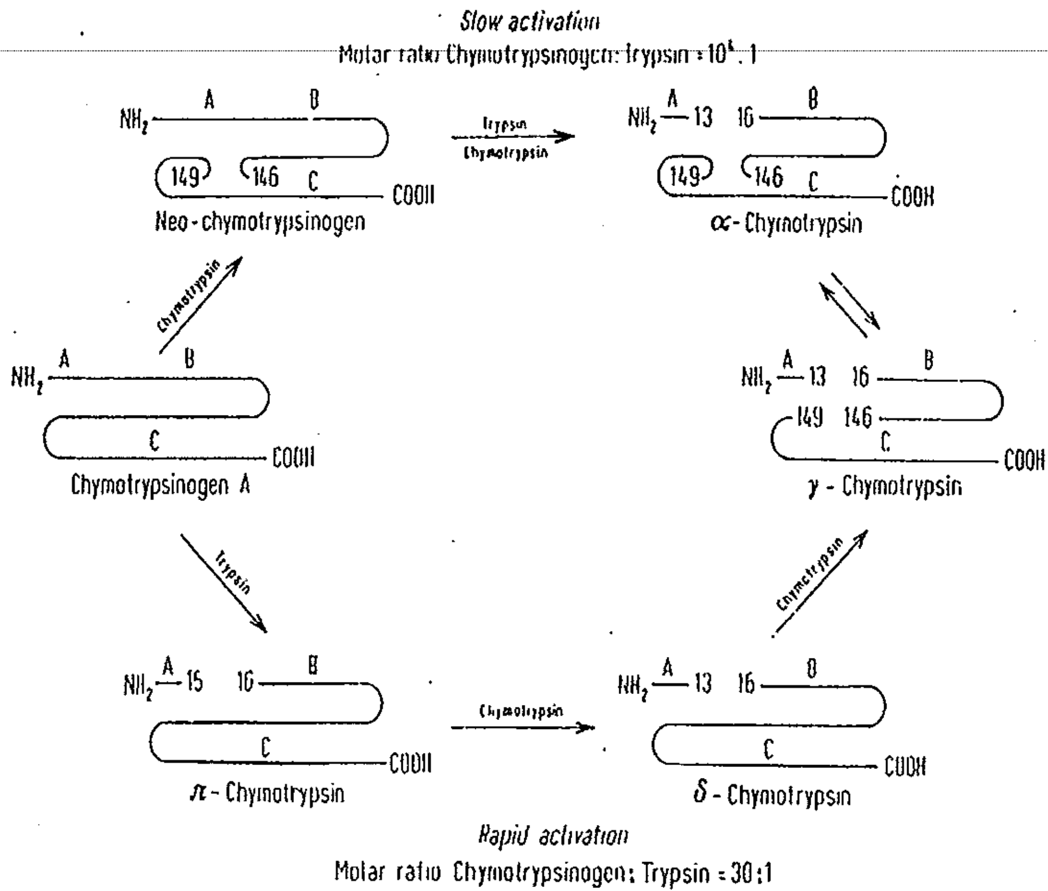


Figure 3 Formation of various forms of chymotrypsin on Activation of chymotrypsin A by trypsin.

Source : Dixon and Webb (1979)

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 ; recover protein from bones ; hydrolysis of blood proteins in meats commodity (Haard, 1992), etc. Chymotrypsin can be extracted from pancreas and stomach of animals which is useful for food industry (Heu *et al.*, 1995) such as fish extract production, baked goods , egg and egg products and cheese curd formation in cheese products (Haard, 1992).

2.1.2. Alkaline proteases

The most useful enzyme is subtilisin produced from bacteria, yeasts and fungi and is an ingredient of powdered detergent and chymotrypsin from *Aspergillus oryzae*. These enzymes have high activity at pH 10.

2.2. Acid proteases

Acid or carboxyl proteases have high activity in acid condition at pH range of 3-4 and have aspartic acid residue at active site, such as pepsin from Atlantic halibut (*Hippoglossus hippoglossus L.*) (Glass *et al.*, 1987), bluefin tuna (*Thunnus thynnus orientalis*) (Tanji *et al.*, 1988), Arctic capelin (*Mallotus villosus*) (Gildberg and Raa, 1983), rainbow trout (Twining *et al.*, 1983), cod (Arunchalam and Haard, 1985), cathepsins D enzyme from sea urchin egg (*Tetrapygus niger*) (Sanchez-Chiang *et al.*, 1986), etc.

2.3. Thiol proteases

There are cysteine residue composition at active site which can be stimulated by reducing compounds such as cysteine and hydrogen cyanide and inhibited by oxidized compounds. The optimum condition

of enzyme activity is pH 7. Enzymes of this group are cathepsin B from viscera of surf clam (Chen and Zall, 1985), papain which has cysteine residue position at 25 of peptide bonds (Haard and Simpson, 1994) and can digest esters and amide materials (Godfrey and Reichelt, 1983), ficin and bromelain (Haard and Simpson, 1994) .

2.4. Metalloproteases

These proteases are most active within the pH range of 6.5-8.0 and are inactivated at pH values lower than 6.0 (Haard and Simpson, 1994). It is necessary to have metal ions to stimulate reaction such as collagenases and calpains which are stimulated by calcium ions extracted from crab, lobster, cod, herring and salmon (Gill, 1992 cited by Haard and Simpson, 1994).

3. Properties of trypsin and chymotrypsin

3.1. Molecular weight

Trypsin from the pyloric caeca of Atlantic cod (*Gadus morhua*) which resolved into three differently charged species by chromatofocusing (pI 6.6, 6.2 and 5.5) that had similar molecular mass of 24.2 kDa (Asgeirsson *et al.*, 1989). They were similar to trypsin from insect (*Trypanosoma brucei*) of 23 - 24 kDa (Hua *et al.*, 1996). Molecular weights of trypsin and chymotrypsin from anchovy were 25.6 and 26.1 kDa, respectively (Heu *et al.*, 1995). Chymotrypsin from pyloric caeca of Atlantic cod (*Gadus morhua*) were purified using a phenyl-sepharose column and chromatofocusing chromatography which gave the apparent molecular weight of 26 kDa (Asgeirsson and Bjarnason, 1991).

Two trypsin (A and B) extracted from the digestive tract of anchovy (*Engraulis encrasicholus*) had the molecular weights of 27 and 28 kDa, respectively (Martinez *et al.*, 1988). Shin and Zall (1986) purified and identified trypsin like enzyme from the pyloric caeca of cod (*Gadus morhua*) with the molecular weight of about 24 kDa. Molecular weights of trypsin and chymotrypsin in marine fish are presented in Table 1 which depend on their protein compositions.

3.2. Amino acid composition

Amino acid composition of trypsin and chymotrypsin can be used to compute the molecular weight of enzyme (Martinez *et al.*, 1988). The amino acid composition of purified trypsin and chymotrypsin from the viscera of anchovy (*Engraulis japonica*), compared to anchovy trypsins (*Engraulis encrasicholus*) (Martinez *et al.*, 1988), bovine trypsin, and bovine chymotrypsin are presented in Table 2. Similar amino acid compositions were clearly observed among anchovy trypsin and trypsins from *Engraulis encrasicholus*. The amino acid profile of anchovy chymotrypsin is similar to that of bovine chymotrypsin (Heu *et al.*, 1995).

Asgiersson *et al.* (1989) showed the N-terminal amino acid sequence of the most prominent trypsin species from Atlantic cod (enzyme I) (Table 3). Its sequence displayed considerable similarly with porcine and rat trypsin (30 identical residues out of 37), bovine trypsin (29 identical residues out of 37) and dogfish trypsin (26 identical residues out of 37), but less so with the crayfish enzyme (6 identical

Table 1 Molecular weights of trypsin and chymotrypsin from some marine fish

Animals	Molecular weight (kDa)		References
	Trypsin	Chymotrypsin	
Atlantic cod (*Gadus morhua*)	24.2	-	Asgeirsson *et al.* (1989)
Atlantic cod	-	26	Asgeirsson and Bjarnason (1991)
anchovy (*Engraulis japonica*)	25.6	26.1	Heu *et al.* (1995)
anchovy (*Engraulis encrasicholus*)	27[a] 28[b]	-	Martinez *et al.* (1988)
Atlantic salmon (*Salmo salar*)	25	-	Outzen *et al.* (1996)
cod	24	-	Shin and Zall (1986)
cod	-	28	Raae (1990)
cunner (*Tautogolabrus adspersus*)	24	-	Simpson and Haard (1985a)

[a] trypsin A

[b] trypsin B

Table 2 Amino acid composition of anchovy trypsin and chymotrypsin compared with anchovy trypsin A and B and bovine trypsin and chymotrypsin

	Anchovy[1]		Anchovy[2] trypsin		Bovine[3]	
	Tryp.	Chymo.	A	B	Tryp.	Chymo.
Asp	32	25	28	29	21	20
Thr	14	21	10	10	11	24
Ser	30	21	30	29	23	19
Glu	24	21	27	28	16	14
Pro	11	15	11	12	13	11
Gly	28	17	33	34	25	23
Ala	11	12	16	17	14	17
Val	16	19	18	18	11	18
Met	3	2	5	6	1	1
Ile	10	10	10	11	12	10
Leu	15	14	16	18	13	15
Tyr	8	3	6	8	7	4
Phe	5	4	5	4	6	5
His	9	10	6	6	4	4
Lys	6	6	5	6	10	14
Arg	6	9	10	10	7	7
Trp	2	6	-	-	3	4
Cys	5	9	10	10	12	11
No of residues	235	234			209	221
Corrected M.W.	25399	25830				
Average hydrophobicity (Kcal/residue)	0.86		0.97			

[1]Values are the nearest integer expressed as amino acid residues/mole based on molecular weight as determined by SDS-PAGE.

[2]Data obtained from trypsins of anchovy (Martinez *et al.*, 1988).

[3]Bovine pancreas α-chymotrypsin (M.W.25,000) and trypsin (M.W.24,000) obtained from Sigma Co. were analyzed with the same condition as anchovy enzymes for amino acid composition comparison.

Source : Heu *et al.* (1995)

Table 3 The N-terminal amino acid sequence of Atlantic cod trypsin (enzyme I) compared to trypsin from other species

Trypsin	Residue at position (1 5 10 15 20 25 30 35 40)	Reference
Atlantic cod	IVGGYQCEAHSQAHQVSLNSGYHYCGGSLIN - -WVVSAA	-
Bovine	IVGGYTCGANTVPY QVSLNSGYHPCGGSLINSQWVVSAA	[1]
Porcine	IVGGYTCAANSVPY QVSLNSGYHFCGGSLINSQWVVSAA	[2]
Rat	IVGGYTCPE HSVPY QVSLNSGYHFCGGSLINDQWVVSAA	[3]
Dogfish	IVGGYECPKHAAPWT VSLNSGYHFCGGSLIAPGWVVSAA	[4]
Crayfish	IVGGYTDVLGEFPY Q LSFQEH FLGFSFHFCGAS I YNENY	[5]

[1] : Walsh and Wilcox (1970) [2] : Hermodsson *et al.* (1973)
[3] : Craik *et al.* (1984) [4] : Titani *et al.* (1970)
[5] : Titani *et al.* (1983)

Source : Asgiersson *et al.* (1989)

residues out of 37). The N-terminal sequence clearly demonstrated the cod trypsin to be a member of the trypsin family of enzymes.

The N-terminal sequence of Atlantic cod (*Gadus morhua*) chymotrypsin was compared with bovine chymotrypsin (Table 4). The results showed the striking resemblance between the N-terminal amino acid sequence of Atlantic cod chymotrypsin and bovine chymotrypsin. Only five substitutions were observed in the first 31 amino acids.

These dedicated that amino acid compositions and partial amino acid sequences of various enzymes revealed the high degree of homology among the structures so far examined. The similarity of amino acid compositions provides a basis for the prediction that most of enzymes from diverse species would possess homologous structures.

Thus, it is probable that they have arisen a common ancestor by a process of divergent molecular evolution (Walsh and Wilcox, 1970).

Table 4 N-terminal sequence of Atlantic cod chymotrypsin compared with bovine α-chymotrypsin where residues 14 and 15 have been removed as the result of autolysis

	Residue at position	
	1 5 10	15 20 25 30
Cod	CysGlySerProAlaIleGlnProValIleSerGly	IleValAsnGlyGluGluAlaValProHisThrTrpTyrTrpGlnVal
Bovine	CysGlyValProAlaIleGlnProValLeuSerGlyLeu	IleValAsnGlyGluGluAlaValProGlySerTrpTyrProGlnVal

Source : Asgiersson and Bjarnason (1991)

3.3. Substrate specificity

The primary sites of cleavage of an oxidized insulin β-chain by trypsin and chymotrypsin determined under identical condition were different and presented in Figure 4. Based on this result, the primary specificity of anchovy trypsin appeared to be similar to that of bovine trypsin, preferring bonds that the carbonyl group was donated by arginine and lysine residues. With extended incubation (6 hr), secondary cleavage at glycine residue was also noted. The preferred specificity of anchovy chymotrypsins appeared to be different with that of bovine chymotrypsin, favored cleavage sites are phenylalanine, tyrosine and leucine residues and after extended incubation (6 hr), secondary cleavage at valine residue was also detected (Heu *et al.*, 1995).

Trypsin

Phe - Val - Asn - Gln - His^{5} - Leu - Cys - Gly - Ser - His^{10} - Leu - Val - Glu -

Ala - Leu^{15} - Tyr - Leu - Val - Cys - Gly^{20} - Glu - Arg ↓ - Gly - Phe ↓ - Phe^{25} -

Tyr - Thr - Pro - Lys ↓ - Ala^{30}

Chymotrypsin

Phe ↓ - Val - Asn - Gln - His^{5} - Leu - Cys - Gly - Ser - His^{10} - Leu - Val - Glu -

Ala - Leu^{15} - Tyr - Leu ↓ - Val ↓ - Cys - Gly^{20} - Glu - Arg - Gly - Phe ↓ - Phe^{25} -

Tyr ↓ - Thr - Pro - Lys - Ala^{30}

Figure 4 Cleavage sites of oxidized β-chain of bovine insulin by anchovy trypsin and chymotrypsin. Reaction was carried out at 37 °C for 30 minutes or for 6 hr with 3 μmol of oxidized insulin β-chain at an enzyme/substrate ratio of 1:200 (mol/mol)

Source : Heu *et al.* (1995)

The effect of two substrates as N-α-benzoyl arginine-*p*-nitroanilide (BAPNA), which is amide substrate, and N-toluenesulfonyl-L-arginine methyl ester (TAME), which is ester substrate, on the activity of trypsin from the digestive tract of anchovy (*Engraulis encrasicholus*) was studied. Trypsin A and B from anchovy had a higher affinity and catalytic activity with ester substrates (TAME) than with amide substrates (BAPNA) giving specific activities of 131.3 and 4.7 U/mg respectively (Martinez *et al.*, 1988).

3.4. Kinetic parameters

Enzyme binds its substrate and formed enzyme-substrate complex which can be cleaved to enzyme and product reversed to enzyme and substrate. Constant values which analyzes the ability of enzyme stimulation of reaction to convert substrate to product were Michaelis-Menten constant (K'_m) was determined by least-squares regression and substrate turnover number (k_{cat}) (Heu *et al.*, 1995). K'_m and k_{cat} values of proteolytic enzymes from some marine fish are presented in Table 5. Kinetic parameters of trypsin were computed when TAME and BAPNA were used as substrates while used BTEE and SAAPPNA for chymotrypsin at the concentrations of 0.02-2.0 mM at pH 8.0 and 30 °C (Heu *et al.*, 1995). Efficiency of enzyme activation is given in the ratio of k_{cat} / K'_m (Martinez *et al.*, 1988).

Heu *et al.* (1995) reported that the k_{cat} for TAME hydrolysis by trypsin was 26 times higher than that for BAPNA hydrolysis and the k_{cat} for BTEE hydrolysis by chymotrypsin was 6.5 times higher than that for SAAPPNA hydrolysis. The k_{cat} / K'_m of trypsin and chymotrypsin was

Table 5 Kinetic parameters of some proteolytic enzymes from marine fish

Marine animals	Enzymes	K′m (mM)	kcat (sec-1)	References
Atlantic cod	Elastase I (8 °C)	1.61	11.4	Gildberg and Overbo (1990)
	Elastase II (25 °C)	1.96	38.5	
	Elastase III (37 °C)	1.56	47.6	
Atlantic cod	Trypsin[a] I	0.029	136.7	Asgeirsson *et al.* (1989)
	Trypsin[a] II	0.021	57.6	
	Trypsin[a] III	0.049	62.8	
	Trypsin[b] I	0.077	4.0	
	Trypsin[b] II	0.094	1.9	
	Trypsin[b] III	0.102	0.7	
	Chymotrypsin[c] A	0.17	329	Asgeirsson and Bjarnason (1991)
	Chymotrypsin[c] B	0.10	276	
	Chymotrypsin[d] A	0.14	0.82	
	Chymotrypsin[d] B	0.11	0.72	
anchovy	Trypsin[e]	0.840	39.67	Heu *et al.* (1995)
	Chymotrypsin[f]	0.397	94.34	
anchovy	Trypsin A	0.83	1.55	Martinez *et al.* (1988)
	Trypsin B	0.66	3.20	
polar cod	Pepsinogen A	0.06	-	Arunchalam and Haard (1985)
	Pepsinogen B	1.33	-	

[a] used TosArgOMe as substrate

[b] used BzArg-NH-Np as substrate

[c] used BzTyrOEt as substrate at 35 °C

[d] used BzTyr-NH-Np as substrate at 35 °C

[e] used TAME as substrate

[f] used BTEE as substrate

higher in esterase reaction (2.84 and 14.3 min^{-1} μM^{-1}, respectively) than amidase reaction (1.84 and 9.97 min^{-1} μM^{-1}, respectively).

The amidase activity of the anionic trypsin from salmon (*Salmo salar*) showed an apparent K'_m value approximately 5-25 times lower than the cationic salmon trypsin and the mammalian trypsin. The maximum turnover, k_{cat}, of anionic salmon and porcine trypsins were about 2-fold higher than that of cationic salmon and bovine trypsins. The catalytic efficiency (k_{cat} / K'_m) of anionic trypsin was about 20-fold higher than bovine trypsin at 37°C and 35-fold higher at 4°C (Outzen *et al.*, 1996).

Comparison of the effect of temperature on kinetic parameters using two synthetic substrates, BzTyrOEt and BzTyr-NH-Np by chymotrypsin (A and B) from Atlantic cod (*Gadus morhua*) and bovine chymotrypsin was conducted. When used 0.5 M BzTyrOEt as ester substrate, the K'_m values of cod chymotrypsin A at 10, 25 and 35°C were similar (0.12, 0.14 and 0.17 mM, respectively) and 0.20, 0.20 and 0.10 mM respectively for cod chymotrypsin B and 0.27, 0.27 and 0.24 mM respectively for bovine chymotrypsin. Using 0.5 M BzTyr-NH-Np as amide substrate, the K'_m values at 10, 25 and 35°C were 0.09, 0.08 and 0.14 mM for cod chymotrypsin A and 0.11, 0.08 and 0.11 mM respectively for cod chymotrypsin B and bovine chymotrypsin gave the K'_m values for 0.11, 0.14 and 0.21 mM respectively. These showed while using BzTyr-NH-Np as substrate gave lower K'_m values than using BzTyrOEt as substrates therefore BzTyr-NH-Np was also employed (Asgiersson and Bjarnason, 1991).

CENTRAL LIBRARY
PRINCE OF SONGKLA UNIVERSITY

Asgiersson *et al.* (1989) studied kinetic parameters of trypsin from the poikilotherm *Gadus morhua* using BzArg-NH-Np and TosArgOMe as substrates gave the K'_m values of 77 and 29 μM respectively and the k_{cat} / K'_m was 7 fold of bovine trypsin.

3.5. Denaturation constants

Denaturation constant (K_D) is calculated according to the method of Hashimoto and Arai (1985 cited by Heu *et al.*, 1995) which is constant values of enzymes when conduct pH and temperature stability. Activation energy (E_a) is calculated from the slope of the line obtained by Arrhenius plot using log specific activity against 1/T (slope = - E_a / 2.303R). The fit of the data to the Arrhenius equation was evaluated by least-squares regression analysis.

Denaturation constants of the enzymes determined at 30 to 55°C revealed that K_Ds up to 45°C of trypsin and chymotrypsin, measured by BAPNA and BTEE respectively, changed insignificantly. However, those over 50°C were measured at higher scores, which indicated that both enzymes were denatured very rapidly over 50°C. The activation energy for hydrolysis of casein by trypsin and chymotrypsin were 16.4 and 5.5 kcal/mole, respectively, and those for hydrolysis of BAPNA by trypsin and BTEE by chymotrypsin were 13.9 and 6.5 kcal/mole, respectively (Heu *et al.*, 1995).

3.6. Inhibition constants

Inhibition constants (K_i) is determined with a Dixon plot on which condition is a various concentration of inhibitors such as N-toluenesulfonyl-L-lysine chloromethyl ketone (TLCK) and N-carbobenzoxy-L-phenylalanine chloromethyl ketone (ZPCK) at 0.2 mM

of substrates (BAPNA and SAAPPNA). K_i values for trypsin was 21 μM of TLCK and 6.6 μM of ZPCK for chymotrypsin (Heu *et al.*, 1995).

The K_i values can be calculated in percentage of relative activity. Jany (1976) studied on the effect of inhibitors indicated that PMSF, TLCK and SBTI totally inhibited trypsin extracted from the stomachless bonefish *Caressius auratus gibellio*, chymotrypsin was inactivated by PMSF and SBTI at 98% and 96% by TPCK.

4. Factors affecting on property and activity of the enzyme

4.1. Temperature

Temperature affects on the general chemical reactions at low temperature (3 - 15°C). Enzymes from cold-adapted organisms are characterized by having a higher turnover number at low temperatures, lower K_m values, and slightly lower free energies of activation when compared to homologous enzymes from warm-blooded organisms (Raae, 1990). Moreover, enzymes from cold-adapted organisms possess a loosely fit tertiary structure and thus enable the enzyme to lower the activation energy when binding to the substrate (Outzen *et al.*, 1996).

Extraction of enzymes should be done at low temperature (0 or 4 °C) to preserve the enzyme. Trypsin extracted from pancreatic tissue of cunner (*Tautogolabrus adspersus*) was frozen in liquid nitrogen and powdered in Waring blender. The powder was stirred with 0.05 M Tris-HCl buffer (pH 7.8) containing 0.5 M NaCl and 0.02 M $CaCl_2$ at 4°C for 3 h, then centrifuged at 3,000×g for 30 minutes at 4°C (Simpson and Haard, 1985a). All centrifugation steps were performed at 4°C (Gelder *et al.*, 1996). The reaction is very temperature-dependent ; the enzyme

activity being inhibited if storage temperature is near -29°C (Magnusson and Martinsdottir, 1995). Most enzymes prefer the lowest temperature allowable : 0°C (on ice, not in a refrigerator) or -20°C with glycerol or sucrose present. If frozen storage above liquid nitrogen or at -70°C is often best (Deutscher, 1990). The storage life of quality frozen fish further depends on its fat content. High quality, low-fat fish stored in the frozen state at -21 to -23°C may retain its quality for as long as 2 years (Potter and Hotchkiss, 1995).

Heu *et al.* (1995) reported that the optimum temperature for trypsin and chymotrypsin activity from anchovy was 45 °C and both enzymes were almost completely inactivated at 60 °C.

Raae (1990) studied the effect of low (3 - 20°C) and high temperatures (25 - 99°C) on chymotrypsin activity from Atlantic cod (*Gadus morhua* L.). Results revealed that at low temperature, activity of enzyme gradually increased to 80 U/mg at 25°C and no enzyme inactivation was detected at temperatures below 50°C and was reduced to 30% relative activity at 85°C. Enzyme was inactivated when incubated at 68°C and rapid inactivation of the native enzymes was observed at 70°C. In addition, because of the great diversity of structures among proteins it follows that enzymes will exhibit a wide range of stabilities to perturbations in heat and pH. Therefore, some proteins such as adenylate kinase and trypsin are relatively heat stable in comparison with the majority of other enzymes (Deutscher, 1990).

Optimum temperature for enzyme activities from some marine fish are presented on Table 6.

Table 6 Optimum temperature and pH on trypsin and chymotrypsin activities from marine fish

Marine animals	Optimum temperature (°C)		Optimum pH		References
	Trypsin	Chymotrypsin	Trypsin	Chymotrypsin	
Atlantic cod	55	-	8	-	Asgeirsson *et al.* (1989)
	-	40	-	7.8	Asgeirsson and Bjarnason (1991)
	-	50	-	7.8	Raae (1990)
Atlantic salmon	40	-	8.5-10.5[a] 10.5[b]	- -	Outzen *et al.* (1996)
anchovy	45	45	8	8	Heu *et al.* (1995)
anchovy	50-55	-	8-9[c] 9.5[d]	- -	Martinez *et al.* (1988)
cod	46-48	-	9.0-9.6	-	Shin and Zall (1986)
cunner	45	-	8.5	-	Simpson and Haard (1985a)

[a] cationic salmon trypsin

[b] anionic salmon trypsin

[c] used BAPNA as substrate

[d] used casein and myofibrillar protein as substrate

4.2. pH

The optimum conditions of enzymes are different based on their characteristics. Trypsin and chymotrypsin give high activities values in neutral-alkaline of pH range (see Table 6). Trypsin and chymotrypsin from fish tend to be stable in alkaline medium and very unstable in acidic conditions. Trypsin and chymotrypsin from mammals, however, are most stable in acidic medium (Haard, 1992).

The optimum pH for activity of trypsin from anchovy was pH 8 - 9 using BAPNA as substrate and was pH 9.5 using casein as substrate (Martinez *et al.*, 1988).

The optimum pH and temperature of cod trypsin activity with molecular weight of about 24 kDa, were pH 9.0 - 9.6 and 46 - 48 °C respectively (Shin and Zall, 1986).

Ramakrishna *et al.* (1987) isolated chymotrypsin from dogfish at optimum pH of 7.8 which Raae (1990) extracted chymotrypsin from cod at the same pH using BTEE as substrate.

Heu *et al.* (1995) purified extracted trypsin and chymotrypsin from anchovy and determined the optimum pH (9.0) from the pH range of 4.0 - 12.0 using casein as substrate and 8.0 when using synthetic substrates including BAPNA, BTEE, ATEE and TAME.

Trypsin from animals can be separated into 2 groups, anionic proteins which found in marine species and cationic proteins found in mammalian. The stability of trypsin at a particular pH may be related to the net charge of the enzyme at that pH. All trypsins isolated from marine organisms, except lungfish trypsin are highly unstable at acidic pH values. Generally the cationic mammalian trypsins are more stable at acid pH values (Martinez *et al.*, 1988).

The property of anionic and cationic of enzymes can be separated and purified by ion chromatography (Walsh and Wilcox, 1970). The exchange groups used include DEAE, a weak base, that will have a net positive charge when ionized and therefore bind and exchange anions ; and CM, a weak acid, with a negative charge when ionized that bind and exchange cations (Deutscher, 1990).

4.3. Substrates

Trypsin and chymotrypsin hydrolyse esters and amides of aromatic amino acids as well as proteins and peptides (Rick, 1974) as presented in Table 7 (Walsh and Wilcox, 1970). The difference of substrates has influence in different enzyme activities caused by their specificity to substrates such as using BAPNA to analyze trypsin activity and BTEE or SAAPPNA to analyze chymotrypsin activity (Heu *et al.*, 1995 ; Glass *et al.*, 1987). Anchovy trypsin had activities toward ester and amide substrates containing arginine residue, but displayed no activity against chymotrypsin substrates, which indicated that anchovy trypsin was not contaminated with chymotrypsin-like enzyme (Heu *et al.*, 1995). Trypsin does not hydrolyse BTEE which used for chymotrypsin (Rick, 1974).

4.4. Salt

Salts affect on solubility and precipitation especially NaCl and divalent ion include Na_2SO_4 and $(NH_4)_2SO_4$. Salts in solution at low ionic strength relative to that of isotonic saline may present a

Table 7 Kinetic constants for some synthetic substrates of bovine trypsin

Substrates	K'_m (mM)	k_{cat} (sec^{-1})	pH	Temp (°C)
Amides				
Benzoyl-L-arginine-*p*-nitroanilide	0.939	0.611	8.15	15
L-lysyl-*p*-nitroanilide	0.364	0.003	8.65	15
Benzoyl-L-argininamide	3.1	1.35	8.35	35
N-α-benzyloxycarbonyl-L-arginine-*p*-toluidide	1.4	0.69	8.0	25
Esters				
Benzoyl-L-arginine ethyl ester	0.0043	14.6	8.0	25
Tosyl-L-arginine methyl ester	0.0125	100.0	8.0	25
α-N-acetyl-*S*-(*β*-aminoethyl) cysteine ethyl ester	1	18.7	8.0	25
α-N-tosyl-*S*-(*β*-aminoethyl) cysteine ethyl ester	0.369	55.6	8.0	25
α-N-acetyl lysine ethyl ester	0.280	80.0	8.0	25
Ethyl-*p*-guanidinophenylacetate	0.0115	0.047	8.0	25
Neutral substrate				
N-acetyl-L-tyrosine ethyl ester	42.0	14.5	8.0	25
N-acetyl-glycine ethyl ester	800.0	0.012	7.0	25
N-α-benzoyl-L-citrulline methyl ester	41.0	0.14	7.0	25
N-benzoyl-L-heptyline methyl ester	0.1	9.4	7.0	25

Source : Walsh and Wilcox (1970)

perturbation that can cause certain proteins to precipitate from solution. Such a protein probably was originally maintained in solution in the tissue because it was "salting in" at the ionic strength of the medium (Deutscher, 1990) with ammonium sulphate, on account of its high solubility in water and absence of harmful effects on most enzymes (Dixon and Webb, 1979). On the other hand, salts present in various high concentrations with ionic strength much greater than that of tissue media will cause the precipitation of many proteins. Precipitation occurs by neutralization of surface charges by the salt, by reducing the chemical activity of the protein, and by diminishing the effective concentration of the water which called "salting out" of proteins (Deutscher, 1990).

Outzen *et al.* (1996) precipitated trypsin extracted from salmon with 30% saturated ammonium sulfate and added various NaCl concentrations (80 - 250 mM) into 20 mM diethanolamide buffer, pH 9.5 while used a Source 15Q column as ion exchanger for better elution.

Trypsin and chymotrypsin from viscera of anchovy (*Engraulis japonica*) was precipitated with the saturation of 30-70% saturated ammonium sulfate (Heu *et al.*, 1995).

4.5. Pressure

The effect of hydrostatic pressure on enzyme reactions can provide important on enzyme-catalysed reactions. The high pressure (of the order of 10,000 to 100,000 pounds per square inch) is a risk that these conditions may cause changes in either the tertiary or the quaternary structure of the enzyme under investigation (Dixon and Webb, 1979).

Pressure has the influence on enzyme recovery by vacuum evaporation systems and ultrafiltration. The 60 - 90% range of recovery yield of the enzyme was apparent using the vacuum evaporation systems and was 90 - 100% in the ultrafiltration systems. There are increasing interest in the purification and concentration of various enzymes by ultrafiltration system since the energy requirements per volume of water removed are substantially lower than other systems including evaporation (Chen and Zall, 1985).

4.6. Chelating agent and metal ions

The contamination of metal ions such as iron, zinc, lead and mercury can inhibited or activated the activity of enzymes including calcium ions has been found to stabilize the structure of cod trypsin and to retard autolysis (Asgiersson *et al.*, 1989). On the other hand, the cod chymotrypsin was reduced by calcium ion (Asgiersson and Bjarnason, 1991). The metal ions were inhibited by adding EDTA and EGTA which were efficient chelators for divalent metal cations (Deutscher, 1990). The 50 mM cysteine which was effective as a SH-group blocking agent or as a chelating agent can inhibited cod chymotrypsin and bovine chymotrypsin with the residual activity of 33 and 75%, respectively (Asgiersson and Bjarnason, 1991).

Shin and Zall (1986) reported that Ca^{++} had no effect on trypsin from cod, for metal ions that reduced enzyme activity were Ag^{+}, Cd^{++}, Co^{++}, Cu^{++}, Ni^{++}, Pb^{++} and Zn^{++} in the values of % residual activity of 5.1, 47.9, 43.6, 57.3, 82.1, 69.2 and 75.2 %, respectively. Rick (1974) mentioned that the stability of α-chymotrypsin can be increased by calcium ions and can prevent the aggregation of the trypsin molecules.

4.7. Inhibitors

The specific enzyme inhibitors are frequently useful as tools : for instance, many of the intermediates in glycolysis and yeast fermentation were discovered largely by the use of inhibitors blocking the various successive steps and allowed the corresponding intermediates to accumulate in sufficient quantities for isolation and identification. The chemical inhibitors were classified into 2 groups including reversible and irreversible inhibitors (Dixon and Webb, 1979).

Choice of an enzyme for food industry is based equally on specificity and heat stability include the resistance of native proteins to proteolysis. A protein may be completely resistant to one enzyme and attacked by another (Kilara, 1985) such as leupeptin inhibited chymotrypsin activity of 89% but had no effect on trypsin activity when using as substrate (Heu *et al.*, 1995). Some inhibitors of trypsin are presented in Table 8.

5. Enzyme applications

Proteases play a vital role in biotechnology, food processing and other industries, as well as in a variety of physiological processes (Table 9) (Haard and Simpson, 1994). In food industry, proteases are used in processing aids for many products including baked goods, beer and wine, cereals, milk and dairy products like cheese, chocolate, eggs and egg products, meat and fish products, legumes, and for production of protein hydrolysate and flavor extracts (Table 10) (Haard, 1992).

Table 8 Some inhibitors of bovine trypsin

	K_i (M)	pH	Temp. (°C)
I. Competitive inhibitors			
p- Aminobenzamidine	8.25×10^{-6}	8.15	15.0
Benzamidine	1.66×10^{-5}	8.15	15.0
β-Naphthamidine	1.46×10^{-5}	8.15	15.0
m-Toluamidine	2.27×10^{-5}	8.15	15.0
p-Toluamidine	2.65×10^{-5}	8.15	15.0
Cyclohexylcarboxamidine	4.27×10^{-4}	8.15	15.0
Phenylguanidine	7.25×10^{-5}	8.15	15.0
α-*N*-Benzoyl-L-arginine	5.8×10^{-3}	8.0	25
1-Propyl-guanidine	5.3×10^{-4}	8.0	25
n-Butylamine	1.7×10^{-3}	6.6	25
Benzylamine	6.0×10^{-4}	6.6	25
Thionine	2.5×10^{-5}	7.6	24
Proflavin	4.0×10^{-5}	7.3	25
II. Active titrants			
Diisopropylphosphofluoridate (DEP)			
1-Chloro-3-tosylamido-7-amino heptanone (TLCK)			
p-Nitrophenyl-*p*′-guanidinobenzoate			
Ethyl-*p*-guanidinobenzoate			
α-*N*-Methyl-*α*-*N*-toluene-*p*-sulfonyl-L-lysine-*β*-naphthyl ester			
p-Nitrophenyl *N*-acetyl-*N*-benzylcarbazate			
p-Nitrophenyl *α*-*N*-benzyloxycarbonyl-L-lysinate			
p-Nitrophenyl ethyl diazomalonate			

Source : Walsh and Wilcox (1970)

Table 9 Some processes facilitated by proteolytic enzymes

Industry / field	Commodity and /or applications
Food	cereals, baked goods, egg and egg products, meats, fish, pulses, dairy, brewing, wine
Biotechnology	leather : bating and dehairing textiles : detergents
Clinical	reduction of tissue inflammation ; dissolution of blood clots ; promotion of wound healing ; digestive aid
Diagnostics	diagnosis of candidosis
Physiological process	cellular processed, e.g.digestion, translocation, activation of enzymes or hormones ; activation of protoxins ; protein turnover

Source : Haard and Simpson (1994)

Table 10 Some uses of proteolytic enzymes in the food industry

Commodity	Applications
Cereals, baked goods	Increase drying rate of proteins ; improve product handling Characteristics ; decrease dough mixing time ; improve texture and loaf volume of bread ; and decrease dough mixing time
Egg and egg products	Improves quality of dried products
Meats	Tenderization ; recover protein from bones ; hydrolysis of blood proteins
Fish	Fish protein hydrolysates, vicosity reduction, skin removal, roe processing
Pulses	Tofu ; soy sauce ; protein hydrolysis ; off-flavor removal ; soy milk
Dairy	Cheese curd formation ; accelerate cheese aging ; rennet puddings
Brewing	Fermentation and filtration aid ; chill proofing
Wine	Clarification ; decrease foaming, promote malolactic fermentation
Cocoa	Facilitate fermentation for chocolate production

Source : Haard (1992)

5.1. Fish sauce and fish silage

During the preparation of fish sauce or fish silage a protein hydrolysate was formed by the action of endogenous enzymes. The production of fish sauce was very simple. Small fish, like sardines, or minced fish material was mixed with 20 – 40 % sea salt and stored at ambient tropical temperature for 6 - 12 months. The amber protein hydrolysate liquid was tapped, filtered and bottled. Protein hydrolysis was caused mainly by trypsin-like enzymes. The high salt concentration in fish sauce partly inhibited the activity of trypsin-like enzymes and the rate of autolysis was low. If the storage temperature was raised to about 45°C for a couple of weeks in the initial storage phase, the total production time may be reduced from 1 year to 2 months (Gildberg, 1993).

The fish sauce from mixed minced fish (long tom, mackerel, sole, anchovy, beam and sardine) gave a maximum protein conversion of 21.76 % was achieved in 35 days using bromelain from pineapple juice incubating at room temperature. However, without additional enzyme supplementation, only 13.42 % of protein conversion was obtained. No microbial spoilage was detected (Poosaran, 1986).

Thawed and chopped sardine was homogenized and hydrolyzed using the commercial proteolytic enzymes (complex enzyme-2000 and alcalase). Optimal pH, enzyme concentration and temperature were 7.0, 7 % (w/w) and 52°C, respectively and those with alcalase were 8.0, 6 % (w/w) and 60°C. Flavor, tase and color of the hydrolysate were improved during the heating proess. The content of free amino nitrogen was 15 %. Total salt was 1,640 mg% (Bae *et al.*, 1990).

Fish silage had many similarities with the fish sauce. During autolysis an aqueous phase rich in small peptides and free amino acids was formed. The silage was normally acidified to pH 3 - 4, which was optimal for protein digestion by the fish pepsins. No salt was added in silage, therefore the autolysis was much faster than in fish sauce. The aqueous phase has a bitter taste and was not suitable for human consumption. The silage was preserved by 2 – 5 % formic acid at pH 4 and stored at 20-40°C (Gildberg, 1993).

Reece (1988) recovered the proteolytic enzymes from fish viscera silage. Procedures were developed for isolating both acidic and alkaline proteases from salmon viscera and acidic proteases from cod and mackerel viscera. The acidic proteases showed optimum activity against haemoglobin at pH 2.6, were inactivated above pH 6.0 and were inhibitied by pepstatin. The alkaline protease from salmon showed optimum activity against casein at pH 8.5 and partially inhibited by PMSF and SBTI.

5.2. Protein hydrolysates

The use of added enzymes was prepared to protein hydrolysates. Fish protein hydrolysate or fish protein concentrate (Haard, 1992) can be used as human food and balanced protein-deficient diets around the world caused by containing high quality protein (Potter and Hotchkiss, 1995). Proteinases from plants or microorganisms have usually been used to prepare fish protein hydrolysates. Fish protein hydrolysates had proved to be excellent as nitrogen sources in microbial growth media and fish peptones were produced commercially in Norway and Japan and food ingredients with special functional properties of feed and fertilizer (Gildberg, 1993).

The protein hydrolysates from Pacific whiting solid wastes using alcalase and neutrase showed optimum enzymes activities at pH 9.5, 60 °C and pH 7.0, 55°C, respectively. Freeze-dried hydrolysate was brownish yellow in color and contained 2.77 % moisture, 79.97 % protein, 13.44 % ash, and 3.83 % lipid (Benjakul and Morrissey, 1997).

Fish protein hydrolysates from herring (*Clupea harengus*) using minced fillets were hydrolyzed with alcalase and papain. Alcalase hydrolyzed samples to a higher degree than papain. Papain hydrolysates were more bitter than those made with alcalase (Hoyle and Merritt, 1994).

Fish hydrolysate made from Atlantic cod (*Gadus morhua*) processing wastes, with an analysis of 1.6-4.2-0.8 (NPK) was compared to Peters 20-20-20 fertilizer on greenhouse grown "Jalapeno" peppers (*Capsicum frutescens*) in Pro-Mix'BX', Metro-Mixed No.350 and top soil at 0, 80 and 160 ppm nitrogen during June 9 to August 21, 1987. Fish hydrolysate fertilizer increased yields by 48 and 76 % at 80 and 160 ppm nitrogen respectively when used at a weekly rate. Fish hydrolysate at 160 ppm applied bi-weekly, consistently increased yield by 123, 97 and 20 % in Pro-Mix'BX', Metro-Mixed No.350 and top soil, respectively as compared to the Peters fertilizer applications (Purohit *et al.*, 1988). The seafood processors can benefit from installing a fish hydrolysate plant through reduced waste disposal costs and by-product revenues (Henderson and Strombom, 1995).

5.3. Ripening by enzyme addition

The whole herring, with or without head, were mixed with salt and stored cool in barrels for a few months before they were washed, filleted and packed. During storage, enzymes from the digestive tract

leaked out and caused partial digestion of the muscle proteins. The fish meat became smooth and pliable and attained a pleasant rich flavour. The high salt concentration (12-15 %) and low temperature (5-10°C) slow down digestion and the maturation process usually took from 2 to 7 months (Gildberg, 1993). Trypsin-like enzymes from the pyloric caeca were the main contributors in the ripening process. The 20 mg cattle and cod trypsins were added in the experiments with herring and squid (1 kg raw material). At 10°C the cod trypsin was more efficient than the cattle enzyme (Simpson and Haard, 1984). The good products by added enzyme can be obtained also with herring caught in early spring and autumn (Gildberg, 1993).

5.4. Carotenoid extraction

The crustacean wastes obtain carotenoids for their bright red color. Since about one third of the dry matter in crustacean shell waste is protein, an proteolytic enzyme process has been developed to recover the protein along with the carotenoid in its native carotenoprotein state (Haard, 1992).

Proteolytic enzymes and a chelating agent included EDTA which appeared to prevent microbial deterioration were applied to facilitate the extraction of a carotenoprotein complex from shrimp processing waste (Simpson and Haard, 1985 ; Cano-lopez *et al.*, 1987). The relating high concentration (153 μg/g) of carotenoids extracted from the crustacean, consisting of 49.4 % of astaxanthin ester, 40.3 % of astaxanthin, and 10.3 % of astacene (Chen and Meyers, 1982).

Cano-lopez *et al.* (1987) extracted carotenoprotein from shrimp process wastes ; heads, shells, and processing water with dissolved and

particulate organic compound (Olsen *et al.*, 1990) with the aid of trypsin from Atlantic cod at 4°C. When 25 mg% cod trypsin was added to extraction medium (0.5 M trisodium EDTA) at pH 7.7, 64 % of the astaxanthin and 80 % of the protein of shrimp waste were recovered as carotenoprotein in 24 h. Simpson and Haard (1985) extracted 80 % of protein and carotenoproteins and 116mg% of astaxanthin from shrimp wastes by using Enzeco ® protease AP-1 enzyme. The stability of carotenoprotein extraction depends on the influence of the antioxidant, butylated hydroxytoluene (BHT) and the protease inhibitor (trasylol) at 30°C.

Chen and Meyers (1982) studied astaxanthin pigment extraction from crawfish waste by using a soy oil process. Aspects, such as particle size, cell disruption by heat and protease treatment, proportion of oil to crawfish waste, and use of the antioxidant ethyoxyquin, were examined to optimize the recovery and to achieve effective pigment concentration maxima. Enzymes using for extraction were fungal protease, bacterial protease, alkaline protease, papain and milezyme ® 8x enzyme. Resulted indicated milezyme ® 8x gave the highest concentration of astaxanthin of 9.8 mg/100g and 10.1 mg/100g at the incubation time of 1 and 3 hr, respectively.

5.5. Fish extract production

Fish extract is a food additive or food flavorant that is produced from tuna condensate using alkaline protease digestion. The protein solubilization occurs mainly due to the action of trypsin enzymes from the fish intestine (Gildberg and Xian-Quan, 1994). Tuna condensate are wastes from the pre-cooking step in canned tuna process which have

high quantity of organic materials including the soluble protein such as gelatin, nitrogen compounds, fat, etc (Sangsri, 1997). The compositions of tuna condensate and fish extract were shown in Table 11 and Table 12, respectively.

Table 11 The compositions of tuna cendensate

	Tropical Canning (Thailand) co., Ltd.		Canned tuna in Spain
	Suwanno (1992)	Jatupornpipat (1994)	Mendez *et al.* (1995)
pH	6.05	6.07 ± 0.12	5.7
COD	157,080 ± 500	73,617 ± 5,942	12,360
Protein	47,600 ± 124	41,525 ± 1,003	28,438
Total solid	82,218 ± 775	68,450 ± 15,166	-[1]
Suspended solid	6,687 ± 124	6,541 ± 1,915	1,930
Phosphate	378 ± 3.2	586 ± 398	180
Chloride	71 ± 0.09	2,625 ± 736	16,790
Magnesium	0.04 ± 0.01	95 ± 23	-[1]
Iron	3.13 ± 0.12	5.80 ± 1.06	-[1]
Grease	32,182 ± 11	19,656 ± 5,657	-[1]

all units are in mg/l except pH values

[1]- not determined

Source : modified from Sangsri (1997)

Table 12 The chemical compositions of fish extract

	Songkla Canning (Thailand) Co, Ltd.	Leelawat and Auprung (1994a,b)
Color	dark brown	dark brown
pH	5.6 - 6.4	8.5 or 6.0
Moisture (%)	32 - 36	92.9
Protein (%)	> 20	0.2
Ash (%)	-[1]	-[1]
Lipid (%)	-[1]	1.73
Salt (%)	12 - 14	1.37
Brix (%)	74 - 76	-[1]
Total plate count	$< 3.0 \times 10^3$	-[1]
Enzyme	Alcalase	papain or Neutrase

[1] - not determined

Objectives

1. To purify trypsin and chymotrypsin from viscera of yellowfin tuna (*Thunnus albacares*)
2. To investigate biochemical characteristics of the two enzymes
3. To study the enzyme application

Chapter 2

Materials and Methods

Materials

1. Raw materials

The viscera of yellowfin tuna (*Thunnus albacares*) and the tuna condensate were kindly provided by Tropical Canning (Thailand) Co., Ltd., Hatyai, Songkhla. and kept in a freezing room (-20°C).

2. Chemicals

Analytical grade chemicals were used for enzyme extraction from yellowfin tuna, assay of trypsin and chymotrypsin activities, protein concentration, purification, gel electrophoresis, characterization and applications (Appendix A and B).

Instruments

1. Spectrophotometer Model U-2000, Hitachi Koki Co., Ltd.
2. Refrigerated centrifuge Model Himac SCR 20B, of Hitachi Koki Co., Ltd.
3. pH meter Model HM-7E, Tokyo TOA Electronic Ltd.
4. Water bath Model W350, Memmert Ltd.
5. Column chromatography, Pharmacia Fine Chemicals
6. Fraction collector Model LKB 2212 Helirac, LKB company
7. Peristatic pump, Bio-RAD Laboratories

8. Electrophoresis Mini - Protein II Dual Slab Cell with power supply Model 1000/500, Bio-RAD Laboratories
9. Soxhlet machine Model ME, Electrothermal company
10. Kjeltech system Model 1002 Distilling Unit, Tecator company
11. Isotemp muffle furnaces 550 series Model 550-14, 550-58, 550-126, Fisher Scientific company

Analytical methods

1. **Soluble protein** was measured by the method of Lowry *et al.* (1951) (Appendix D1).
2. **Total protein and total nitrogen** by Kjeldahl method (modified from A.O.A.C, 1990) (Appendix D2).
3. **Moisture** was measured by the method of A.O.A.C. (1990) (Appendix D3).
4. **Crude fat** was extracted by A.O.A.C. (1990) (Appendix D4).
5. **Ash** was determined by A.O.A.C. (1990) (Appendix D5).
6. **Total solid** was determined by A.O.A.C. (1990) (Appendix D6).
7. **Total amino nitrogen** was measured by A.O.A.C. (1980) (Appendix D7).
8. **Total volatile nitrogen** was determined by the method of Hasegawa (1987) (Appendix D8).
9. **Trimethylamine nitrogen** was determined by the method of Hasegawa (1987) (Appendix D9).
10. **Salt** was determined by the method of A.O.A.C. (1990) (Appendix D10).

11. **Trypsin activity**

An activity of trypsin was determined in a reaction mixture (total volume of 3 ml) consisting 0.1 ml of enzyme solution, 0.3 ml of 10 mM TAME and 2.6 ml of 20 mM Tris-HCl buffer, pH 7.0 containing 0.012 M $CaCl_2$, 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 (modified from the method of Cano-lopez *et al.*, 1987 ; Simpson and Haard, 1984).

Calculation of trypsin activity

$$\text{Units/ml} = \frac{A_{247nm}/\text{min} \times 1000 \times 3}{540 \times \text{Volume of enzyme solution in assay}}$$

$$\text{Units/g} = \frac{\text{Units/ml} \times \text{Volume of extracted buffer (ml)}}{\text{Weight of used viscera (g)}}$$

12. **Chymotrypsin activity**

Chymotrypsin activity was determined in the reaction mixture consisting 0.1 ml of appropriate diluted enzyme solution, 1.5 ml of 1.07 mM BTEE in 50% methanol (v/v) and 1.4 ml of 20 mM Tris-HCl buffer, pH 7.0 containing 0.012 M $CaCl_2$, then incubated at 50°C for 5 minutes. The initial rate of change in absorbance was measured at 256 nm. Activity and specificity of chymotrypsin were expressed in BTEE units per ml and units 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 (modified from the method of Ramakrishna *et al.*, 1987).

Calculation of chymotrypsin activity

$$\text{Units/ml} = \frac{A_{256nm}/\text{min} \times 1000 \times 3}{964 \times \text{Volume of enzyme solution in assay}}$$

$$\text{Units/g} = \frac{\text{Units/ml} \times \text{Volume of extracted buffer (ml)}}{\text{Weight of used viscera (g)}}$$

13. **Molecular weight of enzymes** were determined by the method of Laemmli (1970) (Appendix D12).

14. **Degree of hydrolysis (%)**

A 50 ml of sample was mixed with 20% trichloroacetic acid (TCA) to create 10% TCA-soluble (nonprotein nitrogen) and TCA-insoluble (protein nitrogen) fractions. The mixtures were centrifuged at 12,735×*g* (10,000 rpm) for 20 minutes. The supernatant was analyzed for nitrogen by Kjeldahl method (Hoyle and Merritt, 1994).

Calculation

$$\text{Degree of hydrolysis (\%)} = \frac{\text{10\% TCA-soluble nitrogen in sample} \times 100}{\text{Total nitrogen in sample}}$$

Methods

1. Purification and characterization of trypsin and chymotrypsin

1.1. Extraction of enzyme solution

The 40 samples of yellowfin tuna (*Thunnus albacares*) were weighed their body weights and viscera before and after evisceration, respectively for calculation of the ratio between viscera and body weight. The viscera of yellowfin tuna were kept at -20°C and separated into individual organ ; liver, pancreas, stomach, spleen, intestine and recorded their weights to obtain the ratio to body weight. Each organ and the whole viscera (100 g each) were minced and homogenized in 200 ml of 20 mM Tris-HCl buffer, pH 7.0 containing 5 mM $CaCl_2$ and 0.02% sodium azide. After incubation at 25°C for an hour, the mixture was centrifuged at 12,735×*g* (10,000 rpm) for 30 minutes and each supernatant was used as the crude enzyme solution (Heu *et al.*, 1995). After extraction with buffer at pH 7.0 and centrifugation, each crude enzyme solution was assayed for protein concentration and trypsin and chymotrypsin activities.

1.2. Enzyme purification

1.2.1. Ammonium sulfate precipitation

The crude enzyme solution was precipitated by salt fractionation using 20-40, 40-60 and 60-80 % saturation of ammonium sulfate. Each range, $(NH_4)_2SO_4$ was added gradually with the addition of certain amount of ammonium sulfate (Appendix B2) and stirred well by magnetic stirrer for overnight at 4°C. After fractionation, the solution was centrifuged at 12,735×*g* (10,000 rpm) for 30 minutes at 4°C and the

precipitate was dissolved in 10 ml of 20 mM Tris-HCl buffer, pH 7.0. The assay of trypsin and chymotrypsin activities and protein concentration were conducted.

1.2.2. Dialysis

The enzyme solutions was then dialyzed (using dialysis bags with the molecular weight cut off at 8,000 daltons) in 20 mM Tris-HCl buffer, pH 7.0 in the ratio of 1:50 at 4 °C for 18 hours and stirred by magnetic stirrer. The buffer was changed after 1, 2 and 4 hours. The enzyme solution was then centrifuged at 12,735×*g* (10,000 rpm) for 10 minutes at 4 °C. The supernatant was kept at 4 °C for further purification. The enzyme activities and specific activities were assayed.

1.2.3. Column chromatography

1.2.3.1. Gel filtration column chromatography

Sephadex G-100 was prepared (Appendix B1.1) and packed in a 2 × 27 cm column, equilibrated with 20 mM Tris-HCl buffer, pH 7.0, for 2 bed volumes at the flow rate of 0.20 ml per minute. The enzyme solution was then passed through the column and eluted with the same buffer at the same flow rate. Two ml per fraction was collected by a fraction collector, then measured its absorbancy at 280 nm and assayed for trypsin and chymotrypsin activities. The high enzyme activity fractions were pooled and concentrated by ultrafiltration with PM 10 membrane.

1.2.3.2. Affinity column chromatography

A benzamidine - Sepharose was prepared (Appendix B1.2) and packed in a 2 × 27 cm column, equilibrated by washing with 20 mM Tris-HCl buffer, pH 7.0 for 2 bed volumes at the flow rate of 0.20 ml

per minute. The enzyme solution (from 1.2.3.1) was applied into the column, subsequently washed with the same buffer to elute chymotrypsin which was nonspecifically bound material. The trypsin-like enzyme was eluted from the column using 125 mM benzamidine and 1% NaCl. Two ml per fraction was collected and determined its absorbancy at 280 nm and its enzyme activities. The high enzyme activity fractions were pooled and concentrated by ultrafiltration.

1.2.3.3. Anion - exchange column chromatography

DEAE- Sephadex A-50 was prepared (Appendix B1.3) and applied into a 2 × 27 cm column, then equilibrated with 20mM Tris-HCl buffer, pH 7.0 for 2 bed volumes at the flow rate of 0.20 ml per minute. Trypsin and chymotrypsin solutions were passed through the column and eluted using the same buffer but containing 100 mM NaCl. Both enzymes activities and absorbancies at 280 nm were determined, then concentrated by ultrafiltration.

1.2.4. Gel electrophoresis

The enzymes were examined for purities and molecular weights by gel electrophoresis.

1.2.4.1. Non-denatured polyacrylamide gel electrophoresis (Non-PAGE)

Non-PAGE was carried out as described by Devis (1964). The slab gel with a 12.5% separating gel (9 × 5 × 0.1 cm) and 4.5% stacking gel (9 × 2 × 0.1 cm) was used (Appendix D13).

The enzyme was mixed with a buffer containing 0.5 M Tris-HCl buffer, pH 6.8, 40% glycerol, 0.5% bromophenol blue in the ratio of 3:1 by volume. Standard proteins consisted of α-lactabumin

(14,200 daltons), carbonic anhydrase (29,000 daltons), albumin : chicken egg (45,000 daltons) and albumin : bovine serum (66,000 daltons), were prepared the same as above. The sample and standard proteins were then applied into each well on the stacking gel. Electrophoresis was carried out at room temperature using 0.025 M Tris - 0.192 M glycine buffer, pH 8.3 as an electrode buffer. A constant voltage of 175 volts was supplied until the tracking dye approached the bottom of the gel, then the gel was stained with staining solution for overnight and destained with 40% methanol in 10% acetic acid solution.

1.2.4.2. Sodiumdodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE method was followed as described in 1.2.4.1. using 10% SDS in gel and the electrode buffer consisting of 10% SDS, 40% glycerol, 0.5% bromophenol blue and 2-mercaptoethanol in 0.5 M Tris-HCl buffer, pH 6.8, then boiled for 5 minutes. Hemoglobin, cross-linked (Bovine) consisted of 4 major molecular weights of monomer (16,000 daltons), dimer (32,000 daltons), trimer (48,000 daltons) and tetramer (64,000 daltons) were used as SDS molecular weight markers (Appendix D12) (modified from the method of Laemmli, 1970).

1.3. Characterization of the purified trypsin and chymotrypsin

1.3.1. Effect of pH on enzyme activity and stability

1.3.1.1. Effect of pH on enzyme activity

The effect of pH on the activities of trypsin and chymotrypsin was studied at 50°C by adding each purified enzyme into a reaction mixture containing individual substrate with pH range of 3.0 - 11.0 (0.05 M citrate buffer for pH 3.0, 4.0, 5.0 and 6.0 ; 0.05 M Tris-

HCl buffer for pH 7.0, 8.0 and 9.0 ; 0.05 M carbonate - bicarbonate buffer for pH 10.0 and 11.0). Enzymes activities were expressed as relative activity.

1.3.1.2. Effect of pH on enzyme stability

The effect of pH (in the range of 3.0 - 11.0 as stated in 1.4.1.1.) on the stability of the two enzymes was estimated by measuring residual substrate activity after incubation each enzyme solution at 4°C for different time (0, 30, 60, 90 and 120 minutes).

1.3.2. Effect of temperature on enzyme activity and stability

1.3.2.1. Effect of temperature on enzyme activity

The effect of temperature on enzyme activities was assayed at various temperatures of 0, 4, 10, 20, 30, 40, 50, 60, 70, 80 and 90 °C using the optimum pH buffer (from 1.3.1.1.). Enzymes activities were expressed as relative activity.

1.3.2.2. Effect of temperature on enzyme stability

The thermostability was studied by incubating each enzyme solution (without substrate) for 0, 30, 60, 90 and 120 minutes at various temperatures of 30, 40, 50, 60, 70 and 80 °C and immediately cooled on ice. The remaining activities were then determined according to the standard procedure (incubation temperature at 50°C) but using the buffer with the optimum pH for enzyme activity (from 1.3.1.1.).

1.3.3. Effect of ions and chemicals on enzyme activity

The remaining enzyme activities were measured after the reaction mixture was incubated with the addition of benzamidine,

EDTA, 1-10 phenantroline, 4-aminobenzoic acid, KI, Ag_2SO_4 and $HgSO_4$ dissolved in optimum pH buffer in various concentrations ; 0.1, 1, 10, 100, 1,000 and 10,000 μM. A control assay was included by adding distilled water instead of ions and chemical solutions.

1.3.4. Determination of kinetic parameters

The enzyme solution was incubated with a varying concentrations of substrate (0.02, 0.05, 0.10, 0.20, 0.30, 0.50, 1.0, 1.5 and 2.0 mM in optimum pH buffer). The enzyme activities were assayed every 5 minutes for 30 minutes. The initial velocity was determined from the slope of the straight line by linear regression. K_m and V_{max} values were calculated by Lineweaver-Burk plot (Heu *et al.*, 1995).

2. Enzyme application

The application of crude enzyme from tuna viscera was subjected to fish extract production and the commercial trypsin and α-chymotrypsin were used for comparison. The crude enzyme was prepared by homogenizing 50 g of viscera of yellowfin tuna in 100 ml of 20 mM Tris-HCl buffer, pH 7.0 containing 5 mM $CaCl_2$ and centrifugation at 12,735×*g* (10,000 rpm) for 10 minutes. The supernatant was the crude enzyme which trypsin and chymotrypsin activities were determined. Tuna condensate was incubated at 90°C for 6 hours to remove oil and the supernatant was taken after centrifugation at 12,735× *g* (10,000 rpm) for 10 minutes. The enzyme (0.02%,w/v) was added and the mixture was incubated at 50°C , pH 8.0 for 1 hour. The reaction was stopped by heating at 90°C for 1 hour followed by filtration through a

fine mesh nylon net. Fish extract sample was incubated at 60°C for 1-2 days and cooled to room temperature and left for 2 days to precipitate poorly soluble compounds, then filtered and concentrated by incubation at 50°C for 2 days (also for desalting) and stored at 4°C (modified from the method of Songkhla Canning Co., Ltd.)

The product was determined for moisture (at 105°C for 3 hours), ash (by heating to 600°C for 3 hours), crude protein (the Kjeldahl procedure) and crude fat (by Soxhlet extraction) using petroleum ether, total solid (at 100°C for 4 hours), soluble protein (Lowry *et al.*, 1951), brix (using refractometor), trimethylamine nitrogen, nonprotein nitrogen, total amino nitrogen, degree of hydrolysis, salt, calcium, phosphorus, total plate count (Appendix D14), *Salmonella* (Appendix D15) and *Escherichia coli* (Appendix D16).

Chapter 3

Results and Discussion

1. Purification and characterization of trypsin and chymotrypsin from yellowfin tuna

1.1. Extraction of trypsin and chymotrypsin from yellowfin tuna

The means weight values of total body weight and mixed viscera of yellowfin tuna were about 1,220 ± 110 g and 61.98 ± 3.50 g as determined to the ratio of 5.08% of body weight, respectively. The viscera were separated into individual organ which consisted of stomach, liver, pancreas, spleen and intestine, giving the means weight of 19.05 ± 0.68 g, 12.85 ± 0.58 g, 1.80 ± 0.06 g, 21.30 ± 2.39 g and 3.51 ± 0.35 g, respectively. The percentage values based on the body weight were 1.56, 1.05, 0.15, 1.74 and 0.29 %, respectively (Table 13).

Activity of the enzymes extracted from each viscera organ of yellowfin tuna (*Thunnus albacares*) is given in Table 14. The trypsin activities of spleen, mixed viscera, intestine, stomach, liver and pancreas were 49.26, 44.07, 33.33, 23.52, 12.08 and 8.25 units/ml, respectively and their specific trypsin activities were 13.80, 3.15, 23.81, 4.70, 5.25 and 2.06 units/mg, respectively. The chymotrypsin activity of pancreas, spleen, stomach, mixed viscera, liver and intestine were 4.13, 3.42, 3.06, 3.04, 1.73 and 1.52 units/ml, respectively with their specific activities of 1.03, 0.96, 0.61, 0.22, 0.75 and 1.09 units/mg, respectively.

Table 13 Weight of total body, mixed viscera and individual organ of the viscera of yellowfin tuna (*Thunnus albacares*)

Total body (g)	Organ (g)					
	Mixed viscera	Stomach	Liver	Pancreas	Spleen	Intestine
1,200	60.70	18.39	12.05	1.76	22.86	3.93
1,100	56.43	18.83	13.79	1.86	18.18	3.19
1,200	63.45	19.04	13.54	1.90	20.96	3.08
1,400	65.80	20.39	13.15	1.85	23.14	3.53
1,300	65.43	18.49	12.19	1.75	23.76	3.83
1,400	67.60	20.17	13.29	1.86	22.96	3.48
1,100	58.46	18.93	13.13	1.83	17.16	3.19
1,100	57.89	19.33	12.69	1.73	18.19	3.03
1,200	61.50	18.39	12.49	1.76	22.36	3.86
1,200	62.54	18.49	12.19	1.74	23.46	3.96
**1,220 ± 110	61.98 ±3.50	19.05 ±0.68	12.85 ±0.58	1.80 ±0.06	21.30 ±2.39	3.51± 0.35

** means weight values

Table 14 Comparison on activity of trypsin and chymotrypsin from each organ of yellowfin tuna (*Thunnus albacares*)

Organ	Trypsin		Chymotrypsin	
	Activity (units/ml)	Specific activity (units/mg)	Activity (units/ml)	Specific activity (units/mg)
Mixed viscera	44.07	3.15	3.04	0.22
Liver	12.08	5.25	1.73	0.75
Pancreas	8.25	2.06	4.13	1.03
Stomach	23.52	4.70	3.06	0.61
Spleen	49.26	13.80	3.42	0.96
Intestine	33.33	23.81	1.52	1.09

The best sources for trypsin and chymotrypsin were spleen and pancreas respectively, The highest trypsin activity was 49.26 units/ml with specific activity of 13.80 units/mg protein. The values for chymotrypsin were 4.13 units/ml and 1.03 units/mg protein, respectively. Trypsin specific activity gave the highest value from intestine followed by spleen, liver, stomach, mixed viscera and pancreas, respectively while those of chymotrypsin were intestine following by pancreas, spleen, liver, stomach and mixed viscera, respectively. In this study, although the enzyme solution from intestine gave the highest specificity of trypsin and chymotrypsin, but it was partly due to the activity from microorganisms contained in the intestine. Therefore, spleen was selected as the best source for trypsin and chymotrypsin. Spleen of tuna was previously reported to give the highest protease

activity (53.38 units/ml) and specific activities (2.559 units/mg protein) followed by liver, pancreas and stomach, respectively (Prachumratana, 1998). Spleen and pancreas are organs that normally produce many enzymes such as trypsin in fish and also store enzymes produced from other parts of body. They consequently posses more enzymes than other organs (Hmoejan, 1985). The pyloric caeca was used as source of trypsin from anchovy (*Engraulis encrasicholus*) (Martinez *et al.*,1988), cod (*Gadus morhua*) (Overnell, 1973 ; Shin and Zall, 1986). Marine fish possessing big pyloric caeca normally are plankton eater (Hmoejan, 1985). Tuna have the small pyloric caeca organ because they consume many kinds of food including plankton and most of small marine lives such as small fish, squids and shrimp, etc. (Hmoejan, 1985).

1.2. Enzyme purification

1.2.1. Ammonium sulfate precipitation

After selected the best source of the two enzymes, crude enzyme solution was extracted from spleen of yellowfin tuna and had the trypsin activity of 48.69 units/ml and specific activity of 3.50 units/mg protein (with the protein concentration of 13.90 mg/ml). Enzyme solution from pancreas gave the chymotrypsin activity and specific activity of 3.22 units/ml and 0.73 units/mg protein, respectively with the protein concentration of 4.41 mg/ml. Both enzyme solutions were precipitated by various % saturated $(NH_4)_2SO_4$ and determined for enzyme activity and protein concentration (Table 15). The appropriate salt saturation was at 40 - 60% giving the highest trypsin and chymotrypsin activities of 116.27 and 23.24 units/ml, respectively and their specific activities of

Table 15 Ammonium sulfate precipitation at various salt saturation of trypsin and chymotrypsin from spleen and pancreas of yellowfin tuna (*Thunnus albacares*)

Fraction	Volume (ml)	Protein (mg/ml)	Trypsin		Chymotrypsin	
			A*	SA**	A	SA
Crude enzyme (spleen)	340	13.90	48.69	3.50	2.98	0.21
(pancreas)	340	4.41	20.55	4.66	3.22	0.73
20-40% sat.$(NH_4)_2SO_4$ precipitate						
(spleen)	30	20	66.39	3.32	11.11	0.55
(pancreas)	30	20	54.72	2.88	14.07	0.70
40-60% sat.$(NH_4)_2SO_4$ precipitate						
(spleen)	30	30.20	116.27	3.85	19.61	0.65
(pancreas)	30	31.00	72.61	2.34	23.24	0.75
60-80% sat.$(NH_4)_2SO_4$ precipitate						
(spleen)	30	12.50	43.89	3.51	6.67	0.53
(pancreas)	30	11.40	29.81	2.61	8.44	0.74

* A = Activity (units/ml)

** SA = Specific activity (units/mg protein)

3.85 and 0.75 units/mg protein respectively. Protein concentrations of spleen and pancreas solution were 30.20 and 31.00 mg/ml, respectively (Table 16).

1.2.2. Dialysis

After salt removal by dialysis, spleen solution (20 ml) gave the activity and specific activity of trypsin at 34.13 units/ml and 10.34 units/mg protein, respectively. The total activity of 682.60 units with 4.12 % recovery yield and 2.95 fold of purification factor were achieved. The chymotrypsin activity from pancreas solution (20 ml) was 2.29 units/ml while its specific activity was 1.04 units/mg protein. It gave the total activity of 45.76 units with 41.80% yield and purification factor of 1.42.

1.2.3. Column chromatography

1.2.3.1. Gel filtration column chromatography

The dialyzed enzyme solutions were applied to Sephadex G-100 column and eluted proteins with 20 mM Tris-HCl buffer, pH 7.0. Gel filtration is unique in that fractionation is based on the relative size of protein molecules (Deutscher, 1990). Figure 5 presents gel filtration chromatography of enzymes from spleen of yellowfin tuna. There were four peaks of protein which showed that most trypsin and chymotrypsin activities were at the first two peaks with the values of 1.46 and 0.067 units/ml, respectively. Hence, the other two peaks were discarded. Chymotrypsin was bigger than trypsin as the macromolecules emerge from the column in decreasing order of size (Dixon and Webb, 1979) Fractions of enzyme solution from pancreas (Figure 6) showed two

Table 16 Summary of trypsin and chymotrypsin purification steps

Fraction		Protein conc. (mg/ml)	Activity (units/ml)	Specific activity (units/mg)	Total activity (units)	%Yield	Purity (fold)
Crude	T*	13.90	48.69	3.50	16554.6	100	1.00
	C**	4.41	3.22	0.73	1094.80	100	1.00
40-60% sat.	T.	30.20	116.27	3.85	3488.10	21.07	1.10
$NH_4)_2SO_4$	C.	31.00	23.24	0.75	697.20	63.68	1.03
Dialysis	T.	3.30	34.13	10.34	682.60	4.12	2.95
	C.	2.20	2.29	1.04	45.76	41.80	1.42
Gel filtration	T.	0.110	1.460	13.27	310.98	0.59	3.79
(Sephadex G-100)	C.	0.048	0.067	1.40	15.05	1.37	1.92
Affinity column	T.	0.017	0.350	20.58	87.50	0.53	5.88
(benzamidine-Sepharose column)	C.	0.022	0.106	4.82	18.02	1.65	6.60
Anion column I	T.	0.020	0.417	20.85	75.06	0.45	5.96
(DEAE-Sephadex A-50)	C.	0.038	0.230	6.05	18.40	1.68	8.29
Anion column II	T.	0.020	0.503	25.15	75.45	0.46	7.19
(DEAE-Sephadex A-50)	C.	0.022	0.140	6.36	16.80	1.53	8.71
Anion column III	T.	0.016	0.580	36.25	75.40	0.45	10.36
(DEAE-Sephadex A-50)	C.	0.016	0.120	7.50	11.40	1.04	10.27
Gel filtration (II)	T.	0.012	0.641	53.42	57.69	0.35	15.26
(Sephadex G-100)	C.	0.014	0.142	10.14	11.36	1.04	13.89

*T = Trypsin

**C. = Chymotrypsin

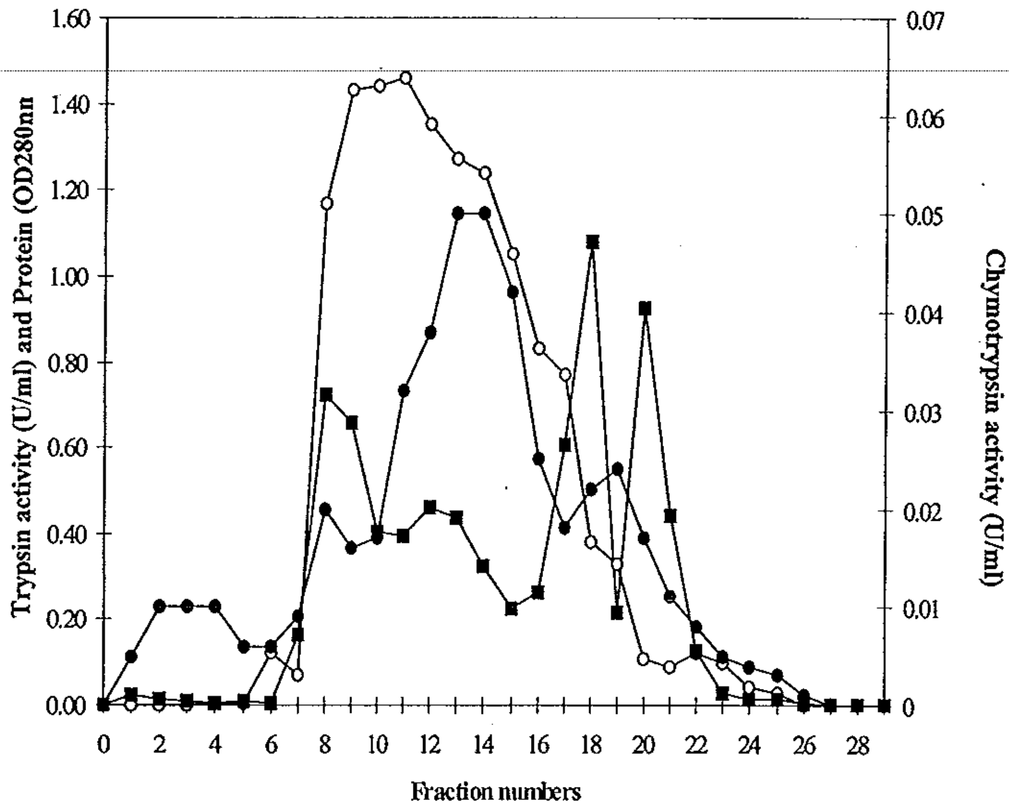


Figure 5 Gel filtration on Sephadex G-100 of trypsin (o) and chymotrypsin (•) from spleen of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was eluted with 20 mM Tris-HCl, pH 7.0 in 2.0 ml fractions at a flow rate of 0.2 ml/min.

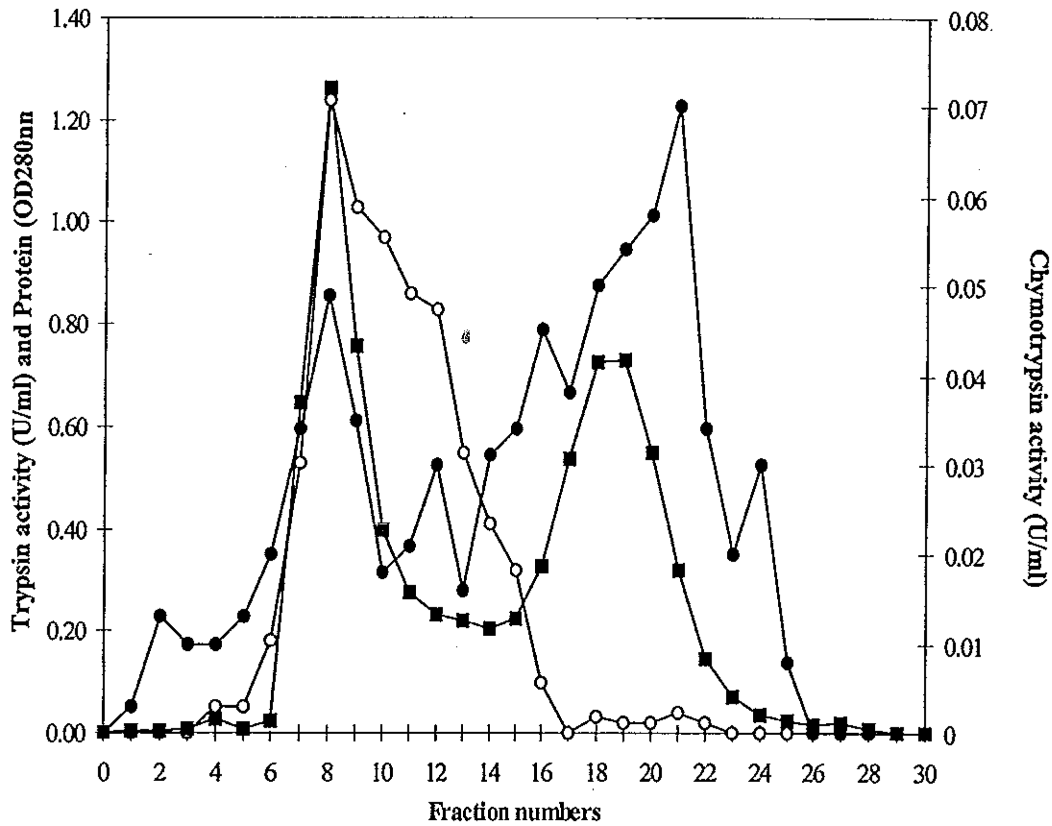


Figure 6 Gel filtration on Sephadex G-100 of trypsin (o) and chymotrypsin (•) from pancreas of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was eluted with 20 mM Tris-HCl, pH 7.0 in 2.0 ml fractions at a flow rate of 0.2 ml/min.

peaks of protein. The peaks of trypsin and chymotrypsin (Figure 5 and 6) were coincided.

The fractions of spleen sample with high trypsin activity (number 7 – 20) and of pancreas sample with high chymotrypsin activity (fraction number 4 - 25) were collected. After gel filtration on Sephadex G-100, the total solution from spleen was 213 ml with the trypsin activity and specific activity of 1.46 units/ml and 13.27 units/mg protein, respectively. The total activity was 310.98 units which gave 0.59% recovery yield and 3.79 fold of purity. The total volume of pancreas sample was 215 ml with the highest chymotrypsin activity and specific activity of 0.067 units/ml and 1.40 units/mg protein, respectively. The total activity was 15.05 units of and 1.37% yield. Its purification factor was 1.92 fold (Table 16).

1.2.3.2. Affinity column chromatography

The enzyme solutions from spleen and pancreas (from 1.2.3.1) were then ultrafiltrated before applying into benzamidine-Sepharose column and eluted with two buffers. Results (Figure 7 and 8) indicated that chymotrypsin and unabsorbed trypsin from both enzyme sources were first eluted from the column by 20 mM Tris-HCl buffer, pH 7.0, followed by eluting the trypsin with the same buffer containing 125 mM benzamidine and 1% NaCl. The fractions number 5 - 25 of both spleen and pancreas sample solutions were pooled as the chymotrypsin solution. The trypsin solution was obtained from the pooled fraction number 76 - 95 of spleen sample and the fraction number 61 - 80 of pancreas sample. After affinity column chromatography, the total volumes of trypsin and chymotrypsin solution were 250 and 170 ml, respectively. Their enzyme activities were 0.350

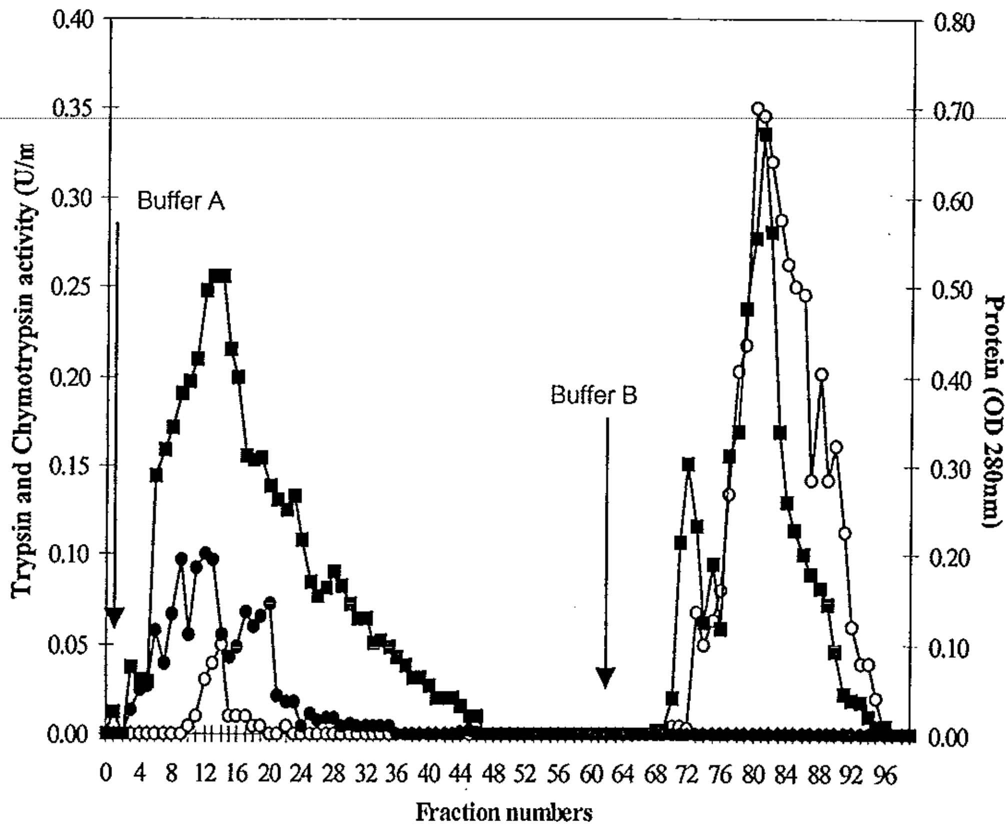


Figure 7 Affinity column chromatography on Benzamidine-Sepharose of trypsin (o) and chymotrypsin (•) from spleen of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was eluted with 20 mM Tris-HCl, pH 7.0 (buffer A) in 2.0 ml fractions at a flow rate of 0.2 ml/min. The bound protein was eluted by the same buffer with 125 mM benzamidine and 1% NaCl (buffer B).

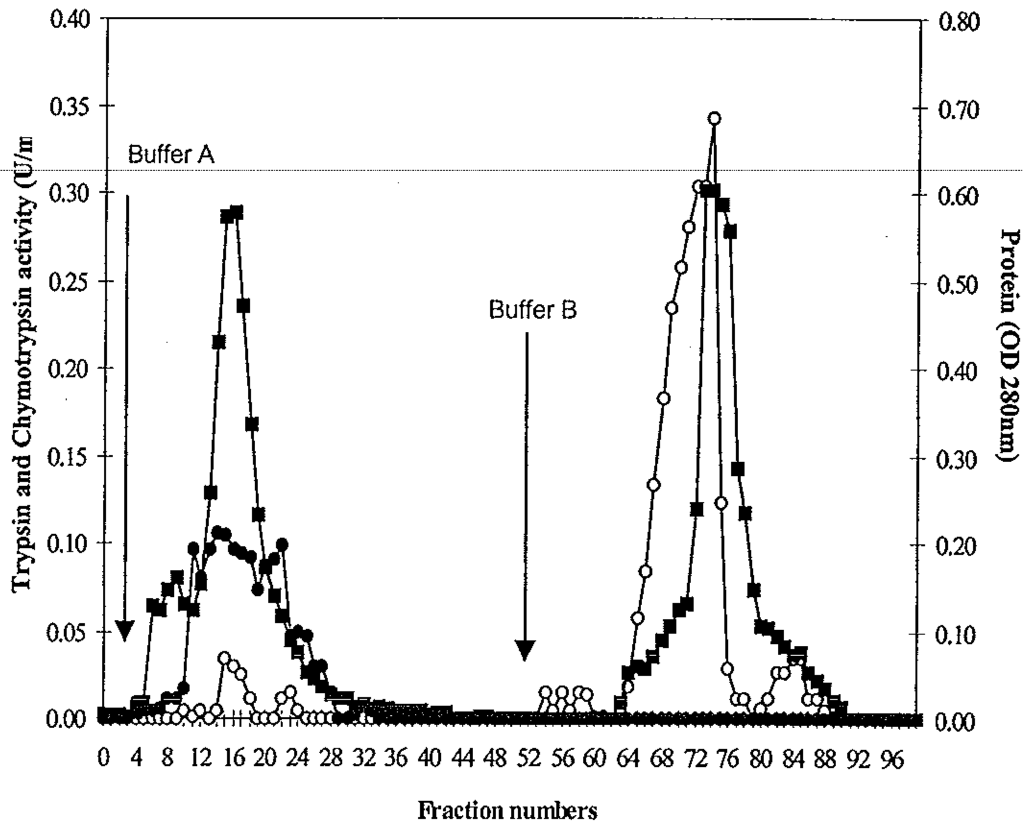


Figure 8 Affinity column chromatography on Benzamidine-Sepharose of trypsin (o) and chymotrypsin (•) from pancreas of yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was eluted with 20 mM Tris-HCl, pH 7.0 (buffer A) in 2.0 ml fractions at a flow rate of 0.2 ml/min. The bound protein was eluted by the same buffer with 125 mM benzamidine and 1% NaCl (buffer B).

and 0.106 units/ml while the specific activities were 20.58 and 4.82 units/mg protein, respectively. Total activity of trypsin solution was 87.50 units and 18.02 units for chymotrypsin. The % yield recoveries of trypsin and chymotrypsin were 0.53 and 1.65, respectively. The purification factors were 5.88 fold and 6.60 fold, respectively (Table 16).

1.2.3.3. Anion exchange column chromatography

Trypsin and chymotrypsin solutions (from 1.2.3.2.) were ultrafiltrated and passed through DEAE-Sephadex A-50 column, an anion exchanger. The other proteins were washed out with 20 mM Tris-HCl buffer, pH 7.0, then eluting the required trypsin and chymotrypsin with the same buffer containing 100 mM NaCl. The high enzyme activity fractions (number 71 - 77 for trypsin in Figure 9 and number 70 - 77 for chymotrypsin in Figure 10) were pooled. The unabsorbed enzymes emerged first from the column and the protein from the fixed charges was displaced by the ability of counterions (salts) (Deutscher, 1990). After DEAE-Sephadex A-50 column I, the total volume of trypsin solution was about 180 ml and 80 ml for chymotrypsin solution. The activities of trypsin and chymotrypsin were 0.417 and 0.230 units/ml respectively, with the specific trypsin activity of 20.85 units/mg protein and 6.05 units/mg protein for chymotrypsin. The total activity and %yield of trypsin were 75.06 units and 0.45%, respectively and 18.40 units with 1.68% respectively for chymotrypsin. The purification factors of trypsin and chymotrypsin were 5.96 and 8.29 fold, respectively.

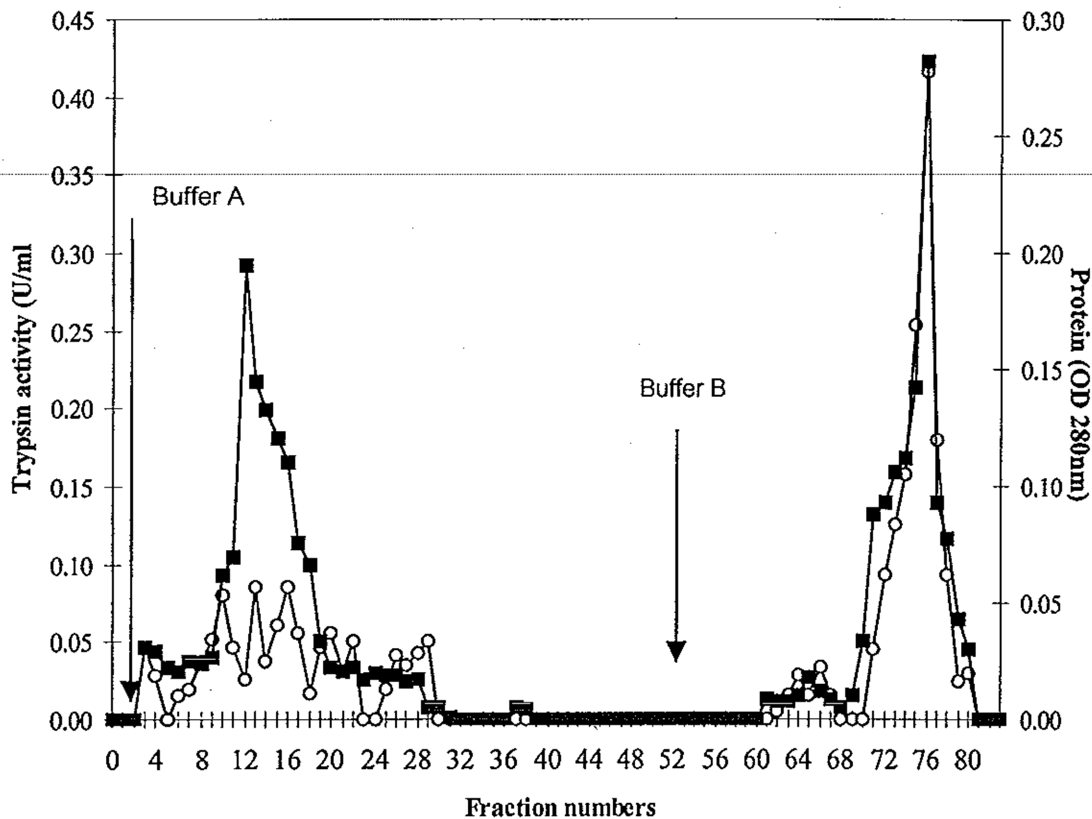


Figure 9 Anion exchange column chromatography on DEAE-Sephadex A-50 of trypsin (o) from yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was washed with 20 mM Tris-HCl, pH 7.0 (buffer A) in 2.0 ml fractions at a flow rate of 0.2 ml/min. The bound protein was eluted by the same buffer with 100 mM NaCl (buffer B).

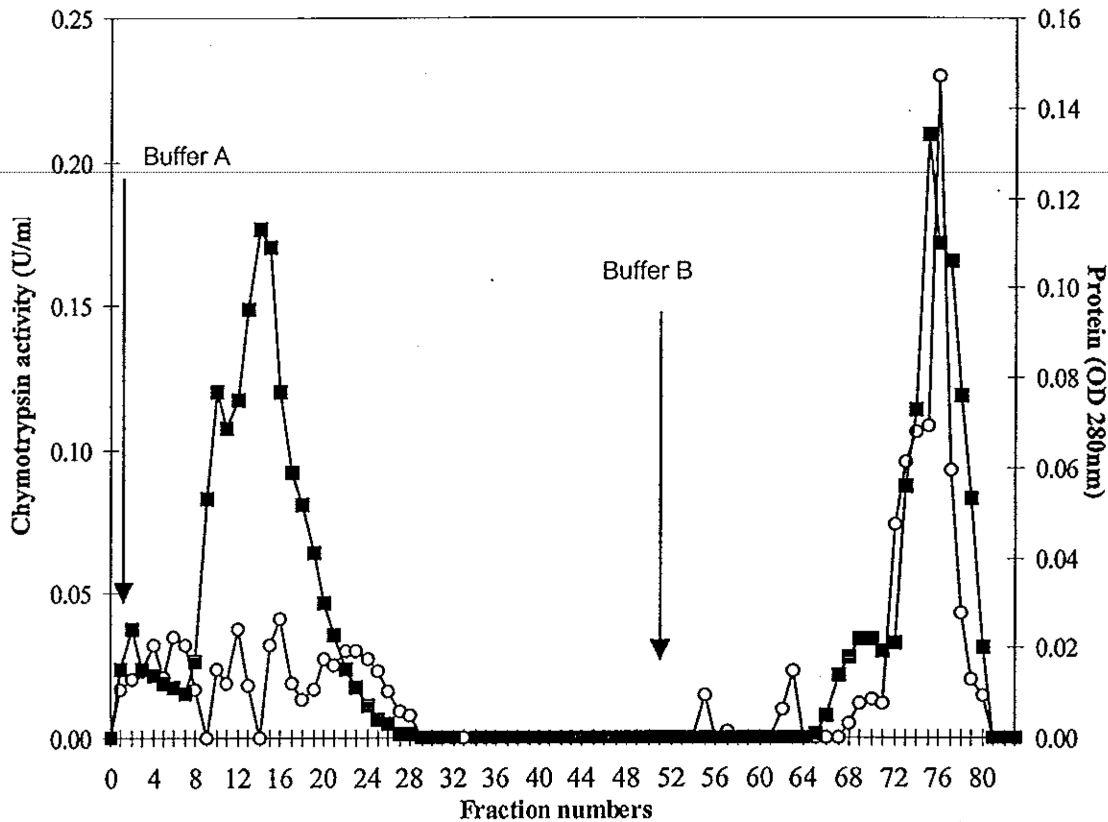


Figure 10 Anion exchange column chromatography on DEAE-Sephadex A-50 of chymotrypsin (o) from yellowfin tuna (*Thunnus albacares*) and protein at OD 280 nm (■). The sample applied on the column (2×27 cm) was washed with 20 mM Tris-HCl, pH 7.0 (buffer A) in 2.0 ml fractions at a flow rate of 0.2 ml/min. The bound protein was eluted by the same buffer with 100 mM NaCl (buffer B).

Each total volume of two enzymes was ultrafiltrated and applied on DEAE-Sephadex A-50 column II and III for higher purity. After DEAE-Sephadex A-50 column II, the total volume of trypsin solution was 150 ml and 120 ml for chymotrypsin solution. Trypsin and chymotrypsin solutions gave the activities of 0.503 and 0.140 units/ml, respectively as well as the specific activities of 25.15 and 6.36 units/mg protein, respectively. Their total activities were 75.45 and 16.80 units, respectively with the recovery yields of 0.46% and 1.53%, respectively. The purification factors of trypsin and chymotrypsin were 7.19 and 8.71 fold, respectively.

DEAE-Sephadex A-50 column III was subsequently conducted, giving total volumes of 130 and 95 ml for trypsin and chymotrypsin, respectively. The solution possessed trypsin activity of 0.580 units/ml with the specific activity of 36.25 units/mg protein. The total activity was 75.40 units (0.45% yield) with its purity of 10.36 fold. The chymotrypsin activity was 0.120 units/ml and its specific activity was 7.50 units/mg protein. The total activity and % recovery yield of chymotrypsin were 11.40 units and 1.04%, respectively, with the purification factor of 10.27 fold.

The enzyme solutions from DEAE-Sepahdex A-50 III column were ultrafiltrated and applied on Sephadex G-100 II column. Trypsin solution (90 ml) had the activity and specific activity of 0.641 units/ml and 53.42 units/mg protein, respectively. Total activity of trypsin was 57.69 units with 0.35% yield and its purification factor was 15.26 fold. For chymotrypsin, total volume (80 ml) contained the activity of 0.142 units/ml as well as the specific activity of 10.14 units/mg protein. Its

total activity was 11.36 units giving 13.89 fold for purity of chymotrypsin. Its % recovery yield was 1.04% (Table 16).

1.2.4. Gel electrophoresis

1.2.4.1. Nondenatured polyacrylamide gel electrophoresis (Non-PAGE)

The enzyme solutions from each step of purification including ammonium sulfate precipitation, dialysis, Sephadex G-100 column, affinity column and DEAE-Sephadex A-50 column were detected for their purity by non-PAGE method which presented the characteristics of the native proteins. Results indicated that after ammonium sulfate precipitaion, dialysis and Sephadex G-100 I column, the enzyme still showed many bands of proteins and appeared as a single band of protein after affinity column chromatography. The estimated molecular weights of purified trypsin and chymotrypsin were found to be almost 23 kDa and 25 kDa, respectively (Figure 11).

1.2.4.2. SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE has proven to be a useful tool for the separation of protein subunits and the determination of enzyme molecular weights. The purified trypsin and chymotrypsin showing the single band proteins (Figure 12 and 13) had the molecular weights of 23 kDa and 25 kDa, respectively compared to the standard proteins (Figure-Appendix D12). These values were similar to those from anchovy (*Engraulis japonica*) of 25.6 and 26.1 kDa, respectively (Heu *et al.*, 1995), those from the pyloric caeca of Atlantic cod (*Gadus morhua*) of 24.2 kDa (Asgeirsson *et al.*, 1989) and 26 kDa (Asgiersson and Bjarnason, 1991), *respectively. Molecular weights of trypsin and*

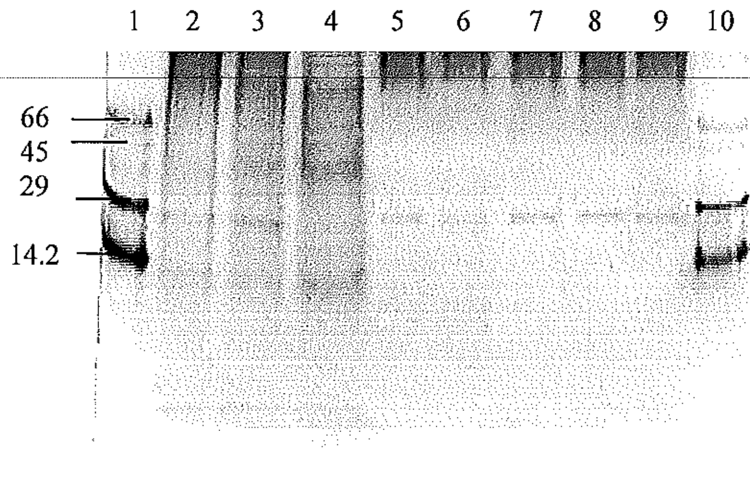


Figure 11 Nondenatured polyacrylamide gel electrophoresis of protein fractions obtained during purification of trypsin and chymotrypsin from yellowfin tuna (*Thunnus albacares*). Lane 1, protein standard ; 2, crude extract ; 3, 40-60% ammonium sulfate fraction ; 4, dialysis ; 5, DEAE-Sephadex A-50 II fraction of chymotrypsin ; 6 and 7, DEAE-Sephadex A-50 II fraction of trypsin, respectively ; 8, Sephadex G-100 II fraction of chymotrypsin ; 9, Sephadex G-100 II fraction of trypsin ; 10, protein standard.

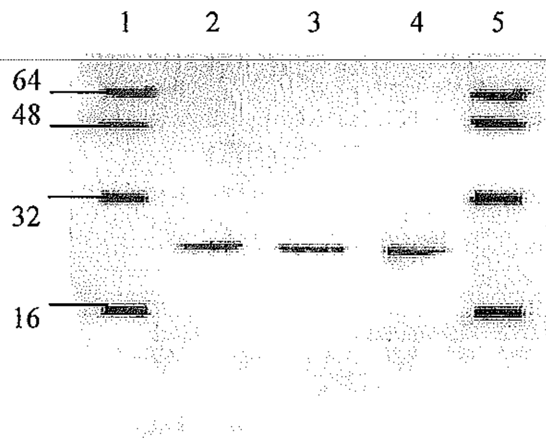


Figure 12 SDS - polyacrylamide gel electrophoresis of protein fractions obtained during purification of trypsin from yellowfin tuna (*Thunnus albacares*). Lane 1, protein standard ; 2, DEAE-Sephadex A-50 II fraction ; 3, DEAE-Sephadex A-50 III fraction ; 4, Sephadex G-100 II fraction ; 5, protein standard.

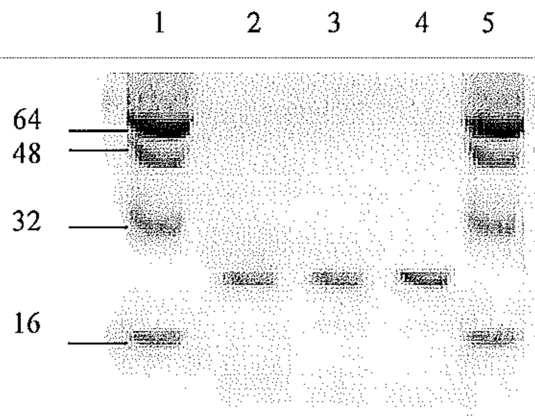


Figure 13 SDS - polyacrylamide gel electrophoresis of protein fractions obtained during purification of chymotrypsin from yellowfin tuna (*Thunnus albacares*). Lane 1, protein standard ; 2, DEAE-Sephadex A-50 II fraction ; 3, DEAE-Sephadex A-50 III fraction ; 4, Sephadex G-100 II fraction ; 5, protein standard.

1.3. Characterization of the purified trypsin and chymotrypsin

1.3.1. Effect of pH on enzyme activity and stability

1.3.1.1. Effect of pH on enzyme activity

The optimum pH for both trypsin and chymotrypsin activities was pH 8.0 (Figure 14). At physiological pH (pH 7.0), the relative activities of trypsin and chymotrypsin were 88.36% and 87.21% of the maximum values, respectively. Apparent pH-activity profile of trypsin from yellowfin tuna (of this experiment) was similar to the pH profiles of trypsin from anchovy (*Engraulis japonica*) (Heu *et al.*, 1995), *Engraulis encrasicholus* (Martinez *et al.*, 1988) and Atlantic cod (*Gadus morhua*) (Asgiersson *et al.*, 1989 ; Overnell, 1973). The pH is usually at about 8 where the serine proteases showed the maximal activity (Walsh and Wilcox, 1970). The optimum pH for trypsin activity from bovine and cationic salmon trypsin was in the range of 8.5 to 10.5 whereas a narrow pH optimum at pH 10.5 was observed for the anionic salmon trypsin (Outzen *et al.*, 1996). This was also similar to the optimum pH for trypsin from the pyloric caeca of cod at pH 9.6 (Shin and Zall, 1986), for trypsin activity from pancreas of cunner (*Tautogolabrus adspersus*) at pH 8.5 (Simpson and Haard, 1985a). However, autolysis on serine proteinases may occur only at pH above 5.0 (Outzen et al., 1996) and at low pH, serine proteases were very unstable under acidic conditions (Simpson and Haard, 1985a).

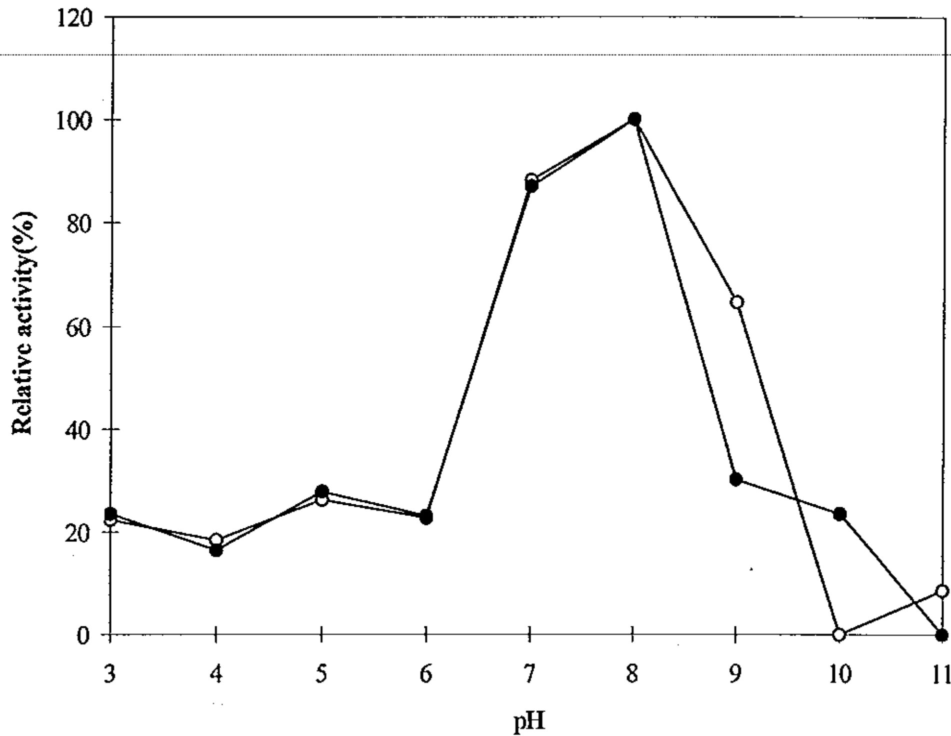
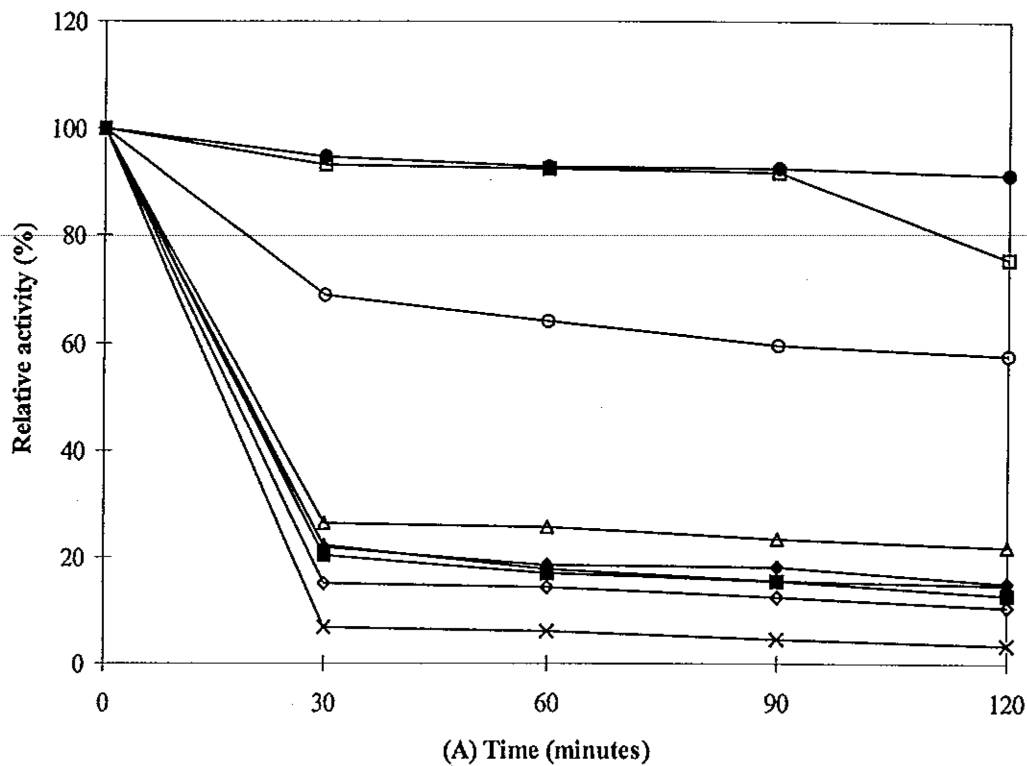


Figure 14 Effect of pH on the trypsin (o)and chymotrypsin (•) activities of yellowfin tuna (*Thunnus albacares*). Buffer used were 0.05 M citrate buffer (pH 3.0-6.0), 0.05 M Tris-HCl buffer (pH 7.0-9.0) and 0.05 M carbonate-bicarbonate buffer (pH 10.0-11.0).

The optimum pH of chymotrypsin in this experiment was similar to the optimum pH of chymotrypsin anchovy (Heu *et al.*, 1995) and cod (Overnell, 1973). Asgiersson and Bjarnason (1991) reported that the chymotrypsin from Atlantic cod had a bell-shaped pH profile for the hydrolysis of BzTyrOEt with the optimum pH at 7.8 and the optimum pH for bovine chymotrypsin was closed to pH 7.8. Therefore, the pH dependence of the two enzymes was very similar as can be expected for enzymes with the same reaction machanism.

1.3.1.2. Effect of pH on enzyme stability

Results on the pH-stability of the purified enzymes extracted from spleen and pancreas are shown in Figure 15 (A and B). The best stability of these enzymes was found to be at pH 8.0, with 93.01 and 90.0 % retained activity, respectively at 60 minutes, and 91.92 and 82.50 %, respectively at 120 minutes. However, they were stable in the pH range of 7.0 - 8.0. Results were similar to the trypsin A and trypsin B from digestive tract of anchovy that were most stable in the range of pH 7 - 8 and 8 - 9, respectively (Martinez *et al.*, 1988). The trypsin-like enzyme from the pyloric caeca of cod were stable from pH 8.8 - 9.6 at 60 minutes incubation (Shin and Zall, 1986). However, the Atlantic cod trypsin showed the maximal activity at pH 8.0 and the enzyme lost activity slowly at 5 °C even at neutral pH (Asgeirsson *et al.*, 1989).



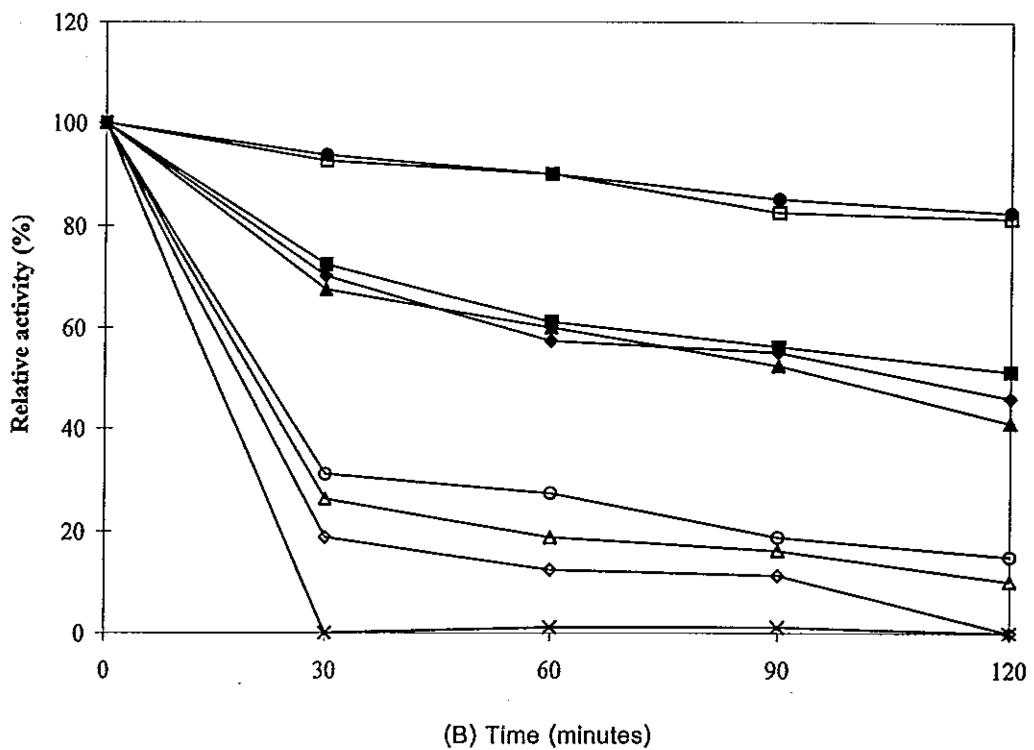


Figure 15 pH stability of trypsin (A) and chymotrypsin (B) from yellowfin tuna. Enzyme solution was dissolved in varying pH (3.0 [♦], 4.0 [■], 5.0 [▲], 6.0 [Δ], 7.0 [□], 8.0 [•], 9.0 [o], 10.0 [◊] and 11.0 [×]) incubated at 4°C.

1.3.2. Effect of temperature on enzyme activity and stability

1.3.2.1. Effect of temperature on enzyme activity

The enzyme activities were assayed at various temperature, 0 - 90°C, using optimum pH buffer at 8.0. While temperature increased, the initial velocity or catalytic activity of enzyme increased simultaneously (Figure 16). At 25°C, the relative activity of trypsin was 59 % while about 52 % for chymotrypsin. The highest relative activities of trypsin and chymotrypsin were achieved at 50°C, and decreased thereafter due to the destruction of the enzymes at high temperatures. Their relative activities were 95.10 and 88.91 % at 60°C and 28.60 and 65.11% at 70°C, respectively. Trypsin activity consequently was reduced more rapidly than the activity of chymotrypsin when temperature heated up to 70°C. The relative activities were decreased at 80°C to 27.33 and 47.59 % respectively and 22.27 and 34.89 % respectively at 90°C. The higher temperatures were reported to cause the instability of the cod trypsin and increase autolysis (Asgeirsson *et al.*, 1989). These are similar to optimum temperature (50°C) of protease activity from skipjack (*Katsuwonus pelamis*), yellowfin (*Thunnus albacares*) and albacore (*Thunnus alalunga*) (Prachumratana, 1998). In addition, anchovy trypsin and chymotrypsin had the maximal activity toward casein and synthetic substrates at 45°C (Heu *et al.*, 1995). A more flexible structure is needed for an enzyme adapted to work at low temperatures (Asgeirsson and Bjarnason, 1991).

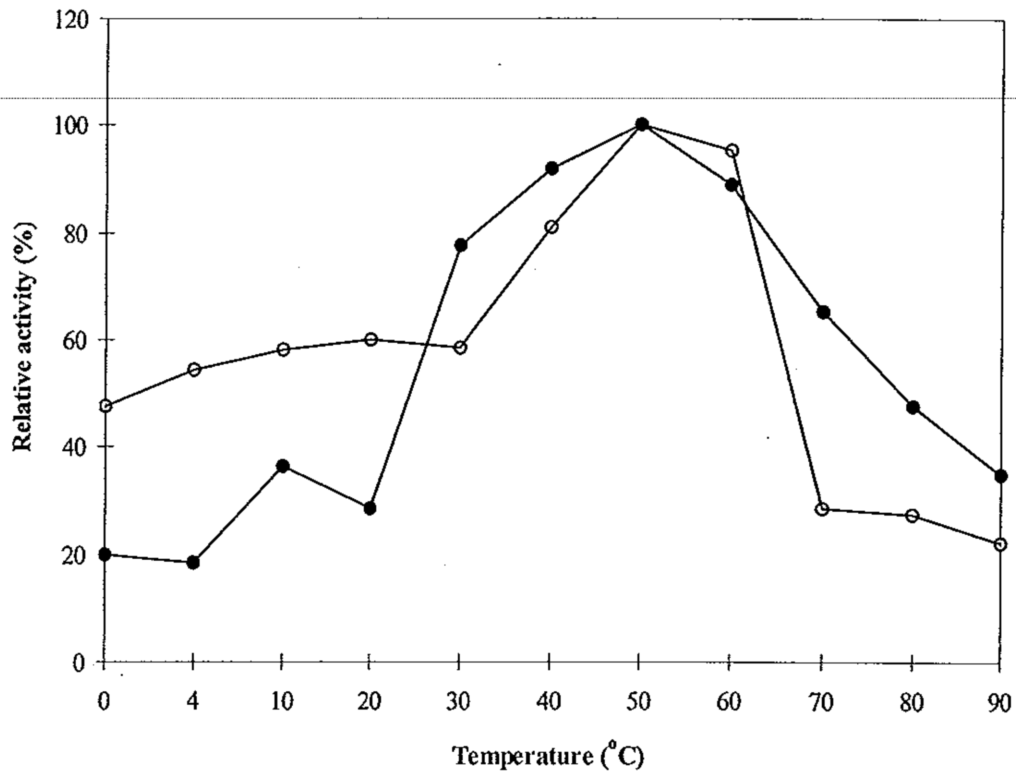
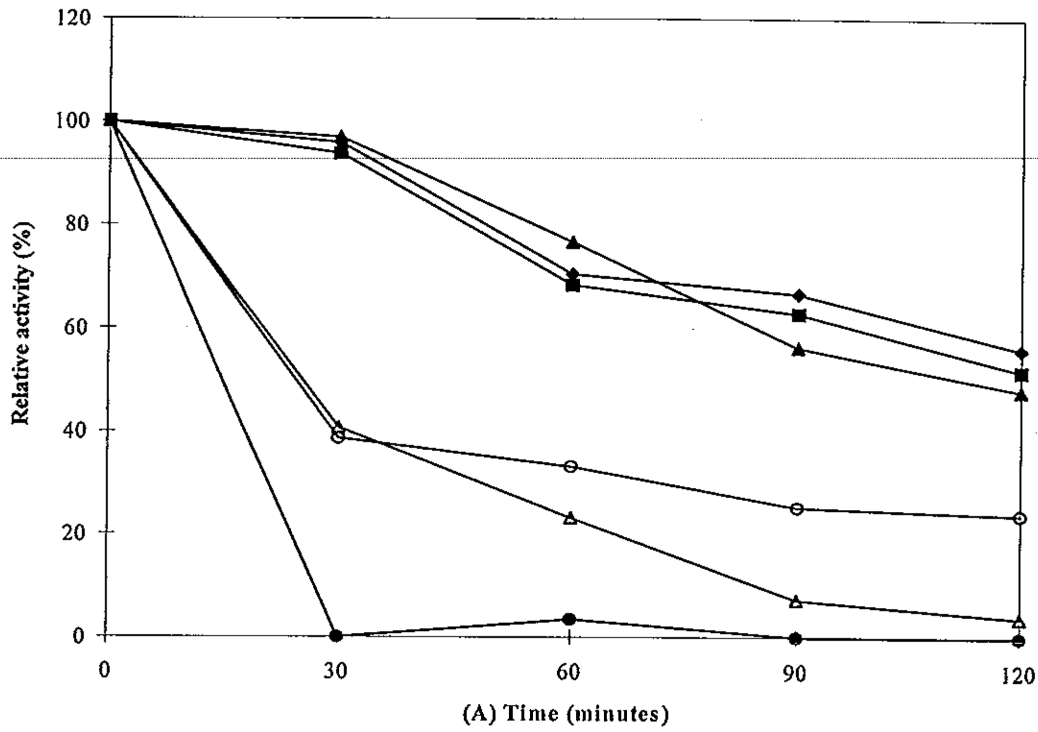


Figure 16 Effect of temperature on the activity of trypsin (o) and chymotrypsin (•) at pH 8.0.

1.3.2.2. Effect of temperature on enzyme stability

The enzyme solution was incubated for 30 - 120 minutes at 30, 40, 50, 60, 70 and 80°C, then determined for the retained activities (Figure 17). Trypsin activity affected slightly at 30°C for 30 minutes incubation. The residual activity of 95.85 % and 55.72 % were obtained after 120 minutes incubation. For trypsin activity, treatment at 40°C and 50°C gave approximately 93.70 and 97.01 % residual activity, respectively for 30 minutes, and 51.58 and 48.09 %, respectively for 120 minutes incubation. Chymotrypsin was stable with 91.30 and 63.0 % residual activity for 30 and 120 minutes, respectively at 30°C. While treatments up to 40°C could remain 91.3% for 30 minutes and 47.1% for 120 minutes. At 50°C the retained activity of 81.2% and 47.8% were achieved after 30 minutes and 120 minutes incubation. Both enzymes were rapidly inactivated at 60°C for 120 minutes giving the residual activities of trypsin and chymotrypsin of 23.88 and 10.9 %, respectively. At 70°C for 120 minutes, the values were 3.81 % residual activity of trypsin and 12.3% for chymotrypsin. Trypsin and chymotrypsin were completely inactivated at 80°C after 90 and 120 minutes, respectively. They had high stability at 50°C for 60 minutes which were similar to the thermal stability of protease from tuna viscera at 37 - 40°C. The enzymes extracted from spleen showed the best thermal stability and the protease activity from the extracted enzyme decreased to a half, after it was incubated at 60°C for more than 120 minutes (Prachumratana, 1998). Chymotrypsin from Atlantic cod (*Gadus morhua*) showed its high thermal stability at 30 to 35°C and rapidly decreased at higher temperature (Asgeirsson and Bjarnason, 1991). However, less or no



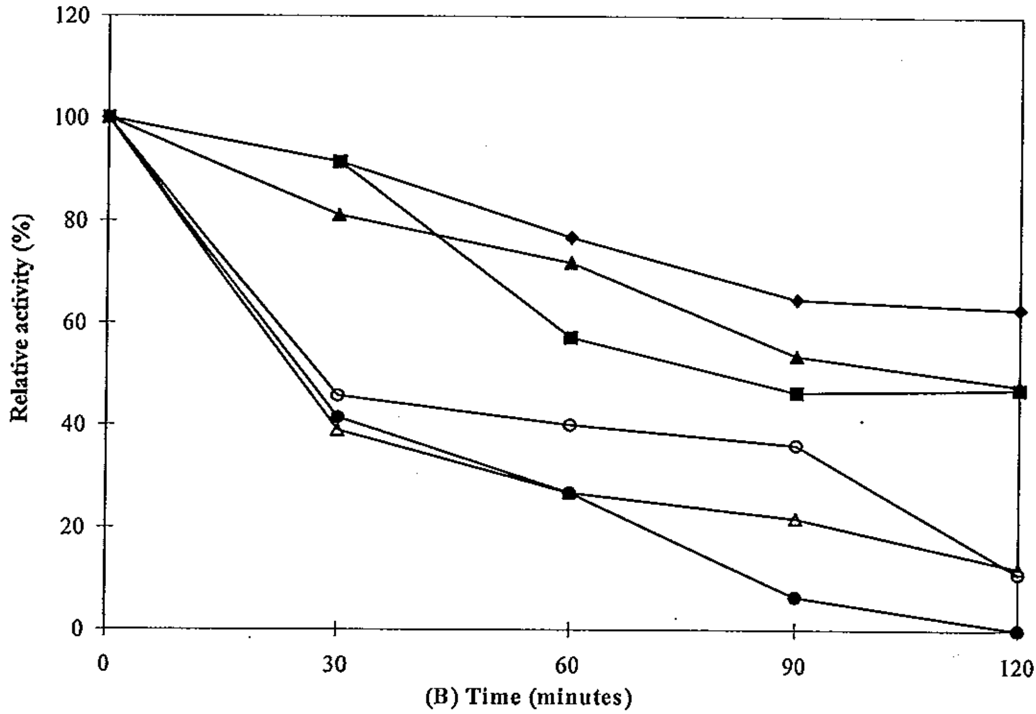


Figure 17 Thermal stability of trypsin (A) and Chymotrypsin (B) at various temperature [30°C (♦),40°C (■),50°C (▲),60°C (o),70°C (Δ) and 80°C (•),]

inactivation of chymotrypsin from Atlantic cod was detected at temperatures below 50°C (Raae, 1990). Cod chymotrypsin practically lost no enzyme activity when incubated for 50 minutes at 50°C but was rapidly inactivated when incubated at 68°C. Moreover, they are similar to the experiment of Heu *et al.* (1995) which indicated that both enzymes were denatured very rapidly over 50°C.

The rapid inactivation of the native enzymes observed at 70°C could therefore be due to thermal distortion of the 3-dimentional polypeptide structure which renders the enzymes susceptible to auto-digestion (Raae, 1990) and the partial unfolding of the enzyme molecule including the strengthening of hydrophobic interactions and disulfide bond in the interior of the protein molecule (Heu *et al.*, 1995).

1.3.3. Effect of ions and chemicals on enzyme activity

The remaining enzyme activities were measured after the reaction mixture was incubated with the addition of ions and chemicals dissolved in buffer pH 8.0 at various concentrations.

Effect of ions and chemicals on trypsin and chymotrypsin activities are demonstrated in Table 17. Benzamidine at the concentration of 86.67 μM inhibited of trypsin and chymotrypsin activities for the residual activity of 70.55 and 92.03 %, respectively. These were slightly lower than the residual activity of trypsin and chymotrypsin from anchovy after treated with 100 μM benzamidine, 83 and 106 % respectively (Heu *et al.*, 1995) and higher than 69% remained activity of cod trypsin after treated with 0.005 mM benzamidine (Asgiersson *et al.*, 1989).

Table 17 Effect of ions and chemicals to trypsin and chymotrypsin activities from yellowfin tuna (*Thunnus albacares*)

Chemicals	Concentration in buffer (2.6ml) (μM)	Final concentration in total vol. (3ml) (μM)	Residual activity (%)	
			Trypsin	Chymotrypsin
None (control)	-	-	100	100
Benzamidine	0.1	0.087	102.25	101.26
	1	0.87	99.59	100.00
	10	8.67	98.77	98.71
	100	86.67	70.55	92.03
	1,000	866.67	67.89	90.62
	10,000	8,666.67	68.51	60.00
EDTA	0.1	0.087	44.17	4.00
	1	0.87	36.74	1.30
	10	8.67	35.79	1.30
	100	86.67	25.15	0.00
	1,000	866.67	24.13	0.00
	10,000	8,666.67	21.06	0.00
1-10 Phenantroline	0.1	0.087	68.30	49.30
	1	0.87	66.87	24.00
	10	8.67	60.33	16.00
	100	86.67	56.03	14.70
	1,000	866.67	54.81	8.00
	10,000	8,666.67	54.81	2.70
4-Aminobenzoic acid	0.1	0.087	43.15	54.70
	1	0.87	40.90	33.30
	10	8.67	36.40	32.00
	100	86.67	34.56	26.70
	1,000	866.67	34.36	21.30
	10,000	8,666.67	33.95	21.30

Table 17 (continue)

Ions	Concentration in buffer (2.6ml) (μM)	Final concentration in total vol. (3ml (μM)	Residual activity (%)	
			Trypsin	Chymotrypsin
K^+	0.1	0.087	38.85	65.30
	1	0.87	33.33	62.70
	10	8.67	33.33	57.30
	100	86.67	29.65	56.00
	1,000	866.67	29.24	52.00
	10,000	8,666.67	26.58	46.70
Ag^+	0.1	0.087	58.49	48.00
	1	0.87	57.87	44.00
	10	8.67	52.35	44.00
	100	86.67	50.92	44.00
	1,000	866.67	46.42	41.30
	10,000	8,666.67	16.97	34.70
Hg^{++}	0.1	0.087	85.89	21.30
	1	0.87	85.07	13.30
	10	8.67	84.66	12.00
	100	86.67	84.25	2.70
	1,000	866.67	84.25	0.00
	10,000	8,666.67	76.69	0.00

EDTA at 0.87 μM reduced trypsin activity with 36.74% remained activity and completely inactivated the activity of chymotrypsin. These were lower the trypsin-like enzymes A and B from anchovy which were readily inhibited by EDTA with 66 and 40% residual activity, respectively (Martinez *et al.*, 1988). Calcium ions consequently stabilize enzyme solutions by preventing autolysis of the enzyme (Shin and Zall, 1986) and the removal of divalent ions by EDTA caused loss of activity in cod trypsin (Asgiersson *et al.*, 1989). This coincides with the results of this study, therefore trypsin and chymotrypsin from viscera of yellowfin tuna required calcium ions as cofactors for their activity. Trypsin and chymotrypsin inhibition by 8,666.67 μM of 1-10 phenantroline gave the retained activity of 54.81 and 2.70%, respectively This residual trypsin activity was higher than the alkaline proteinase from shrimp (*Penaeus indicus*) muscle that remained 41% activity after treated with o-phenantroline (Doke and Ninjoor, 1987) while it obtained lower residual chymotrypsin activity.

The 8,666.67 μM of K^+ inactivated trypsin and chymotrypsin activity with 26.58 and 46.70% residual activity respectively. The Ag^+ at 866.67 μM inhibited 53.58 % of trypsin activity and 58.70% chymotrypsin activity which were higher than the report of Shin and Zall (1986). The cod trypsin was reduced its activity by Ag^+ and Cu^{++} with the residual activities of 5.1 and 57.3 % respectively. Trypsin activity was inhibited partially by 8,666.67 μM Hg^{++} with a residual activity of 76.69 % and completely inhibited chymotrypsin activity. Trypsin activity was partially inactivated by Hg^{++} while chymotrypsin was inhibited as similar as the experiment of Jany (1976) in which 0.87

mM Hg^{++} reduced the activity of trypsin and chymotrypsin with 4 and 9 % remained activity, respectively.

Trypsin and chymotrypsin activity were reduced by 4-aminobenzoic acid at the concentration of 8,666.67 μM with the residual activity of 33.95 and 21.30% respectively.

1.3.4. Kinetic parameters

Kinetic parameters of trypsin and chymotrypsin from yellowfin tuna at 50°C were determined using Lineweaver-Burk plot as given in Figure 18 and 19, respectively. For trypsin, the K_m value was 0.49 mg/ml and the V_{max} was 111.11 μmol/ml/min. For chymotrypsin, the K_m value was 0.27 mg/ml and the V_{max} value of 50 μmol/ml/min. These results were similar to the K_m values of trypsin and chymotrypsin from anchovy (*Engraulis japonica*) which were 0.840 and 0.397 mg/ml, respectively (Heu *et al.*, 1995). Asgiersson *et al.* (1989) studied on the K_m values of trypsin (I, II and III) from Atlantic cod using TosArgOMe substrate, were 0.029, 0.021 and 0.049 mM, respectively and when the BzArg-NH-Np was used as substrate, the values would be 0.077, 0.094 and 0.102 mM, respectively. The K_m value of Atlantic cod chymotrypsin (A and B) was 0.510 mM (Asgiersson and Bjarnason, 1991).

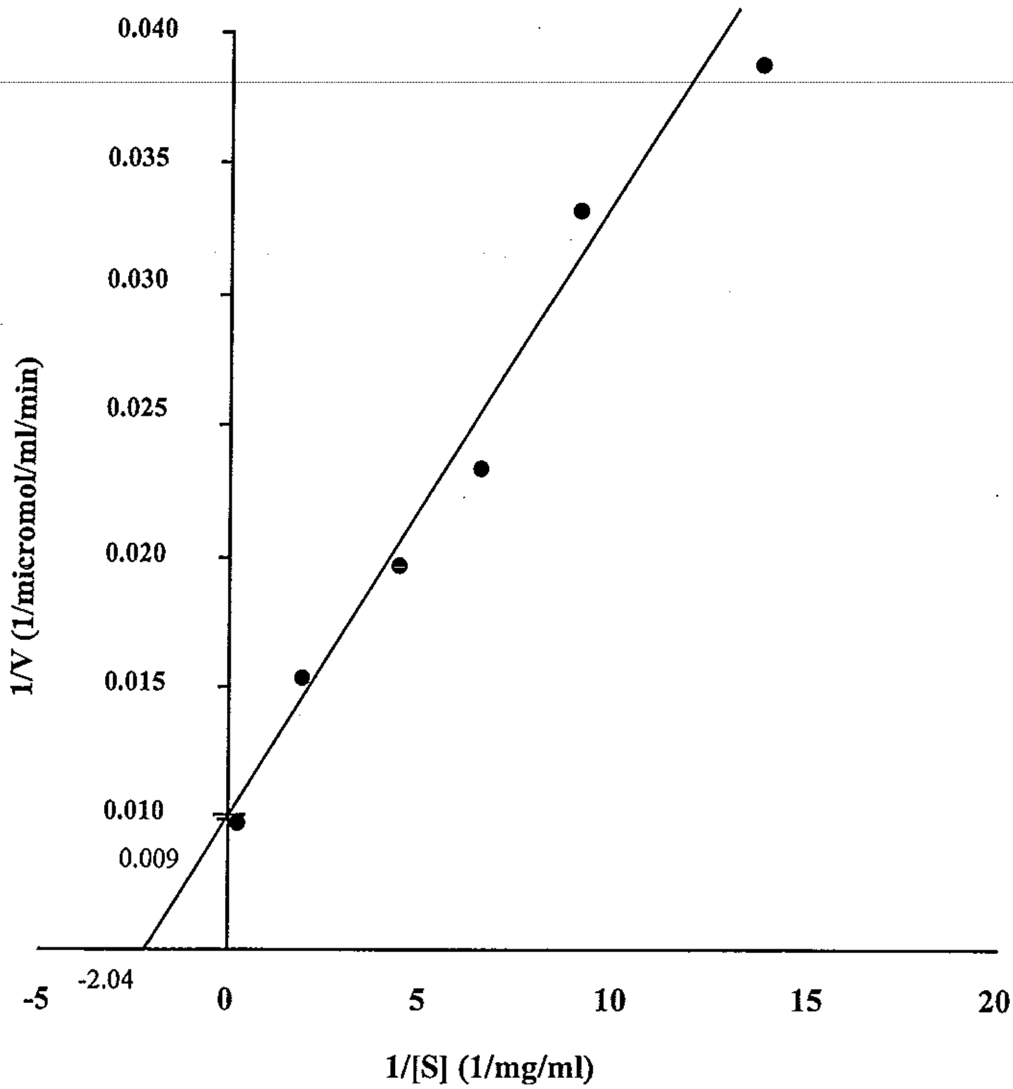


Figure 18 Lineweaver-Burk plot of trypsin activity in various TAME concentrations at pH 8.0, 50°C.

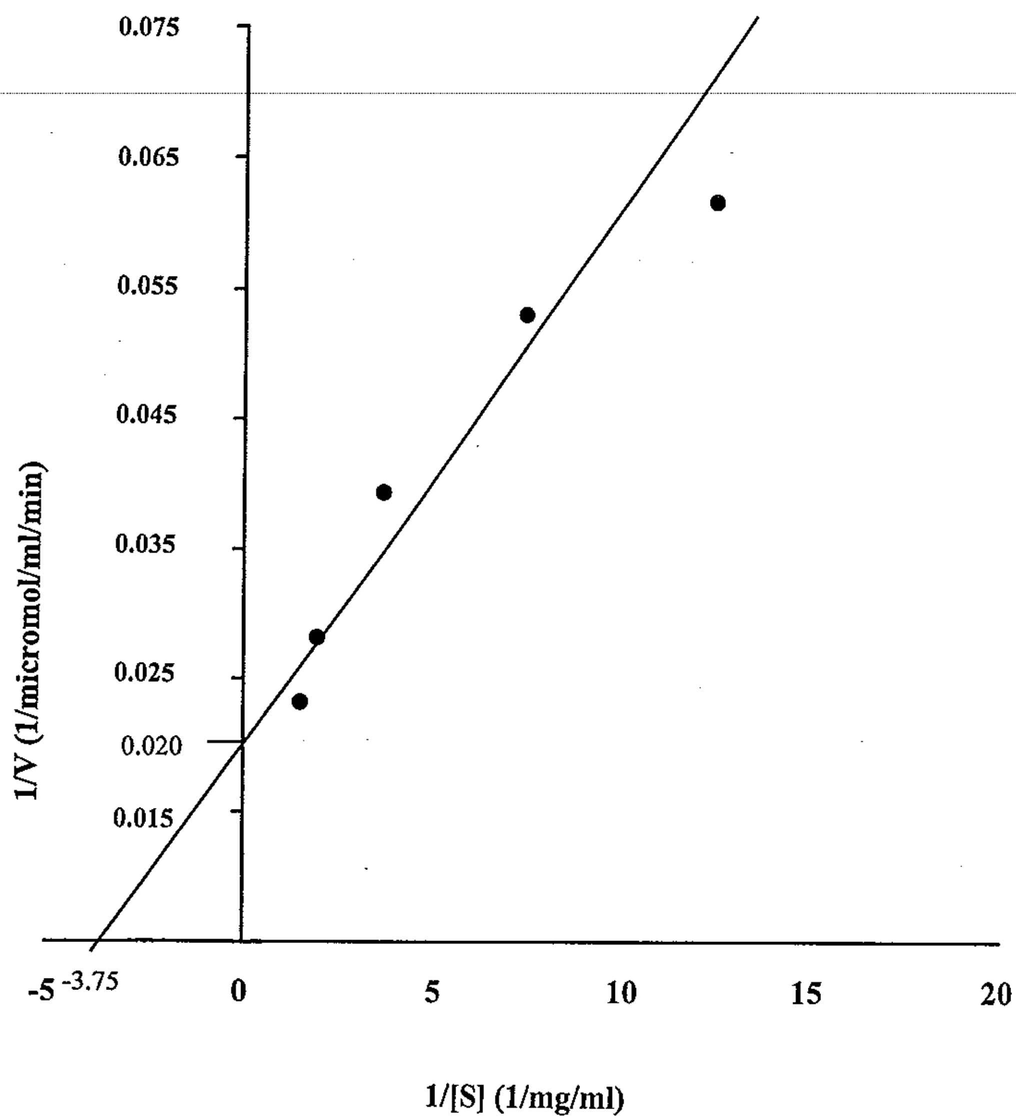


Figure 19 Lineweaver-Burk plot of chymotrypsin activity in various BTEE concentrations at pH 8.0, 50 °C.

2. Enzyme application

Crude enzyme extracted from viscera of yellowfin tuna was used to produce fish extract, a food flavorant or food additive, from tuna condensate and compared with the commercial trypsin and chymotrypsin enzyme. The crude enzyme solution possessed the initial trypsin and chymotrypsin activities 109.44 and 16.29 units/ml with the specific activities of 43.78 and 6.52 units/mg protein, respectively. The crude enzyme solution was mixed with and without 20 % (w/v) skim milk, as a protective agent during freeze dry. Without protective agent, the activities of trypsin and chymotrypsin were 74.63 and 13.07 units/ml, respectively with the specific activities of 3.24 and 0.57 units/mg protein, respectively. In the presence of the protective agent, trypsin and chymotrypsin activities from crude enzyme were 63.33 and 10.58 units/ml, respectively, and 2.75 and 0.46 units/mg protein, respectively for their specific activities. These results indicated that there was no significant difference whether 20 % skim milk was added or not. The decrease on the activity of trypsin and chymotrypsin for crude enzyme powder was in the range of 31.81 - 42.13 and 19.77 - 35.05 %, as well as 92.60 - 93.72 and 91.26 - 92.94 % for their specific activities, respectively, compared to the crude enzyme solution.

Crude enzyme powder and the commercial enzymes were used in fish extract production (Figure 20). Fish extract produced by using the commercial enzymes were slightly clearer than that using the crude enzyme. This may due to the presence of many proteins in the crude enzyme. Chemical composition of fish extract produced was compared to the standard composition (Table 18). Moisture content of the

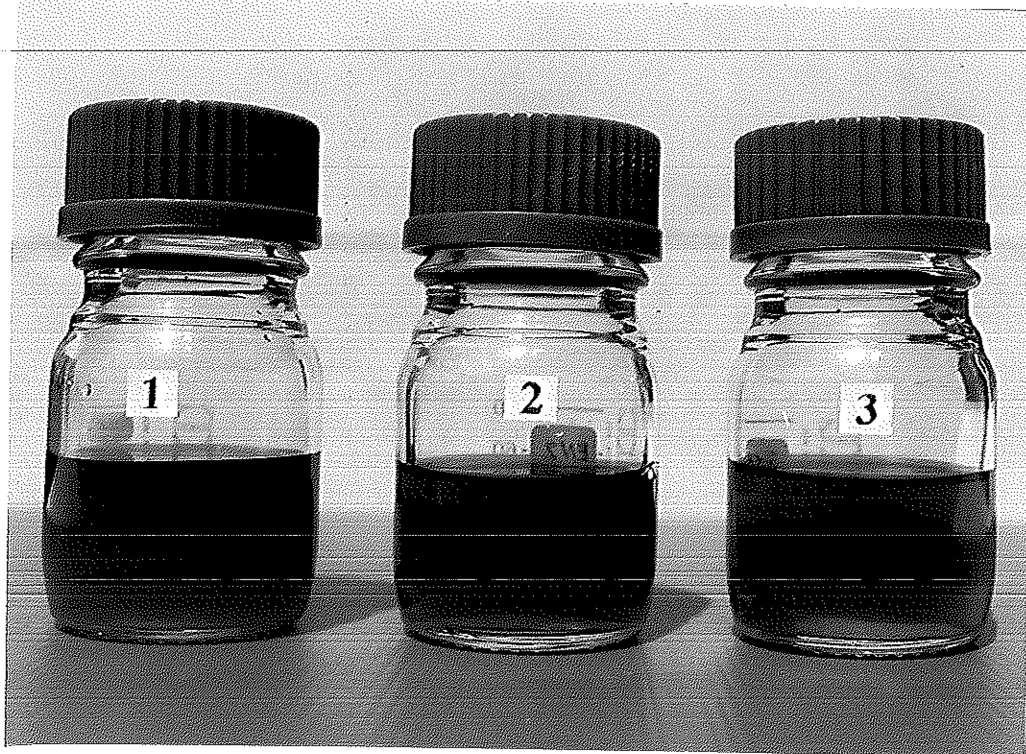


Figure 20 Fish extract before evaporation step using crude enzyme from viscera of yellowfin tuna (1) in comparison with the commercial trypsin (2) and chymotrypsin (3).

Table 18 Comparison on the quality of fish extract produced by crude enzymes from viscera of yellowfin tuna (*Thunnus albacares*) and the commercial enzymes to the standard specification of fish extract

Chemical composition	Tuna condensate	Fish extract			
		Crude enzyme	Trypsin	Chymotrypsin	Standard[1]
Color	yellow-brown	dark brown	light brown	light brown	brown
Smell	tuna fishy	no fishy	no fishy	no fishy	no fishy
pH	6.79	7.53	7.46	7.54	6.45
Moisture (%)	87.56	94.34	94.87	94.52	43.53
Total solid (%)	8.35	6.91	4.97	5.16	56.47
Ash (%)	2.85	0.88	0.31	0.30	17.95
Salt (%)	6.90	4.90	5.20	5.40	10.40
Crude fat (%)	5.00	0.28	0.13	0.12	0.60
Soluble protein (mg/l)	-[3]	15.20	15.00	15.00	-[2]
Total nitrogen (%)	8.92	11.17	11.11	11.25	6.56
Nonprotein nitrogen (NPN) (%)	2.63	5.82	4.77	4.64	5.63
Total amino nitrogen (%)	0.20	0.40	0.17	0.17	1.43
Total volatile nitrogen (TVN) (mg%)	-[3]	28.00	14.00	14.00	309.00
Trimethylamine nitrogen (TMA-N) (mg%)	-[3]	0.00	0.00	0.00	little
Calcium (%)[4]	5.13	5.58	4.94	5.00	0.43
Phosphorus (P_2O_5,%)[5]	15.47	32.64	10.50	9.85	2.77

Table 18 (continue)

Chemical composition	Tuna condensate	Fish extract: Crude enzyme	Fish extract: Trypsin	Fish extract: Chymotrypsin	Standard[1]
Degree of hydrolysis (%)	-[3]	52.10	42.93	41.23	-[2]
Total plate count (CFU/ml)	-[3]	2.15×10^3	4.90×10^2	5.90×10^2	-[2]
Escherichia coli (MPN/100 ml)	-[3]	0.00	0.00	0.00	-[2]
Salmonella	-[3]	0.00	0.00	0.00	-[2]

[1]Source : Indo-Pacific Fisheries Council Regional Studies (1967) cited by Anon (1991)

[2]not shown

[3]not determined

[4]assayed by Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES) method
from Central Equipment Division, Faculty of Science, Prince of Songkla University

[5]assayed by spectroquant NOVA60 (MERCK) spectrophotometer method from Central Equipment Division, Faculty of Science, Prince of Songkla University

fish extract produced by using the commercial trypsin and chymotrypsin were 94.87 and 94.52 %, respectively which was similar to those of fish extract using crude enzyme (94.34) and the fish extract produced by using the immobilized proteases bioreactor (92.90 %) (Leelawat and Anprung, 1994a) and fish sauce (68 %) (Gildberg and Xian-Quan, 1994). However, these products had higher moisture content than the standard value (43.53%). This was due to the lower viscosity of fish extract produced without evaporation step. The total solid of the three fish extract (6.91, 4.97 and 5.16 %, respectively) were consequently much lower than the value specified in the standard fish extract (56.47 %). This was also the result of several times filtration in order to remove any precipitate occured. Using the crude enzyme, the product had ash content (0.88 %) higher than those using the trypsin and chymotrypsin (0.31 and 0.30 %, respectively). On the other hand, they were lower than the ash content of standard (17.95 %) which may be the consequence of lower total solid of the products. Using crude enzyme gave 4.90 % salt in the fish extract and 5.20 and 5.40 % from using trypsin and chymotrypsin, respectively which were lower when compared to salt content in the tuna condensate. This was due to the desalting step during the experiment.

There was less or no fat content in the fish extract since it was removed by heating and skimmed off during the process. This was similar to the study of Gildberg and Xian-Quan (1994). Fish protein extract should contain less than 0.20 % fat for food preservation (Trirekphan, 1997). Furthermore, the lower fat content had resulted in less competitive water binding of proteins to increase solubility from enzyme hydrolysis (Hoyle and Merritt, 1994).

The crude protein concentration of 69.81% when using crude enzyme had no significant difference to those values from using trypsin and chymotrypsin (69.44 and 70.31%, respectively). These values were higher than those of the standard fish extract (41.00 %) and the fish extract (0.2 % protein) produced by immobilized protease bioreactor (Leelawat and Anprung, 1994a). The crude protein contents of these products were higher than that in tuna condensate (55.75 %) since the enzyme which absorbed rapidly onto the insoluble protein particles could cleave the polypeptide chains that were loosely bound to the surface. More compacted core proteins were hydrolyzed more slowly and the rate of enzymatic cleavage of peptide bond controlled the overall rate of hydrolysis (Benjakul and Morrissey, 1997).

Using crude enzyme gave higher nonprotein nitrogen, NPN, (5.82 %) than those from using trypsin and chymotrypsin (4.77 and 4.64 % NPN, respectively). These were similar to the NPN value of standard specification (5.63 % NPN). Nonprotein nitrogen compounds such as amino acids, amines, amides, purines, and pyrimidines affected on protein analysis and food ingredients such as in high temperature processed and stored foods. In the latter case, amino acids have undergone deamination and other chemical reaction including autolysis caused formation of free amino acids from protein, lactate from glycogen, hypoxantines from nucleotides (Morr and Ha, 1995). The NPN must be removed prior to analysis (Morr and Ha, 1995).

The total volatile nitrogen (TVN) value of fish extract (28 mg%) using the crude enzyme was higher than those using trypsin and chymotrypsin (both were 14 mg%). They were similar to those in fish sauce produced by enzyme hydrolysis, 11.89 and 38.55 mg% TVN at

the incubation time of 1 and 7 days, respectively (Poosaran, 1986). However, the values were significantly less than the standard specification (309 mg% TVN). The strong smell of fish sauce was detected due to high total volatile nitrogen (TVN) content (Poosaran, 1986), including trimethylamine nitrogen (TMA-N), ammonia, pyrazine, pyridines and pyrrols which was a better estimator during later stages of spoilage (Hong *et al.*, 1996). TMA-N was generally presented in seawater fish and used as a spoilage indicator (Magnusson and Martinsdottir, 1995). In this experiment, no TMA-N was detected in any products.

Calcium content (5.58 %) in the fish extract using crude enzyme was higher than those from using trypsin and chymotrypsin (4.94 and 5.00 %, respectively). These were similar to the calcium content in tuna condensate but higher than that of standard fish extract. The higher content of calcium in fish extract (5.58 %) using crude enzyme than others (4.94 and 5.00 % using trypsin and chymotrypsin, respectively) was due to the presence of calcium in the buffer used for the extraction of enzymes. For phosphorus, the fish extract using crude enzyme gave higher phosphorus content (36.64 %) than those of using trypsin and chymotrypsin (10.50 and 9.85 %, respectively) as well as that in the standard specification (2.77 %). Most of the phosphorus content of fish extract was from tuna condensate (15.47 %) and in the case of using crude enzyme, viscera was another source of phosphorus content in this experiment. The decrease of calcium and phosphorus in the fish extract using the commercial enzymes may due to the removal by centrifugation.

Degree of hydrolysis (DH) has been used as an indicator for the cleavage of peptide bond (Benjakul and Morrissey, 1997). DH of fish extract using crude enzyme (52.10 %) was higher than those using trypsin and chymotrypsin (42.93 and 41.23 %, respectively). In addition, the DH of fish extract using the two commercial enzymes were in the range of the values obtained in fish protein hydrolysates from herring (*Clupea harengus*) using alcalase and papain (26.8 - 44.7% and 23.84 - 43.14 %, respectively) (Hoyle and Merritt, 1994).

For fish extract using crude enzyme, the total plate count (2.15×10^3 CFU/ml) was higher than those using trypsin and chymotrypsin (4.90×10^2 and 5.90×10^2 CFU/ml, respectively), but similar to the commercial fish extract ($< 3.00 \times 10^3$ CFU/ml). *Salmonella* and *E.coli* were not found in the fish extract.

The fish extract using crude enzyme was then evaporated and compared with the commercial product of Songkla Canning (Thailand) Co., Ltd. that used alkaline protease (Alcalase) in the process (Table 19 and Figure 21). After evaporation and desalting, the decrease of pH of fish extract using crude enzyme was apparent. This was similar to the standard specification (pH 6.45) and the commercial product (pH 5.6 - 6.4). Moisture content was decreased to 40.89 % which was close to the standard (43.53 %) but slightly higher than that of the commercial product (32 - 36 %). Using crude enzyme, the protein content of fish extract was 45.86 % which was the result of protein denaturation and precipitation in high salt concentration and temperature during evaporation step. This was similar to the standard specification (41 %) and higher than 20 % protein content. Salt concentration increased to

14.50 % in the fish extract using crude enzyme which was similar to that of the commercial product (12 - 14 %). Using crude enzyme gave fish extract with 74 % brix and total plate count of 2.00×10^3 CFU/ml which were similar to those specifications of the commercial product (74-76 % brix and $< 3.00 \times 10^3$ CFU/ml).

Table 19 Chemical composition of fish extract using crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) before and after evaporation and desalting step compared to those of the commercial product of Songkla Canning (Thailand) Co., Ltd.

Chemical composition	The commercial product	Fish extract using crude enzyme	
		Before	After
Color	dark brown	dark brown	dark brown
pH	5.6 - 6.4	7.53	6.48
Moisture (%)	32 - 36	94.34	40.89
Protein (%)	> 20	69.81	45.86
Salt (%)	12 - 14	4.90	14.50
Brix (%)	74 - 76	12.20	74.00
Total plate count (CFU/ ml)	$< 3.0 \times 10^3$	2.15×10^3	2.0×10^3

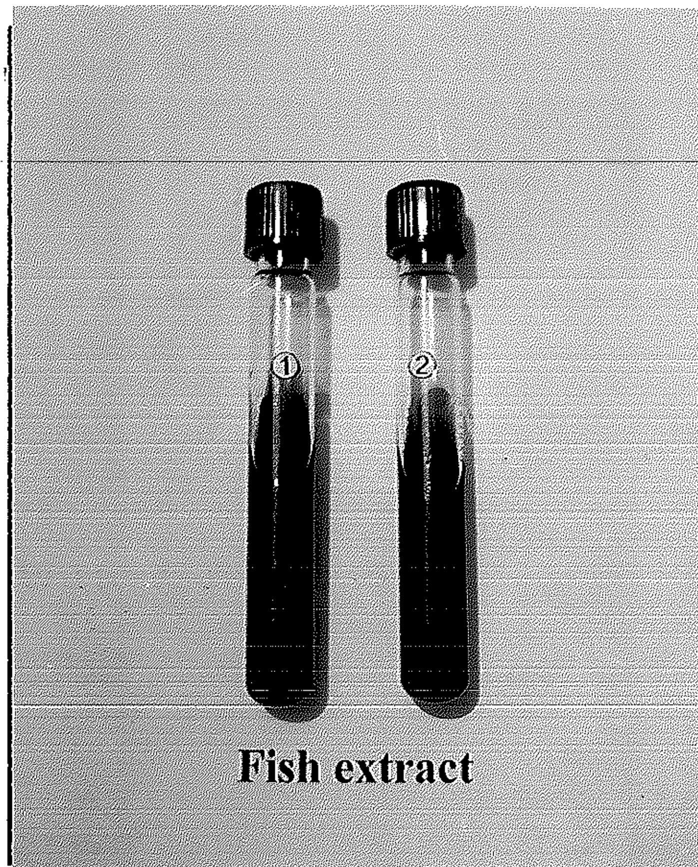


Figure 21 Fish extract using crude enzyme from viscera of yellowfin tuna (*Thunnus albacares*) (2) in comparison with the commercial product (1) of Songkla Canning (Thailand) Co., Ltd.

Chapter 4

Conclusions

1. Extraction of enzymes from tuna viscera

The viscera of yellowfin tuna (*Thunnus albacares*) was used for enzyme extraction. Spleen and pancreas were found to be the best sources for trypsin and chymotrypsin, respectively.

2.Purification of trypsin and chymotrypsin

Trypsin and chymotrypsin were purified from spleen and pancreas of yellowfin tuna respectively by 40-60% sat. ammonium sulfate precipitation, dialysis, gel filtration of Sephadex G-100 column (I), affinity column chromatography of benzamidine-Sepharose column and anion column chromatography of DEAE-Sephadex A-50 column (I, II and III) and gel filtration of Sephadex G-100 column (II). Trypsin and chymotrypsin gave 53.42 and 11.36 units/mg protein, respectively. The purification factors increased about 15 and 14 fold with the yields of 0.35 % and 1.04 %, respectively.

3. Characterization of purified trypsin and chymotrypsin

3.1. The apparent molecular weight of trypsin was 23 kDa and 25 kDa for chymotrypsin from SDS-polyacrylamide gel electrophoresis.

3.2. The trypsin and chymotrypsin had the optimum pH and temperature at pH 8.0 and 50°C, respectively. They were stable in the pH range of 7.0 - 8.0 with the residual activity over 75 % at 120 minutes

and thermostable up to 50°C for 60 minutes with residual activity of 56% for trypsin and 72% for chymotrypsin.

3.3. The trypsin activity was slightly increased upon the addition of benzamidine and was inhibited by EDTA, 1-10 phenantroline, K^+, Ag^+, Hg^{++} and 4-aminobenzoic acid. Chymotrypsin was activated by benzamidine and partially inhibited by 1-10 phenantroline, K^+, Ag^+ and 4-aminobenzoic acid, and completely inhibited by EDTA and Hg^{++}.

3.4. Trypsin and chymotrypsin had the K_m values of 0.49 and 0.27 mg/ml, respectively as well as V_{max} values of 111.11 and 50 μ mol/ml/min, respectively.

4. Fish extract production

Crude enzyme from viscera of yellowfin tuna was used for the production of fish extract from tuna condensate in comparison to those using the commercial trypsin and chymotrypsin. There were no significant difference in chemical composition of the fish extract from two sources of enzymes. After evaporation and desalting step, the fish extract using crude was similar to the commercial product of Songkla Canning (Thailand) Co., Ltd. This indicated that the enzymes from viscera of yellowfin tuna was as efficient in protein hydrolysis as the commercial enzymes.

Suggestions

1. Study on the protection of crude enzyme powder including the comparison on conventional drying including tray drying and other drying methods such as spray-dry and freeze-dry.
2. Production of fish extract by protease immobilized on liposome (Blocher *et al.*, 1999) should be investigated.

References

Anon. 1991. Agro-industrial goods of by product from canned tuna factory. Department of Industrial Promotion, Ministry of Industry.

A.O.A.C. 1980. Official Methods of Analysis of the Association of Official Analytical Chemists 13th ed. The Association of Official Analytical Chemists, Inc. Verginia Alrington.

A.O.A.C. 1990. Official Methods of Analysis of the Association of Official Analytical Chemists 15th ed. The Association of Official Analytical Chemists, Inc. Verginia Alrington.

Arunchalam, K. and Haard, N.F. 1985. Isolation and characterization of pepsin from polar cod (*Boreogadus saida*). Comp. Biochem. Physiol. 80B : 467-473.

Asgiersson, B., Fox, J.W. and Bjarnason, B.1989. Purification and characterization of trypsin from the poikilotherm *Gadus morhua*. J. Biochem.180 : 85-94.

Asgiersson, B. and Bjarnason, J.B. 1991. Structural and kinetic properties of chymotrypsin from Atlantic cod (*Gadus morhua*) comparison with bovine chymotrypsin. Comp. Biochem. Physiol. 99B : 327-335.

Bae, T.J., Han, B.H., Cho, H.D., Kim, B.S. and Lee, H.S. 1990. Conditions for rapid processing of modified fish sauce using enzymatic hydrolysis and improvement of product quality. Bull. Korean Fish. Soc. 23 : 361-372.

Benjakul, S. and Morrissey, M.T. 1997. Protein hydrolysates from Pacific whiting solid wastes. J. Agric. Food Chem. 45 : 3423-3430.

Blocher, M., Walde, P. and Dunn, I.J. 1999. Modeling of enzymatic Reaction in vesicles : the case of α- chymotrypsin. Biotechnol. Bioeng. 62 : 36-43.

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

Chen, H.M. and Meyers, S.P. 1982. Extraction of astaxanthin pigment from crawfish waste using a soy oil process. J. Food Sci. 47 : 892-896.

Chen, H.C. and Zall, R.R. 1985. Concentration and fractionation of clam viscera proteinases by ultrafiltration. Proc. Biochem. 20 : 46-50.

D'Aoust, J.Y. 1981. Update on preenrichment and selective enrichment conditions for detection of *Salmonella* in foods. J. Food Prot. 44 : 369-374.

Deutscher, M.P. 1990. Guide to protein purification. Academic Press, Inc.

Davis, B.J. 1964. Disc electrophoresis methods and application to human serum protein. Ann. NY. Acad. Sci. 121 : 404-427.

Dixon, M. and Webb, E.C. 1979. Enzymes. Longman group Ltd., London.

Doke, N.S. and Ninjoor, V. 1987. Characteristics of an alkaline proteinase and exopeptidase from shrimp (*Penaeus indicus*) muscle. J. Food Sci. 52 : 1203-1208.

Economic agriculture office. 1999. Economic agriculture new. Ministry of agriculture, 45 : 33-36.

Eisen, A.Z., Henderson, K.O., Jeffrey, J.J. and Bradshaw, R.A. 1973. A collagenolytic protease from the hepatopancreas of the fiddler crab, *Uca pugilator*. Purification and properties. Biochem. 12 : 1814-1821.

Gelder, W.V., Huijskes-Heins, M.I.E., Klepper, D., Noort, W.L.V., Cleton -Soeteman, M.I. and Eijk, H.G.V. 1996. Isolation and partial characterization of two porcine spleen ferritin fractions with different electrophoretic mobility. Comp. Biochem. Physiol. 115B : 191-199.

Gildberg, A. and Overbo, K. 1990. Purification and characterization of pancreatic elastase from Atlantic cod (*Gadus morhua*). Comp. Biochem. Physiol. 97B : 775-782.

Gildberg, A. and Raa, J. 1983. Purification and characterization of pepsins from the Arctic fish capelin (*Mallotus villosus*). Comp.Biochem. Physiol. 75A : 337-342.

Gildberg, A. and Xian-Quan, S. 1994. Recovery of tryptic enzymes from fish sauce. Proc. Biochem. 29 : 151-155.

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

Glass, H.J., Macdonald, N.L. and Stark, J.R. 1987. Metabolism in marine flatfish-IV. carbohydrate and protein digestion in Atlantic halibut (*Hippoglossus hippoglossus* L.). Comp. Biochem. Physiol. 86B : 281-289.

Godfrey, T. and Raichelt, J. 1983. Industrial enzymology. Macmillan, London.

Haard, N.F. 1992. A review of proteolytic enzymes from marine organisms and their applicatipn in the food industry. J. Aqua. Food Prod. Technol. 1:17-35.

Haard, N.F. and Simpson, B.K. 1994. Proteases from aquatic organisms and their uses in the seafood industry. Fisheries Processing : Biotech nology applications. London : Chapman & Hall Publishing.

Hasegawa, H. 1987. Laboratory manual on analytical methods and procedures for fish and fish products. Marine Fisheries Research Department. SEAFDEC : Singapore.

Henderson, N.R. and Strombom, D.B. 1995. Model business development and marketing plan for fish hydrolysate fertilizer. Rutgers Cooperative Extension Blake Hall, Rutgers University, USA.

Heu, M.S., Kim, H.R. and Pyeun, J.H. 1995. Comparison of trypsin and chymotrypsin from the viscera of anchovy, *Engraulis japonica.* Comp. Biochem. Physiol. 112B(3) : 557-567.

Hmoejan, W. 1985. Fish biology. Cubook, Chulalongkorn university.

Hong, L.C., Leblanc, E.L., Hawrysh, Z.J. and Hardin, R.T. 1996. Quality of Atlantic mackerel (*Scomber scombrus* L.) fillets during modified atmosphere storage. J. Food Sci. 61 : 646-651.

Hoyle, N.T. and Merritt, J.H. 1994. Quality of fish protein hydrolysates from herring (Clupea harengus). J. Food Sci. 59 : 76-79.

Hua, S.B., To, W.Y., Nguyen, T.T., Wong, M.L. and Wang, C.C. 1996. Purification and characterization of proteosomes from *Trypanosoma breucei.* Mol. Biochem. Parasitol. 78 (1-2) : 33-46.

Jany, K.D. 1976. Studies on the digestive enzymes of the stomachless bonefish *Carassius auratus gibelio* (bloch) : endopeptidases. Comp. Biochem. Physiol. 53B : 31-38.

Kilara, A. 1985. Enzyme-modified protein food ingredients. Proc. Biochem : 149-157.

Kim, H.R., Mayers, S.P., Pyeun, J.H. and Godber, J.S. 1994. Enzymatic properties of anionic trypsins from the hepatopancreas of crayfish, *Procambarus clarkii.* Comp. Biochem. Physiol. 107B :197-203.

King, M. 1995. Fisheries biology, assessment and management. Fisheries News Book, England.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. Nature. 227 : 680-686.

Leelawat, B. and Anprung, P. 1994a. Production of fish extract using immobilized proteases bioreactor. I. Characterization of immobilized papain and neutrase for fish extract production from fish cooking juice. Food 23(2) : 115-127.

Leelawat, B. and Anprung, P. 1994b. Production of fish extract using immobilized proteases bioreactor. II. Chemical properties of dried and concentrated fish extract and their application as flavor enhancer. Food 23(3) : 177-187.

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. 1951. Protein measurement with Folin phenol reagent. J. Biochem. 193 : 265-275.

Magnusson, H. and Martinsdottir, E. 1995. Storage quality of fresh and frozen-thawed fish in ice. J. Food Sci. 60(2) : 273-278.

Martinez, A., Olsen, R.L. and Serra, J.L. 1988. Purification and characterization of two trypsin-like enzymes from the digestive tract of anchovy *Engraulis encrasicholus*. Comp. Biochem. Physiol. 91B : 677-684.

Morr, C.V. and Ha, E.Y.W. 1995. Analyzing food for nutrition labeling and hazardous contaminates. Marcel Dekker, Inc. New York.

Mullally, M.M., O'callaghan, D.M., Fitzgerald, R.J., Donnelly, W.J. and Dalton, J.P. 1995. Zymogen activation in pancreatic endoproteolytic preparations and influence on some whey protein hydrolysate characteristics. J.Food Sci. 60 : 227-232.

Olsen, R.L., Johansen, A. and Myrnes, B. 1990. Recovery of enzymes from shrimp waste. Proc. Biochem. : 67-68.

Outzen, H., Berglund, G.I., Smalas, A.O. and Willassen, N.P. 1996. Temperature and pH sensitivity of trypsins from Atlantic salmon (*Salmo salar*) in comparison with bovine and porcine trypsin. Comp. Biochem. Physiol. 115B : 33-45.

Overnell, J. 1973. Digestive enzymes of the pyloric caeca and of their associated mesentery in the cod (*Gadus morhua*). Comp. Biochem. Physiol. 46B : 519-531.

Panphon, S. 1994. Purification and properties of cellulase and xylanase from Aspergillus niger ATCC 6275. Thesis of master of science (Biotechnology), Prince of Songkla University.

Poosaran, N. 1986. Fish sauce. II : Enzyme hydrolysis. Songklanakarin J. Sci. Technol. 8 : 205-207.

Potter, N.N. and Hotchkiss, J.H. 1995. Food science. Chapmen and Hall, New York, USA.

Prasertsan, P., Wuttijumnong, P., Sophanodora, P. and Choorit, W. 1988. Seafood processing industries within Songkhla-Hatyai region : The survey of basic data emphasis on wastes. Songklanakarin J.Sci. Technol. 10 : 447-451.

Prachumratana, P. 1998. Types and properties of enzymes from viscera of tuna. Thesis of master of science (Biotechnology), Prince of Songkla University.

Purohit, A., Potter, M., Athanas, R.P. and Learson, R.J. 1988. Effects of fish hydrolysate on green house grown *Capsicum frutescens* "Jalapeno". 12th Annual conference of the tropical and subtropical fisheries technological society of the Americas. Florida University, USA. : 675.

Raae, A. 1990. Effect of low and high temperatures on chymotrypsin from Atlantic cod (*Gadus morhua* L.) ; comparison with bovine α-chymotrypsin. Comp. Biochem. Physiol. 97B : 145-149.

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

Reece, P. 1988. Recovery of proteases from fish waste. Proc. Biochem. 23 : 62-66.

Rick, W. 1974. Methods of enzymatic analysis. Academic Press, Inc.

Saila, S.B. and Norton, V.J. 1974. Tuna : status, trends, and alternative management arrangements. Resources for the future, Inc.Washing-ton. D.C.

Sanchez-Chiang, L., Ponce, O., Landsberger, E. and Enriquez, S. 1986. Cathepsin D from sea urgin egg *Tetrapygus niger*- isolation by affinity chromatography and properties. Comp. Biochem. Physiol. 85B : 81-87.

Sangsri, R. 1997. Production of photosynthetic bacteria *Rhodocyclus gelatinosus* R7 from tuna condensate by one and two-stage processes. Master of Science Thesis in Biotechnology, Prince of Songkla University.

Scopes, R.K. 1978. Techniques of protein purification. *In* Techniques in the Life Sciences: Techniques and Enzymes. Biochemistry.(eds. Kornberg, H.L., Metcalfe, J.C., Northcote, D.H., Pogson, C.I. and Tipton, K.F.) p.1-42, Amsterdam : Elesevier/North-Holland Biochemical Press.

Shin, D.H. and Zall, R.R. 1986. Purification and identification of a trypsin-like enzyme from the pyloric caeca of cod. Proc. Biochem. : 11-15.

Simpson, B.K. and Haard, N.F. 1984. Trypsin from Greenland cod as a food-processing aid. J. Appl. Biochem. 6 : 135-143.

Simpson, B.K. and Haard, N.F. 1985. The use of proteolytic enzymes to extract carotenoproteins from shrimp wastes. J. Appl. Biochem. 7 : 212-222.

Simpson, B.K. and Haard, N.F. 1985a. Characterization of the trypsin fraction from cunner (*Tautogolabrus adspersus*). Comp. Biochem. Physiol. 80B : 475-480.

Tanji, M., Kageyama, T. and Takahashi, K. 1988. Three pepsinogens and pepsins purification, characterization and amino-terminal sequences. Proc. Biochem. 177 : 251-259.

Trirekphan, C. 1997. Production of fish protein isolates from fish heads and viscera. Master of science thesis in food technology, Prince of Songkla University.

Twining, S.S., Alexander, P.A., Huibregtse, K. and Glick, D.M. 1983. A pepsinogen from rainbow trout. Comp. Biochem. Physiol. 75B : 109-112.

Walsh, K.A. and Wilcox, P.E. 1970. Serine proteases. Enzymology : 31-112.

Ward, O.P. 1985. Comprehensive biotechnology. Vol.3, moo-Young M. ed. Pergamon Press, Oxford.

Appendices

Appendix A

Buffer preparation

1. Citrate buffer was prepared by the method of Lillie (1948 cited by Deutscher, 1990)

Stock solutions

A : 0.1 M solution of citric acid (21.01 g of $C_6H_8O_7.H_2O$ in 1000 ml)

B : 0.1 M solution of sodium citrate (29.41 g of $C_6H_5O_7Na_3.2H_2O$ in 1000 ml)

X ml of A + *Y* ml of B, diluted to a total of 100 ml

X	*Y*	pH
46.5	3.5	3.0
43.7	6.3	3.2
40.0	10.0	3.4
37.0	13.0	3.6
35.0	15.0	3.8
33.0	17.0	4.0
31.5	18.5	4.2
28.0	22.0	4.4
25.5	24.5	4.6
23.0	27.0	4.8
20.5	29.5	5.0
18.0	32.0	5.2
16.0	34.0	5.4
13.7	36.3	5.6
11.8	38.2	5.8
9.5	41.5	6.0
7.2	42.8	6.2

2. Tris-HCl buffer was carried out the method of Bates and Bower (1956 cited by Prachumratana, 1998)

Stock solutions

A : 0.05 M solution of tris (hydroxymethyl) aminomethane (6.05 g in 1000 ml)

B : 0.05 M HCl

50 ml of A + *X* ml of B

X	pH
46.6	7.0
45.7	7.1
44.7	7.2
43.4	7.3
42.0	7.4
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
12.4	8.6
10.3	8.7
8.5	8.8
7.0	8.9
5.7	9.0

3. Carbonate-Bicarbonate buffer

Carbonate-bicarbonate buffer was prepared by the method of Delory and King (1945 cited by Deutscher, 1990)

Stock solutions

A : 0.2 M solution of anhydrous sodium carbonate (21.2 g in 1000 ml)

B : 0.2 M solution of sodium bicarbonate (16.8 g in 1000 ml)

X ml of A + *Y* ml of B, diluted to a total of 200 ml :

X	*Y*	pH
4.0	46.0	9.2
7.5	42.5	9.3
9.5	40.5	9.4
13.0	37.0	9.5
16.0	34.0	9.6
19.5	30.5	9.7
22.0	28.0	9.8
25.0	25.0	9.9
27.5	22.5	10.0
30.0	20.0	10.1
33.0	17.0	10.2
35.5	14.5	10.3
38.5	11.5	10.4
40.5	9.5	10.5
42.5	7.5	10.6
45.0	5.0	10.7

Appendix B

Column chromatography preparation and $(NH_4)_2SO_4$ fractionation

1. Column chromatography preparation

1.1. Gel filtration

Gel filtration was prepared from the modified method of Cooper (1977 cited by Panphon, 1994). Sephadex G-100 was used as a gel matrix which was preswollen in distilled water for 30 minutes and pooled small suspension out. The 20 mM Tris-HCl, pH 7.0 was then added into the gel and left over night after removed distilled water and washed about 2-3 times with the same buffer, kept at 4 °C until used.

1.2. Affinity column chromatography

A benzamidine-Sepharose was conducted from the modified method of Deutscher (1990). The matrix was swollen in distilled water for 15-60 minutes and washed the matrix with 20 mM Tris-HCl, pH 7.0 in the ratio of 1:200. Benzamidine was added and incubated at room temperature with shaking for 2-4 hours in the ratio of buffer per gel for 2:1. Unused benzamidine was washed out with the same above buffer. The 0.1 M Tris solution was used to block unreacted site on the matrix for 2-4 hours at room temperature, then washed with the same buffer and stored at 4 °C for use in the next step.

1.3. Ion-exchange column chromatography

DEAE-Sephadex A-50 was used and prepared by the modified method of Deutscher (1990). Gel was swollen in distilled

water for 3 hours at room temperature and treated the gel with 1.0 M NaOH for 30 minutes. The gel was washed with water until the pH become the same as water pH. The 0.5 M HCl was used to soak the bead for 30 minutes and excess HCl was removed by washing with distilled water. The gel was kept at 4 °C until used in 20 mM Tris-HCl, pH 7.0 buffer.

2. Ammonium sulfate fractionation

Table-Appendix B2 Final concentration of ammonium sulfate : percentage saturation

AMMONIUM SULPHATE, GRAMS TO BE ADDED TO 1 LITRE

From % ↘ / To % →	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90	95	100
0	27	55	84	113	144	176	208	243	277	314	351	390	430	472	516	561	608	657	708	761
5		27	56	85	115	146	179	212	246	282	319	357	397	439	481	526	572	621	671	723
10			28	57	86	117	149	182	216	251	287	325	364	405	447	491	537	584	634	685
15				28	58	88	119	151	185	219	255	292	331	371	413	456	501	548	596	647
20					29	59	89	121	154	188	223	260	298	337	378	421	465	511	559	609
25						29	60	91	123	157	191	227	265	304	344	386	429	475	522	571
30							30	61	92	126	160	195	232	270	309	351	393	438	485	533
35								30	62	94	128	163	199	236	275	316	358	402	447	495
40									31	63	96	130	166	202	241	281	322	365	410	457
45										31	64	97	132	169	206	245	286	329	373	419
50											32	65	99	135	172	210	250	292	335	381
55												33	66	101	138	175	215	256	298	343
60													33	67	103	140	179	219	261	305
65														34	69	105	143	183	224	266
70															34	70	107	146	186	228
75																35	72	110	149	190
80																	36	73	112	152
85																		37	75	114
90																			37	76
95																				38

Source : Scopes (1978)

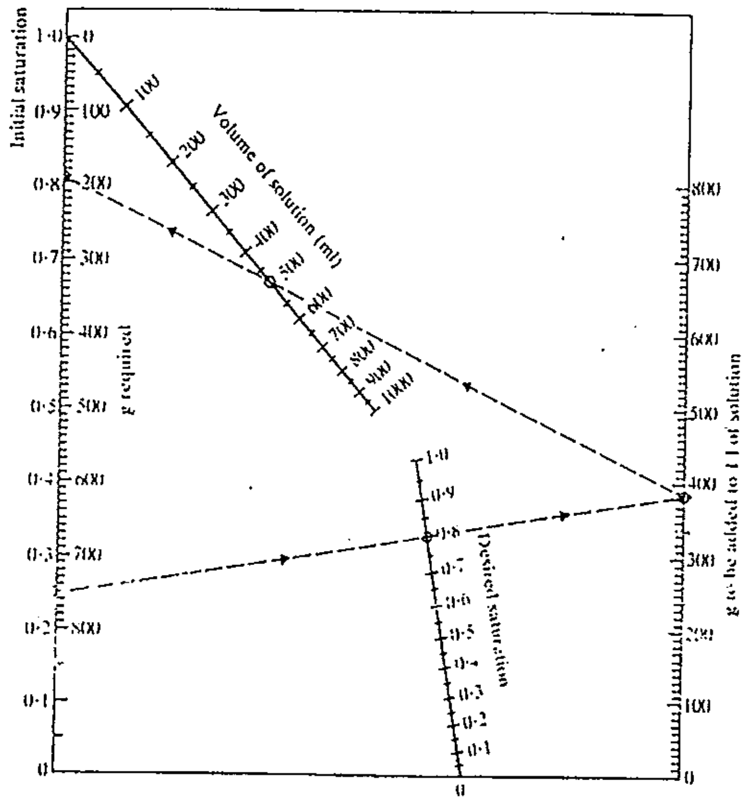


Figure-Appendix B2 Nomogram for obtaining amounts of ammonium sulfate to be added.

Source : Dixon and Webb (1979)

Appendix C

Medium preparation

1. 0.85 % NaCl

NaCl	0.85	g
distilled water	100	ml

2. Brilliant Green Phenol-red lactose sucrose agar (BPLS)

Yeast extract	3	g
Proteose peptone No.3 or Polypepsone	10	g
Sodium chloride	5	g
Lactose	10	g
Sucrose	10	g
Phenol red	0.08	g
Brilliant green 0.25% solution	5	ml
Agar	20	g

Method : dissolved all ingredients in 1,000 ml distilled water, then adjusted pH 6.9 ± 0.2 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

3. EC broth

Pancreatic digest of casein	20	g
Bile salt mixture or Bile salts No.3	1.5	g
Lactose	5	g
Dipotassium phosphate	4	g

Potassium phosphate	1.5 g
Sodium chloride	5 g
Distilled water	1,000 ml

Method : dissolved all ingredients in 1,000 ml distilled water, then adjusted pH 6.9 ± 0.2. Transferred into test tube 10 ml/tube with a durham tube and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

4. Eosin Methylene Blue (EMB) Agar

Peptone (oxoid L37)	10 g
Lactose	10 g
K_2HPO_4	2 g
Eosin Y	0.4 g
Methylene blue	0.065 g
Agar	15 g
Distilled water	1000 ml

Method : dissolved all ingredients by heating, then adjusted pH to 6.8 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes. Decreased temperature to 60°C and added oxidize methylene blue.

5. Lauryl Sulphate Tryptone (LST) Broth

Single strength medium

Tryptone	20 g
Lactose	5 g
K_2HPO_4	2.75 g
KH_2PO_4	2.75 g
NaCl	5 g

Sodium lauryl sulphate	0.1 g
Distilled water	1,000 ml

Method : dissolved all ingredients by heating, then adjusted pH 6.8 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

6. MR-VP medium

Peptone	7 g
Glucose	5 g
Dipotassium phosphate	5 g
Distilled water	1,000 ml

Method : dissolved all ingredients in distilled water, then adjusted pH 6.9 ± 0.2 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

7. Nutrient Broth

Beef extract	3 g
Peptone	5 g
pH	7

Method : dissolved all ingredients in 1,000 ml distilled water, transferred 9 ml/test tube and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

8. Plate Count Agar (PCA) (Standards Methods Agar)

Tryptone	5 g
Yeast extract	2.5 g
Glucose	1 g

Agar	15 g
pH	7.1 ± 0.1

Method : dissolved all ingredients in 1,000 ml distilled water, then mixed well and boiled. Autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

9. Salmonella-Shigella Agar (SS-agar)

Bacto beef extract	5 g
Proteose peptone	5 g
Bacto-lactose	10 g
Bacto-bile salts No.3	8 g
Sodium citrate	8.5 g
Sodium thiosulfate	8.5 g
Ferric citrate	1 g
Bacto agar	13.5 g
Bacto-brilliant green	0.33 g
Bacto-neutral red	0.025 g
pH	7

Method : dissolved all ingredients in 1,000 ml distilled water, boiled for well mixing and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

10. Selenite Cystine Broth (SCB)

Bacto-tryptone	5 g
Bacto-lactose	4 g
Sodium selenite	4 g

di-sodium phosphate	10	g
Cystine	0.01	g

Method : dissolved and boiled all ingredients in 1,000 ml distilled water, then adjusted pH 7.0 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes. Prepared and used immediately.

11. Simmon Citrate Agar

Magnesium sulphate	0.2	g
Ammonium dihydrogen phosphate	0.2	g
Sodium ammonium phosphate	0.8	g
Sodium citrate, tribasic	2.0	g
Sodium chloride	5.0	g
Bromo-thymol blue	0.08	g
Agar	15	g
Distilled water	1,000	ml

Method : dissolved and boiled all ingredients, mixed well and then adjusted pH 7.0. Autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

12. SIM Agar

Beef extract	3	g
Peptone	30	g
Peptonized iron	2	g
Sodium thiosulfate	0.025	g
Agar	3	g
Distilled water	1,000	ml

Method : dissolved all ingredients in 1,000 ml distilled water, then adjusted pH 7.3 ± 0.2 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

13. Triple Sugar Iron (TSI) Agar

Ingredient	Amount	Unit
Bacto-beef extract	3	g
Bacto-yeast extract	3	g
Bacto-peptone	15	g
Proteose peptone, Difco	5	g
Bacto-lactose	10	g
Saccharose, Difco	10	g
Bacto-dextrose	1	g
Ferrous sulfate	0.2	g
Sodium chloride	5	g
Sodium trisulfate	0.3	g
Bacto-agar	12	g
Bacto-phenol red	0.024	g
pH	7.4	

Method : dissolved and boiled all ingredients in 1,000 ml distilled water and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes.

14. Urea Agar base

Ingredient	Amount	Unit
Bacto-peptone	1	g
Bacto-dextrose	1	g
NaCl	5	g

KH_2PO_4	2	g
Agar	20	g
Bacto-phenol red 0.04%	20	ml

Method : dissolved all ingredients in 1,000 ml distilled water, then adjusted pH 6.7 - 6.8 and autoclaved at 15 pound/inch^2 at 121°C for 15 minutes. While temperature decreased to 55°C, added 100 ml 20% urea solution which passed aseptic method by millipore filter. Mixed and poured into test tubes and made slant.

Appendix D

Analytical methods

1. Quantitation of 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 solution 1. and 1 ml of solution 2. Immediately before used.
4. Folin-ciocateus reagent was diluted with distilled water in the ratio of 1:1 rapidly before used.

Procedures

1. A 0.5 ml of appropriated dilution of sample was placed into the tube.
2. A 3.0 ml of alkaline copper was added and incubated at room temperature for 10 minutes.
3. Added 0.3 ml of Folin-ciocatues reagent, vortexed immediately and incubated at room temperature for 30 minutes.
4. Measured the absorbance at 750 nm.

Standard curve of protein

1. Bovine serum albumin 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. Plotted graph of standard curve of BSA concentration and optical density at 750 nm. (Figure-Appendix D1).

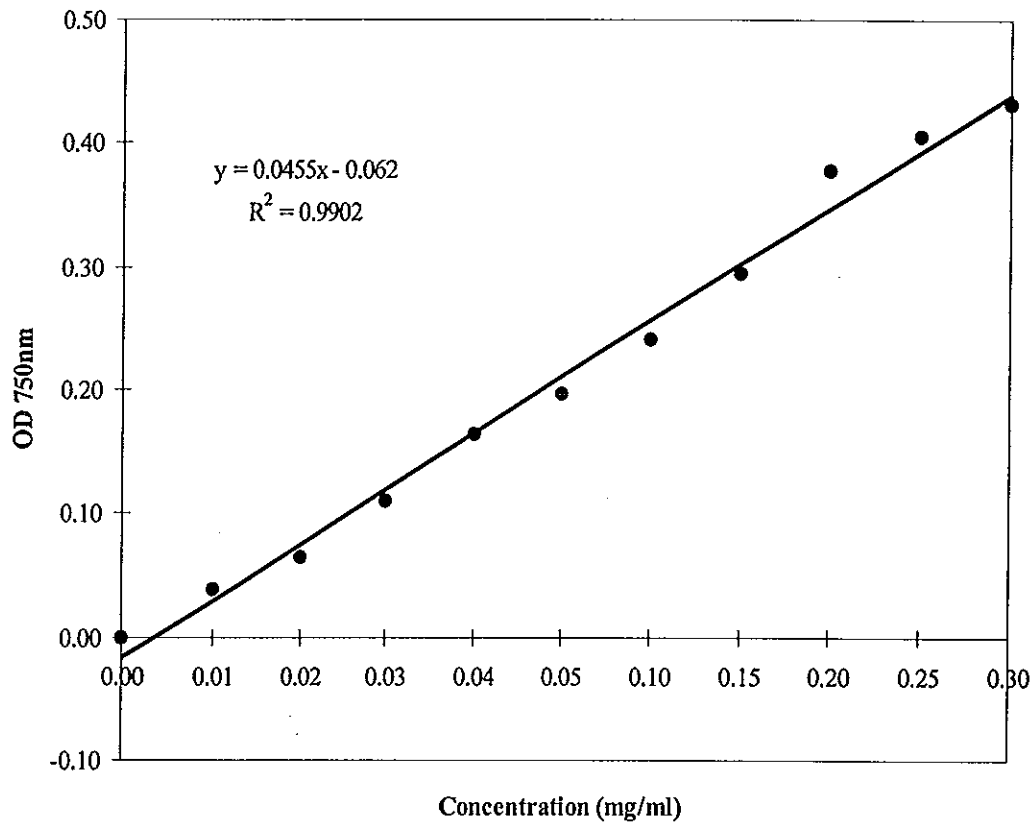


Figure-Appendix D1 Standard curve of BSA at the absorbance of 750 nm.

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

Apparatus

1. Kjeldahl flasks (800 ml 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 $CuSO_4$ and K_2SO_4 in the ratio 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 dissloved 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 litres 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

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

By a = Volume of HCl or H_2SO_4 solution in 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 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 of its weight.

Calculation

$$M = [(W_1 - W_2) \times 100] / W_1$$

by M = % moisture

W_1 = Weight of sample before incubation

W_2 = Weight of sample after incubation

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

Procedures

1. Incubated a 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 membrance, then wrapped it tightly and placed into soxhlet bag which was covered by cotton wool and moved to soxhlet.
3. Poured petrolium 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 of its weight.

Calculation

$$\% \text{ crude fat} = 100 \times \frac{\text{Weight of crude fat after incubation}}{\text{Weight of crude fat before incubation}}$$

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, 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

$$\text{Ash content (\%)} = 100 \times \frac{\text{Weight of ash after heating}}{\text{Weight of sample before heating}}$$

6. Total solid (A.O.A.C, 1990)

Procedures

1. 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 of its weight.
3. A 1-3 g of sample was added into 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 of its weight.

Calculation

$$M = [(W_1 - W_2) \times 100] / W_1$$

by M = % total solid

W_1 = Weight of sample before incubation

W_2 = Weight of sample after incubation

7. Total amino nitrogen (A.O.A.C., 1980)

Reagents

1. 0.2 N $Ba(OH)_2$ or 0.2 N NaOH.

2. Phenopthalene formol mixture consisted of 50 ml 40% HCHO solution and 1 ml 0.05% phenopthalene solution in 50% alcohol.
3. 0.2 N HCl

Procedures

1. Added 10 ml freshly prepared Phenopthalene formol mixture into 20 ml of sample extracted solution.
2. Titrated mixture with 0.2 N $Ba(OH)_2$ to distinct red, added small but known excess 0.2 N $Ba(OH)_2$.
3. Back titrated to neutrality with 0.2 N HCl.
4. Conducted blank titration with same reagents, using 20 ml distilled water.

Calculation

1 ml 0.2 N $Ba(OH)_2$ solution titration = 2.8 mg amino nitrogen

8. Total volatile nitrogen (Hasegawa, 1987)

Reagents

1. Vasaline
2. Indicator (same as indicator of total protein method)
3. Inner ring solution (dissolved 10 g boric acid in 200 ml ethanol and mixed with 10 ml indicator, adjusted to 1,000 ml with distilled water)
4. Saturated K_2CO_3 solution (dissolved 60g K_2CO_3 in 50 ml distilled water, then boiled for 10 minutes and filtered)
5. 4% trichloroacetic acid (TCA) solution
6. 0.02 N HCl

Procedures

1. A 2 g of sample was dissolved in 10 ml 4% TCA solution, left it for 30 minutes and filtered. Sample solution was kept in -20 °C if not used suddenly.
2. Rubbed vasaline on the rim of conway unit
3. Pipetted 1 ml inner ring solution into inner ring of conway unit
4. Pipetted 1 ml of sat. K_2CO_3 solution into outer ring of conway unit.
5. Pipetted 1 ml extracted sample (as stated in 1.) into outer ring of conway unit carefully which not mixed with sat. K_2CO_3 solution.
6. Closed conway unit and mixed slightly, then incubated at 37°C for 1 hour.
7. Titrated solution in inner ring of conway unit with 0.02 N HCl until color changed from green to purple.
8. Blank was tested with same reagents using 1 ml 4% TCA instead of extracted sample solution.

Calculation

$$\text{Total volatile nitrogen (mg/100g) (TVN)} = \frac{(a-b) \times N \times 14 \times V \times 100}{W}$$

By a = Volume of used 0.02 N HCl (ml)

b = Volume of used 0.02 N HCl with blank (ml)

N = Concentration of HCl (N)

V = Total volume of used sample and 4% TCA in sample extraction (ml)

W = Weight of sample (g)

(Molecular weight of nitrogen = 14.007)

9. Trimethylamine nitrogen (Hasegawa, 1987)

Reagents

1. Vasaline
2. Indicator (same as indicator of total protein method)
3. Inner ring solution (dissolved 10 g boric acid in 200 ml ethanol and mixed with 10 ml indicator, adjusted to 1,000 ml with distilled water)
4. Saturated K_2CO_3 solution (dissolved 60g K_2CO_3 in 50 ml distilled water, then boiled for 10 minutes and filtered)
5. 4% trichloroacetic acid (TCA) solution
6. 0.02 N HCl
7. 10% HCOH

Procedures

1. A 2 g of sample was dissolved in 10 ml 4% TCA solution, left it for 30 minutes and filtered. Sample solution was kept in -20 °C if not used suddenly.
2. Rubbed vasaline on the rim of conway unit
3. Pipetted 1 ml inner ring solution into inner ring of conway unit
4. Pipetted 1 ml of sat. K_2CO_3 solution into outer ring of conway unit.
5. Pipetted 1 ml extracted sample (as stated in 1.) into outer ring of conway unit carefully which not mixed with sat. K_2CO_3 solution.

6. Pipetted 1 ml 10% CHOH to mixed with sample in outer ring of conway unit.
7. Closed conway unit and mixed slightly, then incubated at 37°C for 1 hour.
8. Titrated solution in inner ring of conway unit with 0.02 N HCl until color changed from green to purple.
9. Blank was tested with same reagents using 1 ml 4% TCA instead of extracted sample solution.

Calculation

$$\text{Trimethylamine nitogen (mg/100g) (TMA-N)} = \frac{(a-b) \times N \times 14 \times V \times 100}{W}$$

By a = Volume of used 0.02 N HCl (ml)

b = Volume of used 0.02 N HCl with blank (ml)

N = Concentration of HCl (N)

V = Total volume of used sample and 4% TCA in sample extraction (ml)

W = Weight of sample (g)

(Molecular weight of nitrogen = 14.007)

10. Salt (A.O.A.C., 1990)

Reagents

1. 0.2 M $Hg(NO_3)_2$
   1.1. Weighed 68 g $Hg(NO_3)_2 .H_2O$.
   1.2. Dissolved in 800 ml distilled water containing 2 M HNO_3 and adjusted to 1,000 ml with distilled water.

2. Indicator

    2.1. Weighed 0.1 g diphenylcabazone

    2.2. Dissolved in 100 ml ethanol.

3. 0.1 M NaCl

Standardized $Hg(NO_3)_2$ concentration

1. Pipetted 25 ml 0.1 M NaCl into 250 ml flask.
2. Added 1 ml indicator and mixed well.
3. Titrated with 0.2 M $Hg(NO_3)_2$ solution until solution changed to blue-purple in color.

Calculation

$$Hg(NO_3)_2 \text{ concentration} = \frac{\text{NaCl concentration} \times \text{Vol. of NaCl}}{2 \times \text{Vol. of used } Hg(NO_3)_2 \text{ solution}}$$

Procedure

1. Pipetted 25 ml diluted sample (1:100) into 250 ml flask.
2. Added and mixed with 1 ml indicator.
3. Titrated with $Hg(NO_3)_2$ solution (known its concentration) until solution became permanent blue-purple.

Calculation

Formula : M_1V_1 (salt) $= 2\ M_2V_2$ (silver)

$$\text{Salt (NaCl) (g/l)} = \frac{2\ M_2V_2 \times W \times 10}{V_1}$$

by V_1 = Volume of sample solution 25 ml

V_2 = Volume of used $Hg(NO_3)_2$ solution

M_2 = The concentration of $Hg(NO_3)_2$ solution

W = Molecular weight of NaCl = 58.44

11. Calculation of parameters of enzyme

11.1. Specific activity

$$\text{Units/mg} = \frac{\text{Activity of enzyme (units/ml)}}{\text{Protein concentration (mg/ml)}}$$

11.2. Total activity

Units = Volume of enzyme solution (ml) × enzyme activity (units/ml)

11.3. % yield

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

11.4. Purification factor

$$= \frac{\text{Specific activity of enzyme}}{\text{Specific activity of initial enzyme}}$$

11. Determination of molecular weight of enzyme SDS-PAGE as mentioned in Laemmli (1970 ; Mini- PROTEAN® II Electrophoresis Cell Instruction Manual of BIO-RAD Laboratories company)

Stock solution

12.1. Acrylamide / bis (30 %T, 2.67 %C)

The 29.2 g of acrylamide and 0.8 g of N′ N′-bis-methylene-acrylamide were dissolved in 100 ml of distilled water, and stored in an amber bottle about one month after preparation.

12.2. 1.5 M Tris-HCl, pH 8.8

Tris-base about 18.17 g was dissolved in 60 ml of distilled water and adjusted to pH 8.8 with 1 N HCl, then added distilled water to 100 ml, stored at 4 °C.

12.3. 0.5 M Tris-HCl, pH 6.8

A 6.06 g of Tris-base was dissolved in 60 ml of distilled water, then adjusted pH to 6.8 by 1 N HCl and added to 100 ml with distilled water, kept at 4 °C.

12.4. 10% SDS

Dissolved 10 g SDS in 90 ml water with gentle stirring and bring to 100 ml with distilled water.

12.5. Stock sample buffer

consisting of

distilled water	4.8 ml
0.5 M Tris-HCl, pH 6.8	1.2 ml
10% SDS	2.0 ml
40% glycerol	1.0 ml
0.5% bromphenol blue (w/v)	0.5 ml

12.6. SDS reducing buffer

SDS reducing buffer was prepared by mixing of 50 μl of 2-mercaptoethanol and 0.95 ml of stock sample buffer used rapidly.

12.7. 5X-electrode (Running) buffer, pH 8.3

consists of

Tris-base	9.0 g
Glycine	43.2 g
SDS	3.0 g

: diluted to a total of 600 ml by distilled water, kept at 4 °C, warm to room temperature before used if precipitation occured. Diluted 60 ml 5X-stock with 240 ml deionized water for one electrophoretic run.

12.8. Catalyst

consists of

- Ammonium persulfate (APS) 10%, was prepared before used.
- TEMED

12.9. Staining solution (Coomassie Brilliant Blue R-250)

Dissolved Coomassie Brilliant Blue R-250 0.1% (w/v) in 40% methanol and 10% acetic acid.

12.10. Destaining solution

Consists of 40% methanol and 10% acetic acid, adjusted with distilled water to 100 ml.

Preparation of slab gel

1. The glass plate sandwiches was assembled.

2. Prepared 12.5% separating gel which consisting of

distilled water	5.80 ml
30% acrylamide mixture	7.50 ml
1.5 M Tris-HCl, pH 8.8	4.50 ml
10% SDS	0.20 ml
10% APS	0.15 ml
TEMED	0.01 ml

3. Poured gel solution into the assembled gel sandwich and immediately overlayed the solution with water, then allowed the gel to polymerize for 45 minutes to 1 hour.
4. Prepared the stacking gel which consists of

distilled water	3.55 ml
30% acrylamide mixture	0.90 ml
0.5 M Tris-HCl, pH 8.8	1.50 ml
10% SDS	0.05 ml
10% APS	0.15 ml
TEMED	0.01 ml

5. Dried the area above the separating gel with filter paper before pouring the stacking gel.
6. Placed a comb in the gel sandwich and poured stacking gel solution down the spacer nearest the upturned side of the comp, poured until all the teeth have been covered by solution.
7. Allowed the gel to polymerize for 30 - 40 minutes and removed the comb by pulling it straight up slowly and gently.
8. Rinsed the wells completely with running buffer.

Sample preparation

1. Prepared SDS-reducing buffer following using volume by mixing 50 μl of 2-mercaptoethanol and 0.95 ml of stock sample buffer.
2. Mixed the sample at least 1:4 with SDS-reducing buffer, and heat at 95°C for 4 minutes before loading into the gel.
3. Assembled the electrophoresis apparatus.
4. Prepared 300 ml of 5X-electrode buffer.
5. Added approximately 150 ml of buffer to the upper buffer chamber. Filled until the buffer reached a level half way between the short and long plates.
6. Poured the remainder of the buffer into the lower buffer chamber so that at least the bottom 1 cm of the gel was covered.
7. Loaded the sample into the wells under the electrode buffer with pipettor using tips.
8. Placed the lid on top of the lower buffer chamber to fully enclose the cell. The correct orientation was made by matching the colors of the plugs on the lid with the jacks on the inner cooling core.
9. Attached the electrical leads to a suitable power supply (175- 200 volts minimal) with the proper polarity. The usual run times was approximately 45 minutes until marker dye was 1 cm from anodic end of gel.
10. After electrophoresis was completely, turned off the power supply and disconnected the electrical leads.
11. Placed the gel into staining solution, then stained for overnight and destained to remove back ground for several times.
12. Determined the relative mobility (R_f) of a protein by

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The R_f values were plotted against the know molecular weights on semi-logarithmic paper and estimated the molecular weight of unknown protein from calibration curve of standard.

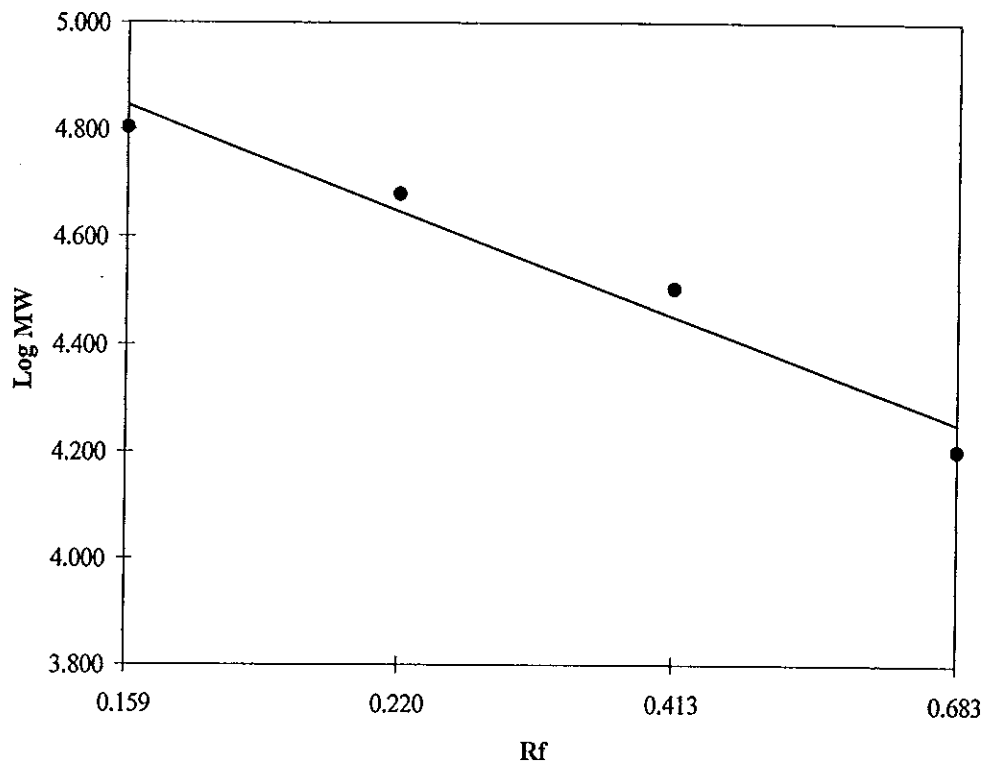


Figure-Appendix D12 Calibration curve of molecular weight protein by SDS-PAGE. The standard consisted of hemoglobin with monomer (16,000 da), dimer (32,000 Da), trimer (48,000 Da) and tetramer (64,000 Da).

13. Non-denatured polyacrylamide gel electrophoresis

Table-Appendix D13 The composition of non-PAGE

	Stacking gel	Separating gel	
	4%	10%	12%
30% acrylamide-bis mixture (ml)	0.65	2.38	2.80
0.5 M Tris-HCl, pH 6.8 (ml)	1.25	-	-
1.5 M Tris-HCl, pH 8.8 (ml)	-	1.82	1.82
10% APS (μl)	25	70	70
TEMED (μl)	5	5	5
distilled water (ml)	3.02	2.66	2.24
Total volume (ml)	5.0	7.0	7.0

Source : Devis (1964)

14. Total plate count (Hasegawa, 1987)

Reagents

1. 0.85% NaCl
2. Plate Count Agar (PCA)

Procedures

1. Pipetted 1 ml sample into 9 ml 0.85% NaCl and mixed well to 10^{-1} dilution.
2. Diluted to 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6} dilution.
3. Pipetted 1 ml of each sample dilution into petridish.
4. Poured warm prepared PCA into petridish and incubated at 37 °C for 24 hour.
5. Checked number of isolated white colony.

17. *Salmonella* (modified from D'Aoust, 1981)

Medium

1. Nutrient Broth (NB)
2. Selenite Cysteine Broth
3. Salmonella-Shigella (SS) Agar
4. Brilliant-green phenol-red lactose sucrose agar (BPLS)
5. Triple Sugar Iron (TSI)
6. Lysine decarboxylase medium
7. Urea media
8. Simmon citrate medium

Procedures

1. Pipetted 1 ml sample into 9 ml NB and incubated at 35-37°C for 24 hour.

2. Pipetted 1 ml sample (from 1.) into 10 ml SCB and incubated at 35°C for 24 hour.
3. Streaked on BPLS and SS agar, then incubated at 35°C for 24 hour.
4. Salmonella colony

   BPLS agar : pink colony on black colony

   SS agar : no color or pink colony
5. Tested biochemical properties of Salmonella colony by TSI, lysine decarboxylase, urea medium and Simmon citrate medium.

16. *Escherichia coli* (Hasegawa, 1987)

Medium

1. Lauryl Sulphate Tryptose (LST) Broth + durham tube
2. EC medium + durham tube
3. Eosine Methylene Blue (EMB) Agar
4. Nutrient Broth (NB)
5. MR-VP medium
6. Simmon citrate agar

Procedures

1. Inoculated sample into LST tubes (10 ml in each of 5 large tubes, 1 ml in each of 5 tubes and 0.1 ml in each of 5 tubes) then incubated at 35-37°C for 48 hour.
2. Observed for gas formation in each tube. If a tube showed more than 10% gas, it was considered positive. Recorded results for each tube in 24 hr.

3. Transferred 1 loop of LST that appeared gas in durham tube into EC broth and recorded results of happened gas in tube of each dilution for opening MPN table.
4. Incubated at 45°C for 48 hour.
5. Transferred 1 loop of EC broth that happened gas in durham tube, and streaked on EMB agar.
6. Incubated at 35°C for 18-24 hour.
7. *E. coli* colony was black (have/ no metallic sheen)
8. Transferred *E. coli* colony into NB and incubated at 35°C for 18-24 hour.
9. Tested biochemical properties by transferring 1 loop from 7. Into MR-VP medium, SIM medium and Simmon citrate agar.

Table-Appendix D16 Most probable number (MPN) of *E. coli* per 100 ml.

No.of positive tubes out of :				No.of positive tubes out of :				No.of positive tubes out of :			
Five	Five	Five	MPN/ 100 ml	Five	Five	Five	MPN/ 100 ml	Five	Five	Five	MPN/ 100 ml
0	0	0	0.00	3	0	0	8	5	0	0	23
0	0	1	2	3	0	1	11	5	0	1	31
0	0	2	4	3	0	2	13	5	0	2	43
0	1	0	2	3	1	0	11	5	0	3	60
0	1	1	4	3	1	1	14	5	0	4	75
0	1	2	6	3	1	2	17	5	1	0	33
0	2	0	4	3	1	3	20	5	1	1	46
0	2	1	6	3	2	0	14	5	1	2	63
0	3	0	6	3	2	1	17	5	1	3	85
1	0	0	2	3	2	2	20	5	1	4	115
1	0	1	4	3	3	0	17	5	2	0	49
1	0	2	6	3	3	1	20	5	2	1	70
1	0	3	8	3	4	0	20	5	2	2	94
1	1	0	4	3	4	1	25	5	2	3	120
1	1	1	6	3	5	0	25	5	2	4	150
1	1	2	8	4	0	0	13	5	2	5	175
1	2	0	6	4	0	1	17	5	3	0	79
1	2	1	8	4	0	2	20	5	3	1	110
1	2	2	10	4	0	3	25	5	3	2	140
1	3	0	8	4	1	0	17	5	3	3	180
1	3	1	10	4	1	1	21	5	3	4	200
1	4	0	11	4	1	2	26	5	3	5	250
2	0	0	5	4	2	0	22	5	4	0	130
2	0	1	7	4	2	1	26	5	4	1	170
2	0	2	9	4	2	2	30	5	4	2	220
2	0	3	12	4	3	0	27	5	4	3	280
2	1	0	7	4	3	1	33	5	4	4	350
2	1	1	9	4	3	2	40	5	4	5	425
2	1	2	12	4	4	0	34	5	5	0	240
2	2	0	9	4	4	1	40	5	5	1	350
2	2	1	12	4	4	2	45	5	5	2	540
2	2	2	14	4	5	0	40	5	5	3	920
2	3	0	12	4	5	1	50	5	5	4	1,600
2	3	1	14	4	5	2	55	5	5	5	>2,400
2	4	0	15								

Source : Hasegawa (1987)

Publication

• Jantaro, S., Prasertsan, P. and H-Kittikul, A. 1999. Partial purification and characterization of trypsin and chymotrypsin from viscera of yellowfin tuna (*Thunnus albacares*). The 5th Asia-Pacific Biochemical Engineering Conference 1999 and The 11th Annual Meeting of the Thai Society for Biotechnology on 15-18 November 1999, Phuket Arcadia Hotel & Resort, Phuket, Thailand.

Vitae

Name Ms. Saowarath Jantaro

Birth date 31 August 1974

Education Attainment

Degree	Name of Institution	Year of Graduation
Bachelor of Science (Biotechnology) First Class Honors	Prince of Songkla University	1997

Scholarship Awards during Enrollment

- National Science and Technology Development Agency (NSTDA) Scholarship provided from June 1997 to May 1999