PURIFICATION OF CHYMOTRYPSIN FROM FISH WASTE USING REVERSE MICELLES

by

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Submitted in partial fulfilment of the requirements
for the degree of Master of Science

at

Dalhousie University
Halifax, Nova Scotia
November 2011

DALHOUSIE UNIVERSITY

DEPARTMENT OF PROCESS ENGINEERING AND APPLIED SCIENCE

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Dated: November 23rd 2011

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DALHOUSIE UNIVERSITY

DATE: November 23rd 2011

AUTHOR: Liang Zhou

TITLE: PURIFICATION OF CHYMOTRYPSIN FROM FISH WASTE USING REVERSE MICELLES

DEPARTMENT OR SCHOOL: Department of Process Engineering and Applied Science

DEGREE: MSc CONVOCATION: May YEAR: 2012

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TO:

My parents

Xueou Zhou and Zhengman Zhu

My Fiancé

Constant Ma

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ABSTRACT

Reverse micelles systems AOT/isooctane was used for the concentration chymotrypsin from crude aqueous extract of red perch (intestine). The effects of pH and AOT concentration in the forward extraction step and pH and KCl concentration in the backward extraction step on the enzyme activity, purification fold and recovery yield were studied. The optimum conditions for the forward extraction were AOT concentration 20 mM and pH of 7.0 and optimum conditions for backward extraction were KCl concentration 1.0 M and pH of 7.5 which gave a good recovery yield (102.24%) and a purification (32.24-fold). The addition of 15% v/v alcohol in backward extraction dramatically improved recovery yield by 4.5 times and purification by 2.5 times. The enzyme activity and recovery yield obtained using reverse micelles method under its optimal conditions were 2 fold higher than those obtained using the ammonium sulphate precipitation method, while purification fold were 3 fold higher.

LIST OF ABBREVIATIONS AND SYMBOLS USED

A_E: activity of enzyme

AEBSF: benzamidine, 4-(2-Aminoethyl)-benzenesulfonyl fluoreide

AOT: sodium di-2-ethylhexyl sulfosuccinate

ATEE: N-acetyl-L-tyrosine ethyl ester

BSA: bovine serum albumin

BTEE: benzoyl-tyrosine ethyl ester

ChT: chymotrypsins

Cp: protein concentration

CTAB: cetyl trimethylammonium bromide

DEAE: diethylaminoethyl

DHA: docosahexaenoic acid

DOPLA: dioleyl phosphoric acid

E: enzyme

EDTA: ethylenediaminetetraacetic acid

EPA: eicosapentaenoic acid

ES: enzyme-substrate complex

ES': intermediate (acyl-enzyme) which is found in every case

IOP: intraocular pressure

IPA: isopropyl alcohol

K_1, K_2 the first order rate constants for the acylation and deacylation step

K_m: the Michaelis constant

NaDEHP: bis(2-ethylhexyl)phthalate

nPA: n-propyl alcohol

NSAID: non-steroidal anti-inflammatory drug

P: protein

PBA: phenyl-butyl-amine

Pefabloc SC: 4-(2-aminoethyl)-benzenesulfonyl fluoreide

PF: purification fold

pI: isoelectric point

PMSF: phenylmethanesulfonyl fluoride

PMSF: phenyl-methyl-sulphonyl-fluoride

PMSF: phenyl-methyl-sulphonyl-fluoride.

PVS: polyvinyl sulfonate

RY: recovery yield

SA: specific activity

SAAPPNA: N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide

SBTI: soybean trypsin inhibitor

SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis

TA: total activity

TBP: tributyl phosphate

TLCK: leupeptin, benzamidine, ela-statinal or tosyl-L-lysine chloromethyl ketone

TPCK: N-toluenesulfonyle-phenylalanine chloromethyl ketone

TPCK: tosyl-phenylalanine chloromethyl-ketone

TPCK: tosyl-phenylalanine chloromethyl-ketone.

TV: total volume

ZGGPCK: N-carbobenzoxy-(Gly)2-phenyl-alanine chloromethyl ketone

ZPCK: N-carbobenzoxy - L-phenylalanine chloromethyl ketone

ACKNOWLEDGEMENTS

I would like to express my deepest thanks to my supervisors Dr. Abdel Ghaly, (Professor of Biological Engineering, Department of Process Engineering and Applied Science) and Dr. Suzanne M. Budge (Associate Professor of Food Science, Department of Process Engineering and Applied Science) for their guidance, assistance and kindness. Their advice and support are highly appreciated. I also want to thank the other member of my guiding committee: Dr. Su-Ling Brooks (Associate Professor of Biological Engineering, Department of Process Engineering and Applied Science), for her fresh ideas and contributions.

I would like to give special gratitude to Mr. John Thompson and Ms. Anne Timmins (Food Science Technicians) for their excellent technical assistance with my laboratory work. The assistance given by Dr. Deepika Dave (Postdoctoral Fellow) at the start-up phase of the project is appreciated. Thanks are also extended to all the friends and staff in Food Science Program for their support and friendship in the past two years.

Finally, specially thanks to my parents for their love, patience, encouragement and financial support to complete my study.

The financial support provided by the Natural Sciences and Engineering Research Council (NSERC) of Canada though strategic grant provided to Dr. Abdel Ghaly is highly appreciated.

CHAPTER 1. INTRODUCTION

Canada has 25% (244,000 km) of the world's coastline and 16% of the world's fresh water (755,000 Km^2) (AAC, 2008). In Canada, 80% of total fish landings comes from the Atlantic fishery (mostly lobster, crab and shrimp and scallops) while the Pacific fishery accounts for up to 16% (mostly salmon, clams, groundfish and herring roe). Canada's total seafood and fish income in 2007 was more than $5 billion and the exports were around $3.9 billion (AAC, 2008).

A large portion of the fish landed in Canada is processed. The most common fish processing operation includes three steps: (a) removing the viscera, (b) removing the head, tail, fins and skin and (c) removing the frame and producing fillets. Fish waste is generated from the unwanted parts of the fish which can generally be divided into two streams: (a) solid waste including heads, tails, fins, frames, offal (guts, kidney and liver) and skin and (b) wastewater from cleaning the fish and equipment (Bechtel, 2003; IFC, 2007). Fish processing waste can account for up to 80% of material from production of surimi, 66% from production of fillets and 27% from production of headed and gutted fish (Bechtel, 2003; IFC, 2007). In the tuna canning production, 24-30% of solid waste is generated from bones, viscera, head and skin, while another 30-35% of fish waste is generated in a liquid form from condensate and blood.

Currently, in Canada, fish waste is an approved substance for disposal at sea and the Canadian fish industry is dumping all fish waste into the sea because there is no economical way of utilizing the waste off shore and it is costly to transport the large amount of fish waste to meal plants or land-based waste disposal systems (PPRC, 1993; AMEC, 2003). The large volumes of wastes lower the level of dissolved oxygen in the water and generate toxic by-products during decomposition (Bechtel, 2003; Gumisiriza et al., 2009).

Since fish processing waste components (the heads, viscera, skin and frames) are removed in separate processes, each waste stream can be collected separately and used for the production of value added products. Fish processing wastes contain high value protein which can potentially be reused to produce valuable by-products such as fertilizers, fishmeal and silage (IFC, 2007). Fish wastes are also known to contain highly valued fatty acids including omega-3 fatty acids such as docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), enzymes (pepsin, trypsin, chymotrypsin), collagen and oil (Byun et al.,2003; Swatschek et al., 2002; Kim et al., 2006). These valuable compounds can be used in the medical and food industries while the oil can be converted into biosel and used in the transportation industry. Therefore, proper utilization of fish wastes can result in a tremendous commercial value (Chong et al., 2002).

Currently, chymotrypsin has wide application in food, leather processing, chemical and clinical industries. Industrially, chymotrypsin is always produced from fresh cattle or swine pancreas and is commonly made in either tablet forms for oral consumption or as a liquid for injection. The price of chymotrypsin is related to the purity of the products. Using fish waste, rather than fresh cattle or swine pancreas, could dramatically lower the cost of chymotrypsin production. There have been many studies on chymotrypsin from higher vertebrates such as cattle and swine but there is little information available on fish chymotrypsin (Einarsson et al., 1996). Currently, ammonium sulphate is used to precipitate crude chymotrypsin from raw material which is further purified with chromatography. The aim of this study is to evaluate the possibility of using reverse micelles for the purification of fish chymotrypsin.

CHAPTER 2. OBJECTIVES

The main focus of this study was to evaluate the effectiveness of the reverse micelles method for purifying chymotrypsin from fish processing waste. The specific objectives were:

1. Determine the optimal conditions for using the reverse micelles method for purifying crude chymotrypsin from fish waste.

a) Study the effect of sodium di-2-ethylhexyl sulfosuccinate (AOT) concentration (1, 5, 10, 15, 20, 35, and 50 mM) on enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

b) Study the effect of pH (6.0, 6.5, 7.0, 7.5, 8.0, and 8.5) in the forward extraction step (pH-1) on enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

c) Study the effect of pH (6.5, 7.0, 7.5, 8.0 and 8.5) in backward extraction stage (pH-2) on enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

d) Study the effect of salt concentration (0.5, 1, 1.5 and 2M) in backward stage on enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

2. Study the effect of alcohol in backward extraction step on enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

3. Compare the effectiveness of reverse micelles under optimal extraction conditions to the ammonium sulphate precipitation method on the basis of enzyme activity, protein concentration, specific activity, purification fold and recovery yield.

CHAPTER 3. LITERATURE REVIEW

3.1 Function and Structure of Chymotrypsin

Chymotrypsin, an endopeptidase, is a digestive enzyme existing in pancreatic tissues of vertebrates and inverbrates which is secreted into the duodenum (Geiger, 1983). Chymotrypsin primary hydrolyzes amide, ester and peptide ponds in protein with a carboxyl side as an amino acid residue at P_1 position (the first amino acid residue in the N-terminal direction from the cleaved bond). Other peptide amide bonds like tryptophanyl-, tyrosyl- and phenylalanyl- bonds are attacked by chymotrypsin. It is also reported that leucyl and glutamyl bonds could be cleaved by chymotrypsin (Appel, 1986; Geiger, 1983). Appel (1986) indicated that the hydrolyzing power increases according to the type of substrate in the following sequence: proteins < peptides < amides < esters < N-tyrosine esters.

Chymotrypsin has 245 amino acid residues and 5 pairs of disulfide linkages with a molecular mass around 24000. Generally, the single polypeptide molecular weight is beween 25 and 28 kDa (Simpson, 2000). Chymotrypsin exists in three inactive forms (chymotrypsinogens A, B and C) in the zymogen granules (dense, membrane-bound bodies that are derived from the Golgi body of cells) of the pancreas (Smith et al. 1951; Geiger, 1983; Raae et al., 1995; Leth-Larsen et al., 1996). The three types of chymotrypsin (A, B, C) have been found in mammals but only two types of chymotrypsin (A and B) have been found in fish (Yang et al., 2009).

As one of the main digestion proteases, chymotrypsin has been widely reported in the gut of a variety of fish including: discus (*Symphysodon sp*.), carp (*Cyprinus carpto*), rainbow trout (*Oncorhyncus mykiss*), coho salmon (*O. Kistuch*), chinook salmon (*O. tshawytscha*), gilthead seabream (*Sparus awrata*) and dentex (*Dentes dentex*) (Dimes et al., 1994, Alarcón et al., 1998; Chong et al., 2002). Chymotrypsin concentration is 10^5

times higher in the gut than in non-gut tissue but the activity and concentration vary according to fish species and the environment in which the fish live (Elert et al., 2004; Parra, 2007).

Fish chymotrypsin usually has two different forms: cationic (chymotrypsin B) and anionic (chymotrypsin A) (Yang et al., 2009). The isoelectric points of fish chymotrypsinogens A and B are at pHs of 9.1 and 5.2, respectively. The amino acid sequences of chymotrypsins A and B are very similar with only minor differences and the activated enzymes contain three polypeptide chains. The two isoforms of chymotrypsin (A and B) have been found in and extracted from rainbow trout (Kristjansson and Nielsen, 1992), grass carp (Fong et al., 1998), Altantic cod (Ásgeirsson and Bjarnason, 1993), anchovy (Heu et al., 1995), Monterey sardine (Castillo-Yañez et al., 2006) and crucian carp (Yang et al., 2009).

The inactive form of chymotrypsin (chymotrypsinogen) can be activated by trypsin which partially cleavs it into two parts while still maintaning an S-S bond. In the enzymogen activation process, chymotrypsinogen goes through different forms of intermediate products (α-chymotrypsin, γ-chymotrypsin, δ-chymotrypsin, π-chymotrypsin) before forming chymotrypsin A. However, the type of intermediate products generated depend on trypsin concentration and reaction velocities as shown in Figure 3.1 (Geiger, 1983).

3.2 Chymotrypsin Specificity, Mechanism of Hydrolysis and Inhibitors

3.2.1 Specificity

Chymotrypsin hydrolyzes peptide bonds with various α-amino acid carbonyl groups and attacks larger nonpolar aromatic groups such as tyrosine, phenylalanine and tryotophan (Folk, 1970 Appel, 1986; Galvão et al., 2001). It also attacks nonpolar groups

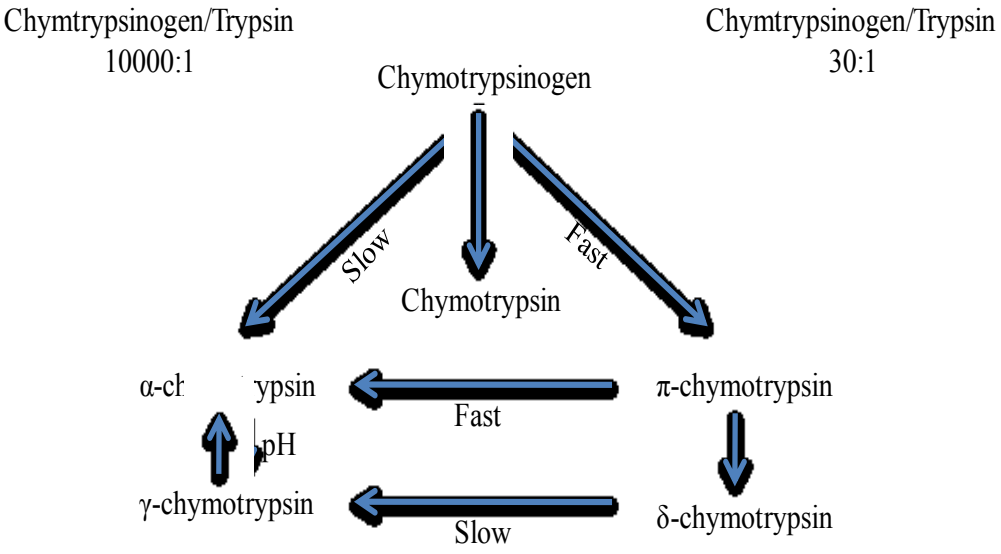

Figure 3.1. Activation of chymotrypsinogen. Different chymotrypsins result as reaction products, depending on relative concentrations and reaction velocities (Modified from Appel, 1986).

like leucine but the reaction is slower. Hudáky et al. (1999) reported that chymotrypsins A and B have similar specificities in hydrolyzing peptides with Phe, Trp, Leu and Tyr residues. Raae et al. (1995) found specificity differences between two types of chymotrypsins (ChT1 and ChT2) isolated from cod and pointed out that ChT1 can hydrolyze 8 peptide bonds [Leu(6)-Cys(7), Leu(15)-Tyr(16), Tyr(16)-Leu(17), Arg(22)-Gly(23), Phe(25)-Tyr(26),Phe(1)-Val(2), Leu(17)-Val(18) and Tyr(26)-Thr(27)] while ChT2 has only 4 major cleavage sites [Tyr(16)-Leu(17), Arg(22)-Gly(23), Phe(25)-Tyr(26), the Tyr(26)-Thr(27)] and 2 minor cleavage sites which are peptide bonds between [(Phe(1)-Val(2) and the Phe(24)-Phe(25)].The difference in the hydrolysis specificity of chymotrypsin can be used to determine the enzyme activity utilizing specific substrates (Geiger, 1983 ; Srinivas and Prakash, 2009). However, most substrates used can also be hydrolyzed by other proteases like trypsin. Because of this, the use of highly sensitive substrates such as Suc-$(Ala)_2$-Pro-Phe-4-NA and Suc-phe-4-NA has been recommended (Geiger, 1983).

3.2.2 Mechanism of Catalyzed Hydrolysis

The process of chymotrypsin-catalyzed hydrolysis (Figure 3.2) can be divided into two steps in which acyl-enzymes play a role as intermediates (Parker and Wang, 1968; Kallies and Mitzner, 1996). In the first step, the substrate binds with the pocket structure of chymotrypsin. The side chain Ser-195 and His-57 hydrolyzes the substrate to form an intermediate. In the second step, solvent water cleaves the acyl-enzyme (ester intermediate) to form carboxylic acid and reform the enzyme (Kallies and Mitzner, 1996). The overall reaction is described by the following equation (Bender and Ferbnj, 1964).

$$E + S \underset{}{\overset{K_m}{\longleftrightarrow}} ES \xrightarrow{K_1} \begin{matrix} ES' \\ + \\ P_1 \end{matrix} \xrightarrow{K_2} E + P_2 \qquad (1)$$

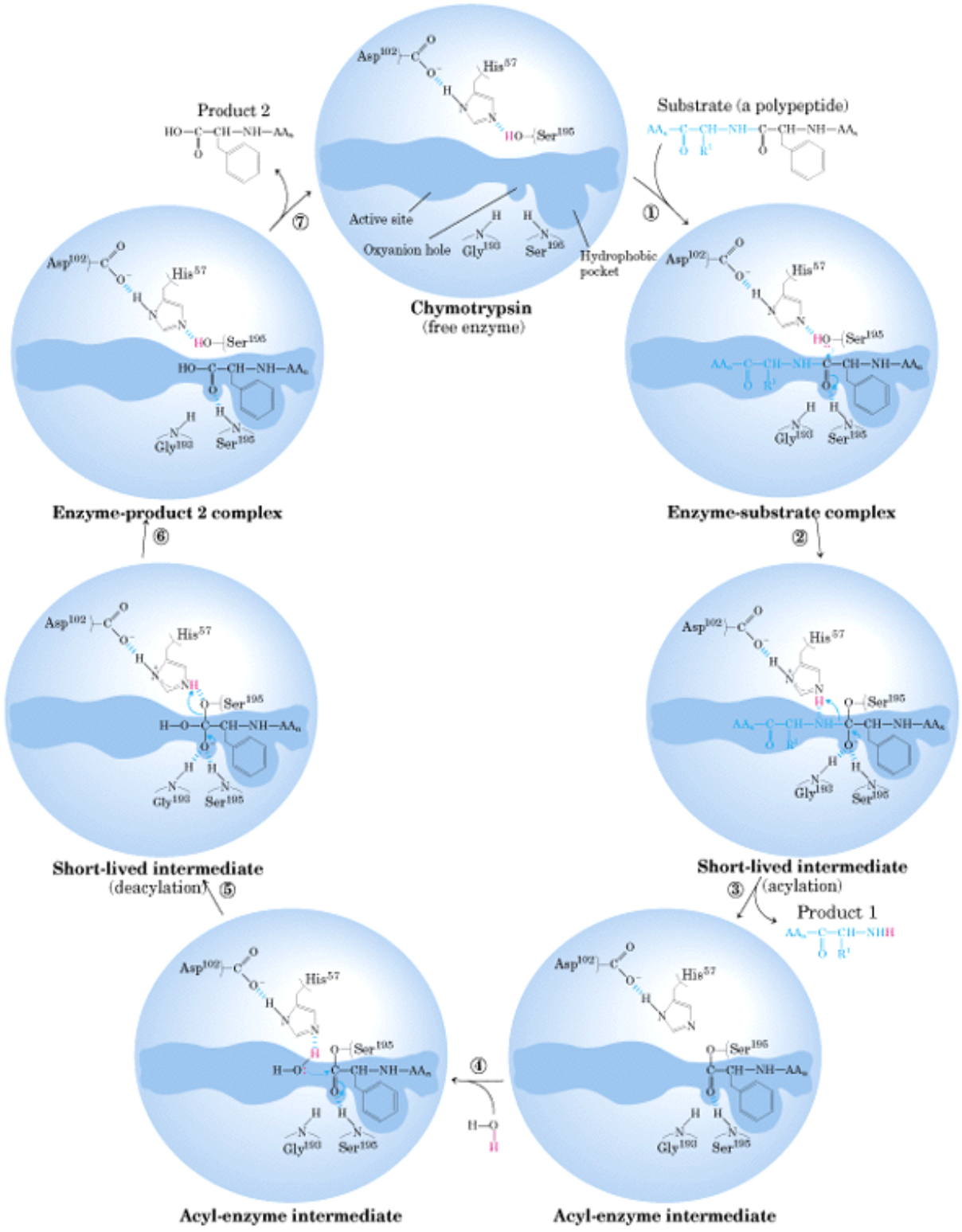

Figure 3.2. The catalytic mechanism of chymotrypsin (Nelson and Cox, 2008).

Where:

E	is the enzyme
S	is the substrate
ES	is the enzyme-substrate complex
ES'	is the intermediate (acyl-enzyme) which is found in every case
K_1, K_2	are the first order rate constants for the acylation and deacylation step
K_m	is the Michaelis constant
P_1, P_2	are products (proteins)

The reaction is pH dependent and the optimal pH is approximately 7. Parker and Wang (1968) reported that K_2 dramatically increased with an increase in pH when the pH was lower than 7 but once the pH was greater than 7 no significant differences were observed. Similar observations were reported by Fersht and Requena (1971), Lucas et al. (1973) and Leslie and Wang (1968).

3.2.3 Inhibitors

Synthetic and natural inhibitors can decrease the ability of chymotrypsin to hydrolyze proteins or peptides by forming enzyme-inhibitor structures (Guha and Sinha, 1984; Makkar et al., 2007). Natural chymotrypsin inhibitors are found in plants such as phaseolus lunatus, black gram, macrotyloma axillare, solanum tuberosum, pigeon pea, chick pea, erythrina caffra and patato (Makkar et al., 2007) or animal sources such as egg whites (Guha and Sinha, 1984). Natural inhibitors have a wide tolerance range of pH but are heat sensitive (Guha and Sinha, 1984; Makkar et al., 2007). Synthetic chymotrypsin inhibitors such as N-toluenesulfonyle-phenylalanine chloromethyl ketone (TPCK), N-carbobenzoxy-(Gly)2-phenyl-alanine chloromethyl ketone (ZGGPCK), N-carbobenzoxy - L-phenylalanine chloromethyl ketone (ZPCK) and chymostatin are chymotrypsin specific and can dramatically inhibit the catalysis power of chymotrypsin (Sabapathy and Teo, 1995; Heu et al., 1995; Tokumoto, 1999; Santigosa et al., 2008). The effects of

Table 3.1. Effect of various proteinase inhibitors and metal ions on chymotrypsins (Castillo-Yàñea et al., 2009; Yang et al., 2009).

Chemical	Concentration (mM)	Residual enzyme activity (%)				
		Chy A	Chy B	Chy I	Chy II	Bovine Chy
None		100	100	100	100	100
TPCK	0.05	35.6	17.5	56	60	53
Chymostatin	0.01	0	0	-	-	-
Benzamidine	5	60.3	62.5	103	71	117
Pefabloc SC	1	1.2	1.1	-	-	-
PMSF	1	0.4	0.3	1	2	4
SBTI	0.5	-	-	0.5	0	17
Pepstatin	0.03	95.9	92.3	-	-	-
EDTA	1	99	97	98	65	96
	10	98	100	-	-	-
$CaCl_2$	1	107	101	-	-	-
	5	115	103	-	-	-
$MgCl_2$	1	102	105	-	-	-
	5	111	110	-	-	-
$MnCl_2$	1	98.5	51.1	-	-	-
	5	85.3	48.9	-	-	-
$CdCl_2$	1	86.8	59.1	-	-	-
	5	76.8	49.7	-	-	-
$FeSO_4$	1	26.4	27.8	-	-	-
	5	14.6	21.4	-	-	-
$CuCl_2$	1	19.8	21.1	-	-	-
	5	0	2.5	-	-	-

Chy I and II: 2 types of chymotrypsin isolated from Monterey sardine.
TPCK: tosyl-phenylalanine chloromethyl-ketone.
PMSF: phenyl-methyl-sulphonyl-fluoride.
SBTI: soybean trypsiin inhibitor.
-: not tested

inhibitors on chymotrypsin activity are shown in Table 3.1. Serine proteinase inhibitors such as phenylmethanesulfonyl fluoride (PMSF) and 4-(2-aminoethyl)- benzenesulfonyl fluoride (Pefabloc SC) effectively inhibit the activity of chymotrypsin (Elert et al., 2004; Yang et al., 2009) while asparatic proteinase inhibitors such as pepstatin and the metalloproteinase inhibitor EDTA have slight influences on chymotrypsin (Heu et al., 1995; Castillo-Yàñea et al., 2006; Yang et al., 2009). Metal ions such as Mn^{2+}, Cd^{2+}, Cu^{2+} and Fe^{2+} can inhibit chymotrypsin but their inhibition abilities are different (Castillo-Yàñea et al., 2006; Yang et al., 2009).

3.3 Chymotrypsin Activity and Stability

The pH, temperature, source and storage are the most significant factors influencing the activity and stability of chymotrypsin.

3.3.1 pH

The pH range of chymotrypsin activity is 7.5-9.0 (Kristjansson and Nielson, 2002). Castillo-Yañez (2009) studied the effect of pH on activity of sardine and bovine chymotrypsins and found that the activity of chymotrypsin was significantly inhibited when the pH was <6 and the enzyme was denatured at a pH of 4 but was stable at a pH of 8 (Figure 3.3). Similar results were obtained by Tsai (1986) for chymotrypsin isolated from *P. monodon hepatopancreas*. Sabapathy and Teo (1995) found the optimal activity of rabbitfish chymotrypsin to be within the pH range of 7.5-8.5. Lam et al. (1999) reported that the optimal activities for 2 types (CTR1 and CTR2) of chymotrypsin extracted from *Locusta migratoria* were in the pH ranges of 8-10 and 8-11, respectively. Similar results were reported for European sea bass (Alliot et al., 1974), Dover sole (Clark et al., 1985), skipjack (Joakimsson and Nagayama,1990), *Siganus canaliculatus*, (Sabapathy and Teo, 1995), anchovy, (Heu et al., 1995) and Bluefin tuna, (Parra et al., 2007).

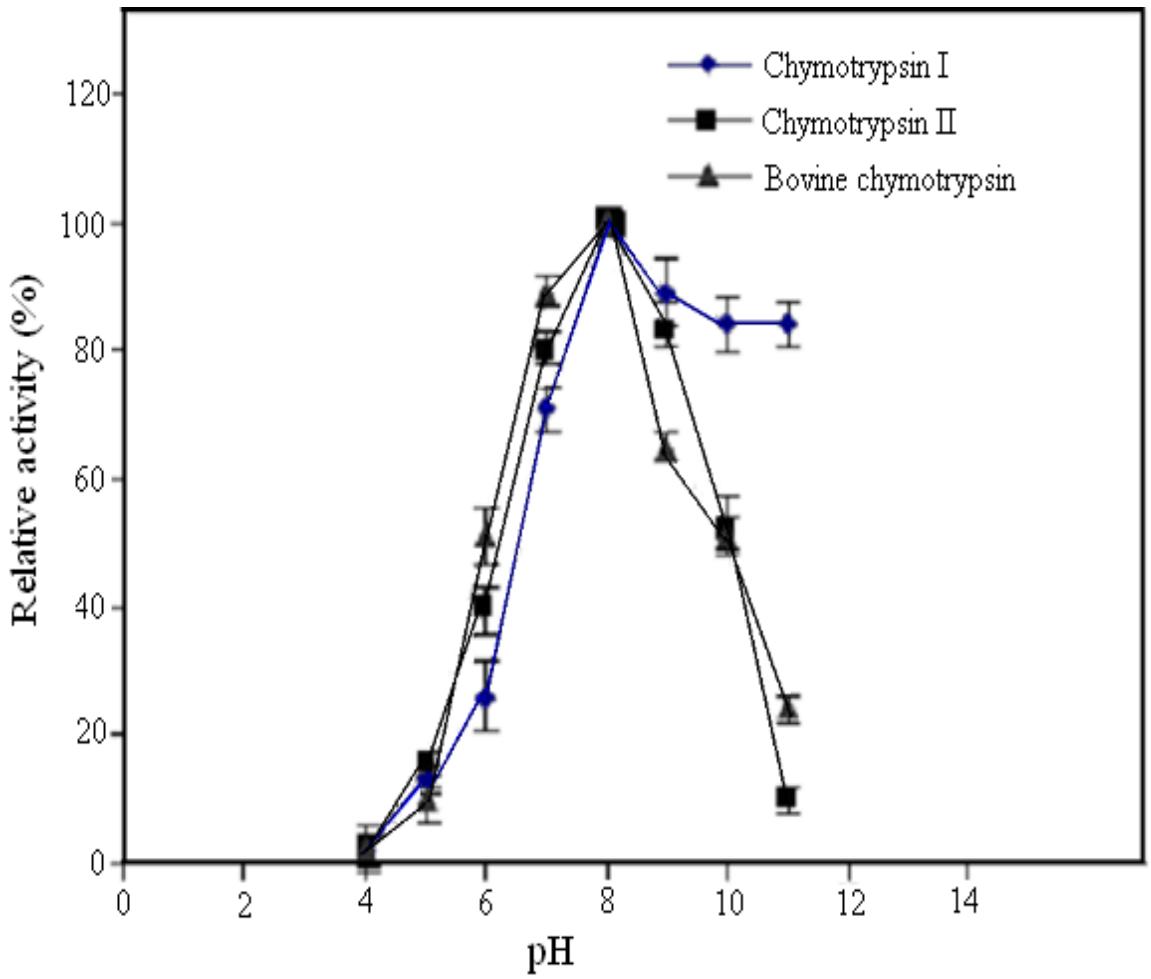

Figure 3.3. Effect of pH on chymotrypsin activity at 25°C (Castillo-Yañez et al, 2009).

3.3.3 Source

Enzyme activities differ depending on the source from which the enzymes were isolated. Chymotrypsins extracted from marine animals such as the Altantic cod (*Gadus morhua*), herring (*Clupeas harengus*), capelin (*Mallotus villosus*), rainbow trout (*Oncorhynchus mykiss*), anchovy (*Engraulis japonica*), and spiny dogfish (*Squalus acanthias*) contain higher enzyme activity but are less heat stable than mammalian chymotrypsins (Kristjansson and Nielson, 1992). Carp (warmwater fish) chymotrypsin has similar physical and kinetic properties as mammal chymotrypsins (Cohen and Birk, 1981 a; Cohen and Birk, 1981 b). The K_{cat}/K_m (results in the rate that measures catalytic efficiency) of carp is 15 while bovine, anchovy, rainbow trout and monterey sardine chymotrypsins I and II are 21, 165, 62.8, 251 and 100 (Heu et al., 1995; Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009).

3.3.4 Temperature

Temperature has a significant effect on the stability of chymotrypsin. Tsai et al. (1986) studied the thermal stability of one type of chymotrypsin isolated from *P. monodon* and *E. superb* and found denaturation of the enzymes when the temperature was above 56ºC and 45ºC, respectively (Figure 3.4). Similar results were reported by Heu et al. (1995) for a chymotrypsin extracted from anchovy. Sabapathy and Teo (1995) reported an optimal temperature range of 45ºC -55ºC for chymotrypsin extracted from rabbitfish and observed denaturation of the enzyme at a temperature of 60ºC. Similar changes were detected for chymotrypsins extracted from *Siganus canaliculatus* (Sabapathy and Teo, 1995), anchovy (Heu et al., 1995), bluefin tuna (Parra et al., 2007) and Monterey sardine (Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009).

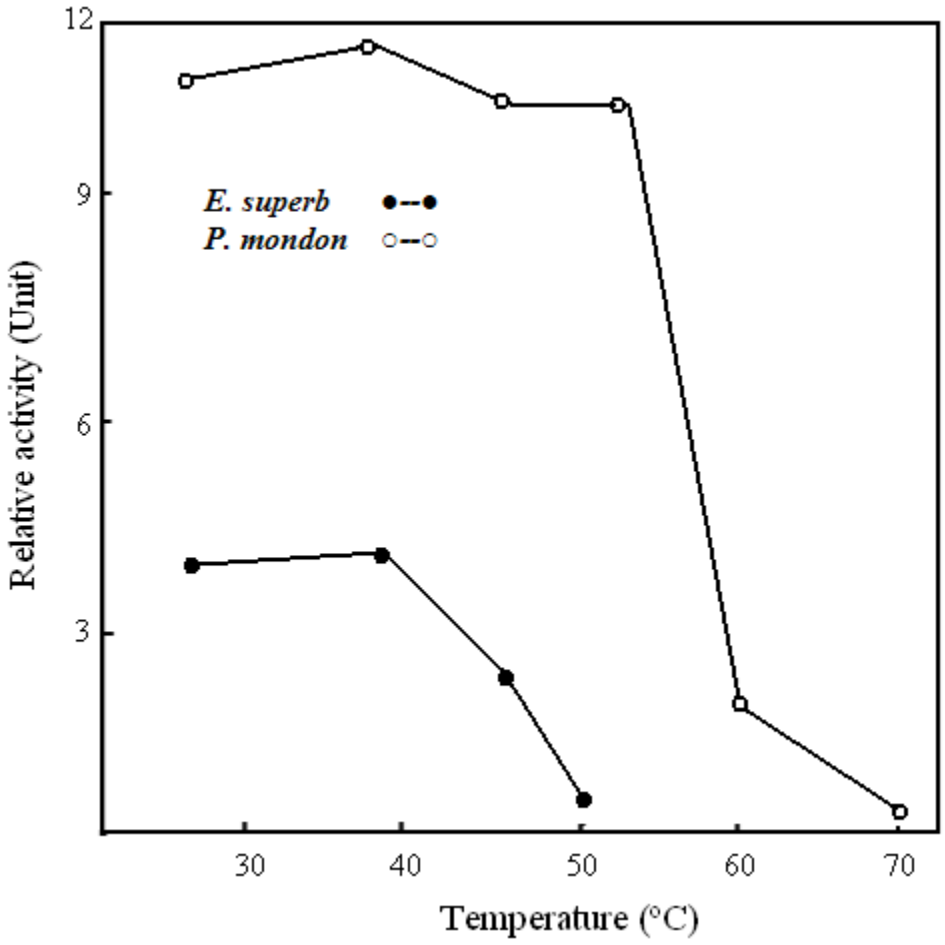

Figure 3.4. Thermostability of chymotrypsin activities from two hepatopancreases (Tsai et al., 1986).

3.3.5 Storage

Folk (1970) stated that dry, purified chymotrypsin powder can maintain its activity up to 4 years under frozen conditions without significant losses. He also claimed that chymotrypsin in water or Tris buffer (pH= 6.0-8.5) solution can be stored up to several weeks in a sterile environment. However, under acidic pH conditions, enzyme activity is rapidly lost. Tsai (1986) and Castillo-Yañez (2009) reported a 12 % loss in chymotrypsin activity in 20 minutes at a pH of 3. Castillo-Yañez (2006) evaluated chymotrypsin storage in different buffers under various pH conditions and found that the activity was most stable under a pH of 8.0 and in the pH range of 5.0-9.5 no significant differences in activity were observed. No literature reported on the effect of light during storage.

3.4 Differences between Fish and Animal Chymotrypsin

Chymotrypsins isolated from fish and animals such as bovine and swine have four major differences: pH stability, temperature stability, enzyme activity and molecular weight. However, their amino acid compositions are still similar (Heu et al., 1995; Castillo-Yañez et al., 2009).

3.4.1 pH Stability

The optimum pH for fish chymotrypsin and mammal chymotrypsin are quite similar. Castillo-Yañez et al. (2009) claimed that two types of chymotrypsin isolated from Monterey sardines had optimum pH similar to that of bovine chymotrypsin (8.0). Sabapathy and Teo (1995) found that the optimum pH for rabbitfish chymotrypsin was 8.0. However Castillo-Yañez et al. (2009) reported that fish chymotrypsin was stable only under alkaline pH condition but animal chymotrypsin was stable at both alkaline and acidic pH. For instance, bovine chymotrypsin was found to be stable at a pH of 3.0 (Wilcox, 1970; Bender and Killheffer 1973) and rat chymotrypsin was found to be stable at a pH range of 2.0-4.0 (Kumar and Pattabiraman, 1996). In contract, no activity was found for two chymotrypsins isolated from Monterey sardine at pH <4.0 (Castillo-Yañez

et al., 2009) and cod chymotrypsin was unstable at a pH <5.0 (Heu et al., 1995). Castillo-Yañez et al. (2006) reported that sardine chymotrypsin was more stable in basic environments than acidic ones. Chymotrypsin from tuna was stable within the pH range of 7-10 and only 20% residual activity was found within a pH range of 3-5 (Parra et al., 2007).

3.4.2 Temperature Stability

The chymotrypsins isolated from marine fish living in cold environments such as carp, rainbow trout, menhaden, anchovy, sardine and Atlantic cod, have higher catalytic activity at low temperature conditions and are more cold stable (but less heat stable) than mammal chymotrypsins (Cohen and Birk, 1981a; Martinez et al., 1988; Raae and Walther, 1989; Ásgeirsson and Bjarnason, 1993; Kristjansson and Nielsen, 1992; Heu et al., 1995; Castillo-Yañez et al., 2006). Castillo-Yañez et al. (2009) stated that the optimum temperature for sardine chymotrypsin was 45ºC, ten degrees lower than bovine chymotrypsin. Castillo-Yañez et al (2006) reported that when sardine chymotrypsin was incubated at 55ºC for 15 min no residual activity was observed but bovine chymotrypsin was inactivated when the temperature was >60ºC. Ásgeirsson et al. (1995) reported that calf chymotrypsin was stable at 50ºC while cod chymotrypsin denatured at a temperature of 40ºC. Kumar and Pattabiraman (1996) reported that rat chymotrypsin was stable at 50ºC for one hour but 92 % of activity was lost at 60ºC in 5 min.

3.4.3 Activity

It is well known that the catalytic activities of fish chymotrypsins are higher than warm-blooded animal chymotrypsin under optimal condition (Cohen and Birk, 1981a; Cohen and Birk, 1981b; Kristjansson and Nielsen, 1992; Castillo-Yañez et al., 2009). Ásgeirsson and Bjarnason (1993) found that the Kcat/Km of cod chymotrypsin was 3-4 fold higher than bovine chymotrypsin. Fish chymotrypsin not only maintained higher activity than mammal chymotrypsin but also had higher specific activity than mammal

chymotrypsin (Raae and Walther, 1989; Heu et al., 1995; Castillo-Yañez et al., 2006). Castillo-Yañez et al (2009) reported that Kcat/Km was 21 for bovine chymotrypsin compared to 15, 165, 62.8, 251 and 100 for chymotrypsins from crap, anchovy, rainbow trout and Monterey sardine, respectively.

3.4.4 Molecular Weight

The molecular weight of chymotrypsin isolated from various fish ranges from 22,000 to 30,000 Da and the amino acid composition is slightly different among chymotrypsins. The amino acid composition of fish and animal chymotrypsin is shown in Table 3.2. These are 245 amino acid residues in chymotrypsin of all mammals (bovine, dog, human and rat) and the sequences are very similar. Numbers of amino acid residues in fish chymotrypsins vary slightly. For instance, the numbers of residues in anchovy chymotrypsin and cod chymotrypsin B are 234 and 247, respectively. The sequence of Atlantic cod chymotrypsins A and B are very close but the differences are significant between the two types. Two amino acids Asn and Gln which exist in Atlantic cod chymotrypsin are absent in anchovy chymotrypsin.

3.5 Factors Affecting the Concentration and Activity of Fish Chymotrypsin

Trypsin is an important protease which can activate chymotrypsin during the digestive process. Therefore, the factors that influence the activity of trypsin will affect the activity of chymotrypsin. Both internal factors (trypsin phenotypes and fish life stage) and external factors (water temperature and fish starvation, feeding and nutrient condition) affect the activity of chymotrypsin.

Table 3.2. Number of amino acid in Atlantic cod and anchovy chymotrypsin compared with chymotrypsin from mammal species.

Amino	Atlantic cod[a]		Anchovy[b]	Bovine[c]		Dog[d]	Human[e]	Rat[f]
Acid	A	B		A	B	B	B	B
Ala	21	20	12	22	23	21	22	19
Arg	9	9	9	4	5	4	6	3
Asn	14	16	-	14	7	9	9	8
Asp	9	9	25	9	13	12	15	14
Cys	10	10	5	10	10	10	10	10
Gln	10	11	-	10	10	13	9	12
Glu	9	9	21	5	8	5	6	9
Gly	25	22	17	23	23	24	23	23
His	6	6	10	2	2	4	3	2
Ile	10	13	10	10	9	11	12	11
Leu	18	15	14	19	19	20	19	6
Lys	8	7	6	14	11	13	15	14
Met	6	6	2	2	4	3	2	4
Phe	6	4	4	6	7	9	7	8
Pro	11	14	15	9	13	13	13	14
Ser	18	24	21	28	22	21	23	21
The	18	15	21	23	23	21	17	20
Trp	8	9	6	8	8	8	8	8
Tyr	5	4	3	4	3	1	2	2
Val	24	24	19	23	25	23	24	27
Total	245	247	234	245	245	245	245	245

[a] Guðmundsdóttir et al. (1994)
[b] Heu et al. (1995)
[c] Bell et al. (1984)
[d] Pinsky et al. (1983)
[e] Smillie et al. (1968)
[f] Tomita et al. (1989)

3.5.1 Water Temperature

Haard (1995) reported that water temperature affects the chymotrypsin activity in fish intestine. Rungruangsak-Torrissen et al. (2006) studied the relationship between water temperature and chymotrypsin activity and found the chymotrypsin activity of Atlantic salmon to be significantly higher at 6°C than at 10 ºC (Kofuji et al., 2005). Approximately 95 % of the volume of the Atlantic Ocean is colder than 5°C (Hultin, 1980). Therefore, using Atlantic fish wastes for production of chymotrypsin will offer higher chymotrypsin concentrations because of the high latitude and low water temperature.

3.5.2 Fish Species

Fish species has a significant influence on the concentration of chymotrypsin and its activity. Based on their food habit, fish are separated into carnivores, omnivores and herbivores. Fish food habit would affect their gut weight to body weight ratio and the proteolytic activity (Chakrabarti, et al., 1995; Hidalgo et al., 1999). Hofer and Schiemer (1981) reported that proteolytic activity relates to different feeding habits. Although carnivorous species have smaller guts compared to herbivore species, they have higher activity. Chakrabarti et al. (1995) and Hidalgo et al. (1999) reported similar observations.

3.5.3 Fish Age and Mass

Fish age will influence the chymotrypsin activity and amount in fish. Chymotrypsin activity is very low (0.085Umg/protein) when the fish hatches, increases very slowly in the first 2-3 weeks, then dramatically increases in the next three days and finally increases at a constant rate (Cuvier-Peres and Kestemont, 2001; Chakrabarti et al., 1995; Chakrabarti et al., 2006). The total chymotrypsin activity is positively related to fish mass which is also correlated to fish age (Dabrowski and Glogowski, 1976; Rungruangsak-Torrissen et al., 2005). However, after fish weight was over 100g, no significant

difference in total activity was observed. Practically, in chymotrypsin manufacturing, fish weight can be a parameter in selecting raw material.

3.5.4 Starvation

Chymotrypsin will accumulate in the pancreas tissue when there is no food in the gut and the level of chymotrypsin in the intestinal digestion system decreases with starvation. Einarsson et al. (1996) reported that chymotrypsin activity of Atlantic salmon (in pyloriccaeca/pancreas) increased in the first few days but was reduced in the following days. This was explained by the reduced secretion of chymotrypsin from the pancreatic tissue to the intestinal digestion system and the accumulation in the pancreatic tissue. On the other hand, Dabrowski and Glogowski (1976) reported that starving rainbow trout showed higher chymotrypsin activity in the intestine than the fed fish. Therefore, the effect of starvation on chymotrypsin activity will depend on the type of fish (Chakrabarti et al., 1995; Dabrowski and Glogowski, 1976).

3.6 Industrial Applications of Chymotrypsin

Chymotrypsin has been used extensively in food processing, leather production, chemical and medical industries.

3.6.1 Food Industry

Chymotrypsin can be used to improve the nutritional value of proteins and to lower the protein denaturation temperature and cleavage specificity (Yamashita et al., 1976; Haard, 1992). It is used in meat tenderization, fermentation, protein hydrolysate production and bone protein removal (Haard, 1998). In the dairy industry, chymotrypsin is used together with trypsin to hydrolyze casein, the main protein in milk. When hydrolyzed by chymotrypsin, casein functional properties (such as antioxidant activity, angiotensin converting enzyme inhibition and antibacterial ability) are improved (Srinivas and Prakash, 2009). Chymotrypsin is used as an additive with trypsin to control

hydrolysis of cheese whey proteins and β-lactoglobulin in the cheese production industry (Galvão et al., 2001).

3.6.2 Leather Production

In the leather industry, chymotrypsin has been used in production processes including dehairing, bating and soaking. In the bating process, the specific catalytic ability of chymotrypsin has been used in hydrolyzing nonleather-forming proteins such as mucoids, globulin and albumins in raw materials. It can also help tanning materials and other chemicals to penetrate into fibers in order to obtain the desired texture (Kamini et al., 1999; Nathalie et al., 2004; Sandhya et al., 2005; Cera, 2008).

3.6.3 Chemical Industry

In the detergent industry, chymotrypsin is added into laundry detergent or dish detergents to enhance the decontamination ability of the detergent. The enzyme remains stable in both ionic and non-ionic surfactants and maintains around 80 % of its catalyzing ability after one hour of incubation with chemical detergent (Espósito et al., 2009). Due to the specificity of chymotrypsin on proteins and peptides, it can be used to break down proteinaceous contaminants (blood and foods) on cloth efficiently. Another advantage of using chymotrypsin is its ability to work under mild conditions such as low water temperature or natural pH environments which may lower the damage to cloth and body (Kamini et al., 1999). A US patent for detergent (Patent Number: 5,269,959) lists chymotrypsin as a Liquid Deep Cleaning Detergent (Schreibman, 1993). Another US patent (Patent Number: 20090281010) uses chymotrypsin in an eco-friendly laundry detergent comprising natural essence (Carter et,al., 2009). Gupta et al. (2002) reported that at least 25 % of extracted proteases is used as additives in laundry detergents every year. Espósito (2009) isolated chymotrypsin from fish processing waste and added it to laundry detergent to demonstrate high temperature tolerance and high enzyme stability (only 15% enzyme activity lost after 30 minutes incubation under 60ºC).

3.6.4 Medical Applications

Chymotrypsin has been used in the treatment of dyspesia and anorexia, cataract extraction, infertility and snakebites and as an anti-inflammation drug. It has been applied in several ways including: oral, local injection and atomized inhalation.

3.6.4.1 Dyspepsia and anorexia

Dyspepsia and anorexia are defined as disturbed, difficult or painful digestion. The incomplete protein digestion is dangerous because it may result in allergies or even production of toxic materials by bacterial breakdown of the incompletely digested protein (Bland, 1993). Chymotrypsin can contribute to proper digestion of proteins (Sims, 2001). Normally, people do not need to supplement with additional proteolytic enzymes because the body can produce them but pancreatic insufficiency does occur because of chemotherapy, physical injuries, chronic stress, cystic fibrosis and acute pancreatitis so extra chymotrypsin supplication may be required (Sims, 2001).

3.6.4.2 Anti-inflammation and prevention of wound infection

Chymotrypsin has been reported to have significant inhibition against the early stages of inflammation. It is widely used both orally and by injection as a non-steroidal anti-inflammatory drug (NSAID) in the treatment of athletic injuries, wound infections, sciatica and hand trauma (Seppa, 1980). Chymotrypsin dissolves soft fibrin and cleans the proteinecious debris at inflammation sites (Swamy and Patil, 2008). Latha (1997) used a trypsin-chymotrypsin combination in the ratio of 6:1 as an anti-inflammation agent to treat burn patients and found superior results compared to the use of trypsin alone.

3.6.4.3 Cataract extraction

Alpha chymotrypsin is widely used to separate cataractous lens from the zonular attachment sites (Hill et al., 1960; Rhee et al., 1999; Rich et al., 1974). Chymotrypsin

decreases the manipulating force required during the surgery to make it easy to remove the cataract which guarantees higher success rates in surgery (O'Malley et al., 1961). It has been shown that α-chymotrypsin specially attacks certain components of the zonules and does not cause any damage to other tissues in the eye (Hill et al., 1960). Yorston and Kiku (2000) claimed that chymotrypsin was the fastest and cheapest way to treat the cataract. Using α-chymotrypsin during cataract surgery can help avoid significant intraocular pressure (IOP). Rapidly raised IOP may cause pain and corneal oedema to patients (Rich et al., 1974; Passo et al., 1985).

3.6.4.4 Infertility

α-chymotrypsin can play a significant role in the process of semen liquefaction. It can shorten the semen liquefaction time and make it less viscous without influencing sperm motility. Combined with intrauterine insemination, α-chymotrypsin has been used in infertility treatment experiments on 38 infertile patients and 23 % of them became pregnant. Zhang (2007) claimed that twice-weekly injection of 5 mg chymotrypsin in 61 cases of infertile patients led to a cure rate is 83.6 %. The advantages of using chymotrypsin are shorter and more effective treatment of infertility at a lower cost.

3.6.4.5 Snakebite

Chymotrypsin has been used to treat patients with snakebite with good results (Omogbai, 2002). Zhang et al. (2005) studied the effect of local chymotrypsin injection on patients with bites from Chinese cobra and the results showed that it had less primeval effect than local injection of antivenom but better effects than other snakebite drugs. The toxins of snakes usually contain neurotoxin and cytotoxin which can change the permeability of cell membranes and the concentration of ions like Na^+, K^+ or Ca^{2+} causing the death of cells. The low amount of proteases kuronidase in snake toxin can accelerate this process (Zhang et al., 2005). Local chymotrypsin injection can slow down

or stop this process because chymotrypsin can cleave the snake toxin protein. The hydrolyzed protein loses its toxic properties and becomes harmless to humans (Zhang et al., 2005). Omogbai (2002) reported that the use of chymotrypsin to treat snakebite patients was effective, especially for those patients with tissue inflammation and edema.

3.7 Extraction and Purification of Chymotrypsin

Generally, the initial recovery steps of chymotrypsinogen (the inactivated zymogen precursor of chymotrypsin) involve: (a) extraction which includes preparation of crude material, homogenization using a buffer to extract crude chymotrypsin (chymotrypsinogen) from the prepared material and centrifugation to separate the crude chymotrypsin and (b) precipitation or fractionation to collect chymotrypsinogen. The processes that can be used to extract and purify chymotrypsin from fish waste (ammonium sulphate, reverse micelles and chromatography) are shown in Figures 3.5-3.7.

3.7.1 Extraction

The fish stomach and intestines are removed from fish and separated as soon as the fish has been killed and washed with cold water or isotonical saline solution to remove blood in the tissue. The inhibitors in blood can reduce chymotrypsin activity (Chong et al., 2002; Boeris et al., 2009). The intestine is then chopped into small pieces and mixed with 50 mM Tris-HCl buffer having a pH of 7.5. The homogenized mixture is centrifuged at 10000 rpm and 4ºC for 15 minutes. Crude chymotrypsin (chymotrypsinogen) is extracted as a supernatant and stored at -80ºC till further purification is carried out (Heu et al., 1995; Chong et al., 2002; Li et al., 2005; Castillo-Yañez et al., 2009; Yang et al., 2009).

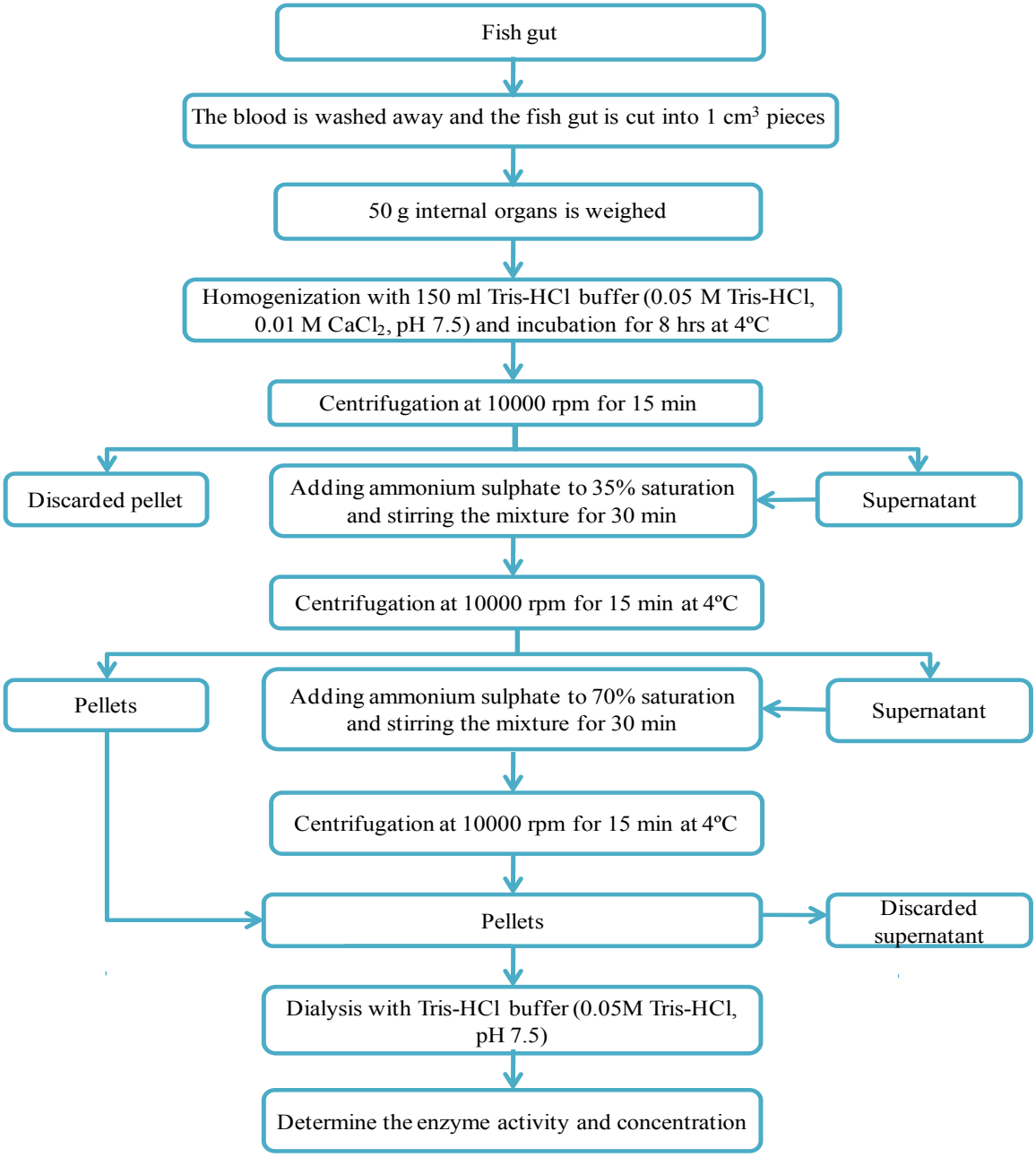

Figure 3.5. Extraction of chymotrypsin from fish waste using the ammonium sulphate method.

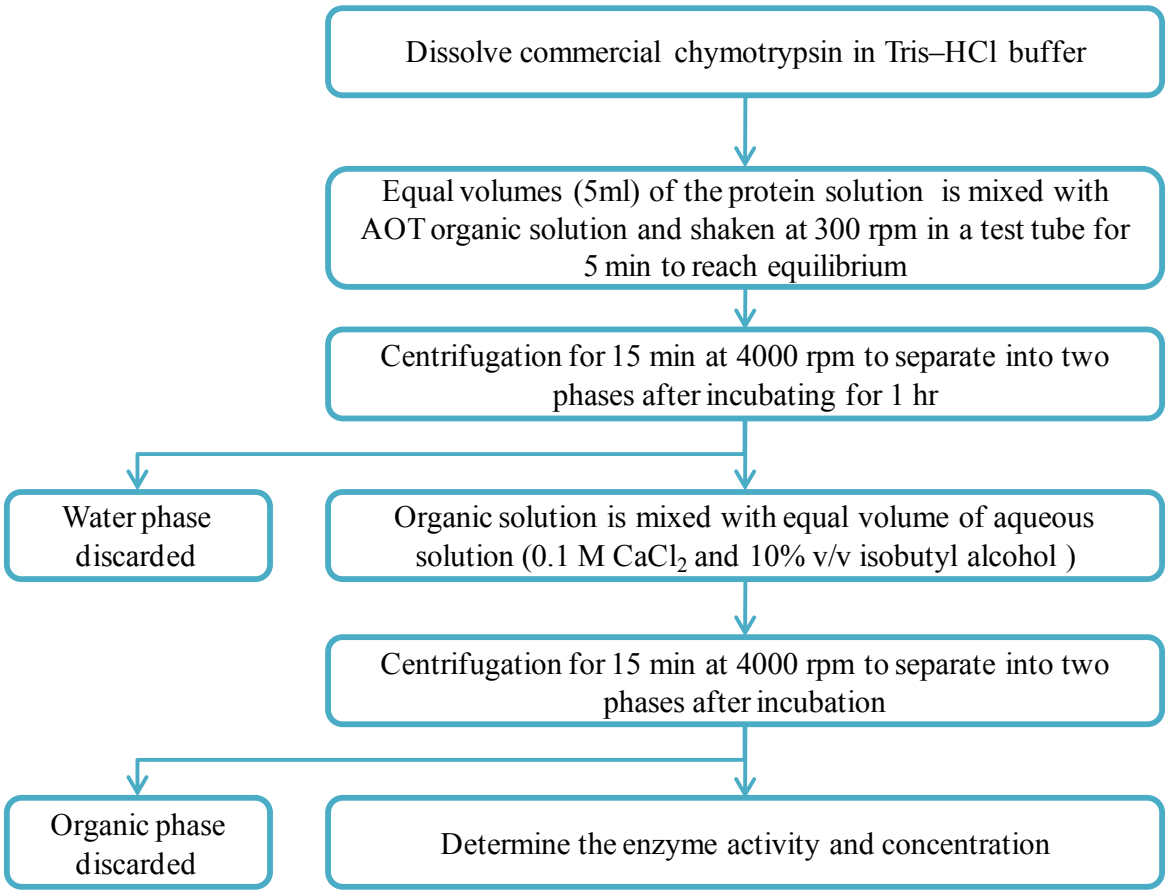

Figure 3.6. The extraction of chymotrypsin from fish waste using the reverse micelles method.

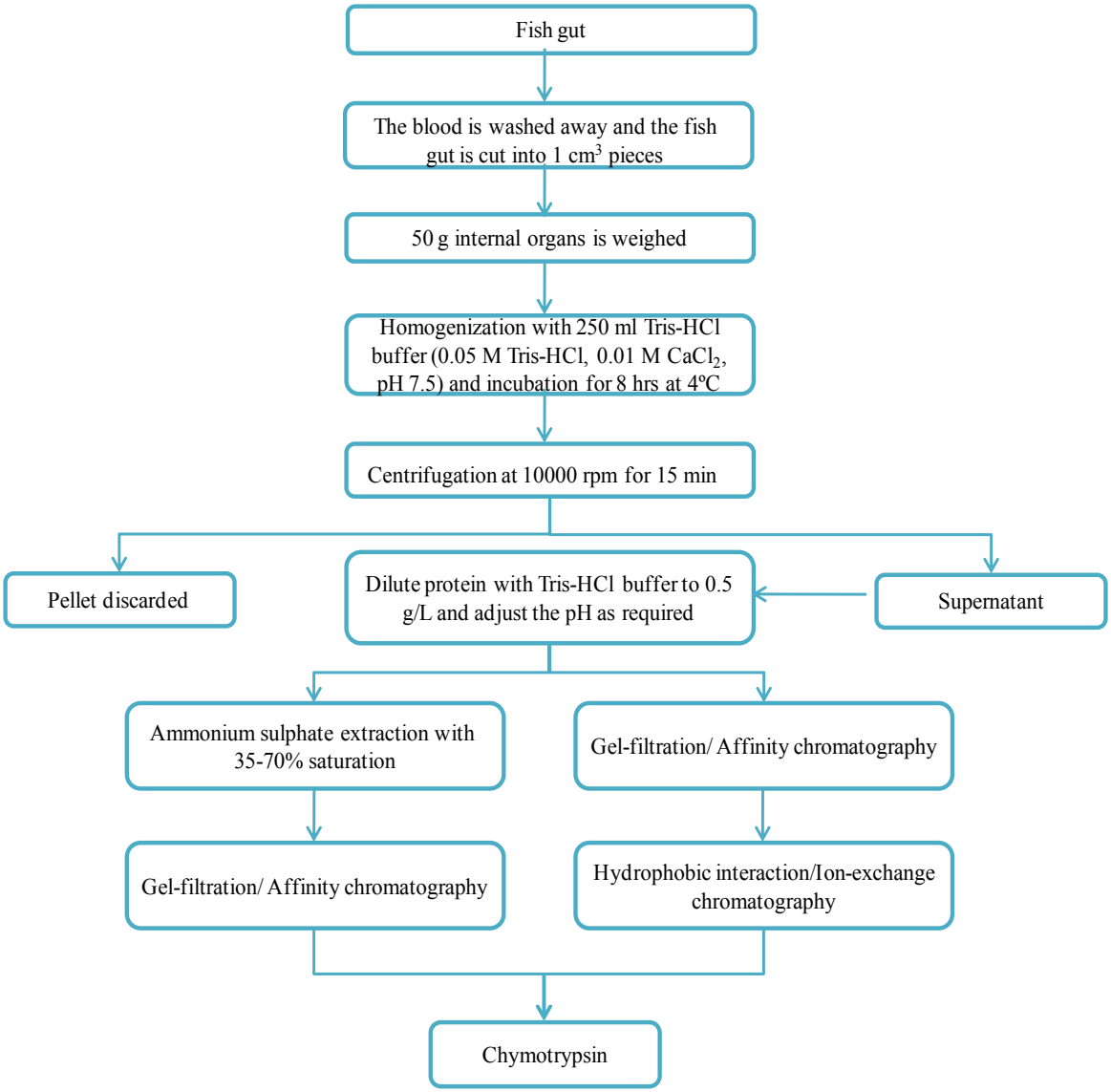

Figure 3.7. The extraction of chymotrypsin from fish waste using chromatography.

3.7.2 Purification

The chymotrypsinogen purification processes accounts for up to 80% of the total cost of chymotrypsin production. Precipitation is the technique most widely used in the chymotrypsin production industry because it can effectively purify and concentrate chymotrypsin at a very low cost (Matsudo et al., 2003; Boeris et al., 2009). Generally, chymotrypsin precipitation is used to obtain crude chymotrypsinogen and can be combined with chromatography techniques for further purification. Purification can be accomplished either by precipitation (using Tris-HCl) and ammonium sulfate fractionation or formation of reverse micelles (Möckel and Barnard, 1969a; Simpson, 2000; Castillo-Yañez et al., 2009; Yang et al., 2009).

3.7.2.1 Ammonium sulfate precipitation method

Ammonium sulfate is the most reported polyelectrolyte in chymotrypsinogen production (Chatterjee et al., 2004). In the precipitation process, poly-charged molecules which contain opposite electrical charges to the chymotrypsinogen are added into protein solutions to form a chymotrypsinogen-polyelectrolyte complex and generate insoluble aggregates. The materials used in the process are salts, non-ionic polymers, organic solvents and polyelectrolytes which are easy to bioseparate in the downstream process (Chatterjee et al., 2004; Boeris et al., 2009). Boeris (2009) used polyvinyl sulfonate (PVS) as polyelectrolytes during the purification process to produce a chymotrypsinogen-PVS precipitate complex in acidic pH which decreased the recovery rate of chymotrypsin. Also, increasing the PVS concentration lead to a lower recovery rate but produced a higher purity.

3.7.2.2 Reverse micelles method

Reverse micelles are thermodynamically stable molecules that can extract large biomolecules like proteins through electrostatic interaction that attracts soluble proteins

into the inner layer of the reverse micelles (Jolivalt et al., 1990; Hu and Gulari, 1996). Reverse micelles are useful because they can form amphiphilic structures in polar organic media which can be used to extract large amounts of proteins in the aqueous phase without denaturation. In the purification process, selection of surfactants plays a significant role in protein stabilization. Sodium di-2-ethylhexyl sulfosuccinate (AOT) is the most common surfactant used in chymotrypsin purification. (Jolivalt et al., 1990; Hentsch et al., 1992; Hu and Gulari, 1996).

pH influences ionic molecular interactions in solution and, therefore, influences the efficiency of extraction by reverse micelles (Jolivalt et al., 1990). The yield of chymotrypsin will increase with an increase in pH and the maximum extraction yield can be reached at an isoelectric point around a pH of 8.5 (Hu and Gulari, 1996). However, the pH of protein aqueous solutions should be held near a pH of 3 because at that pH the autohydrolysis rate is minimized and chymotrypsin is most stable (Hu and Gulari, 1996). Jolivalt et al. (1990) and Hentsch et al. (1992) reported that increasing chloride ion concentration will decrease chymotrypsin yield by competing with chymotrypsin in the extraction process and the effect is particularly significant at low ionic strength.

The whole extraction process with reverse micelles can be divided into two steps: forward extraction and back extraction. During the forward extraction process, the aqueous and organic phases are separately prepared and homogenized with an orbital stirrer at 250 rpm for 90 minutes. After extraction (protein transfer from aqueous to organic phase), the phases are separated by centrifugation at 1500-2000 rpm for 10 minutes (Jolivalt et al., 1990; Hu and Gulari, 1996). The concentration of protein can be measured with UV spectroscopy at 280 nm. Back extraction transfers proteins from reverse micelles to aqueous solutions. Back extraction is usually very slow and $CaCl_2$ is added into the aqueous phase to assist the process (Hu and Gulari, 1996). The limitations of using reverse micelles in the back-extraction are due to: (a) the difficulty in separating

proteins from AOT reverse micellar phase and (b) the excessive time involved in the process (Goto et al., 1998; Hu and Gulari, 1996).

Co-surfactants have been used to improve the extraction properties of AOT and the most common is dioleyl phosphoric acid (DOPLA). This can be added to AOT to form AOT-DOLPA for use in chymotrypsin extraction. Goto (1998) reported that the effectiveness of mixed reverse micelles increased with increasing amount of DOLPA added. He also found that a 4:1 ratio of AOT: DOLPA was best for chymotrypsin extraction and that 10 mM of mixed reverse micelles had higher extraction ability than 200 mM of AOT. At low concentrations, the mixed reverse micelles are, therefore, very effective in separating and enriching chymotrypsin. Hu and Gulari (1996) and Goto et al.(1998) reported that mixed reverse micelles not only make chymotrypsin extraction more complete but it also shortens the back extraction time from 24 hour to 2 hour.

3.7.2.3 Chromatography method

Chromatography is always the last step in the chymotrypsin purification process. Chromatography columns are used to concentrate and ultrafilter the fluid passing though them. The fluids can then be dialysed against a buffer (Heu et al., 1995). There are four types of chromatography that have been used in the chymotrypsin purification process: ionic chromatography, gel chromatography, affinity chromatography and hydrophobic interaction chromatography. Ionic chromatography is particularly useful because it can be used to both separate cationic and anionic forms of chymotrypsin A and B and to separate chymotrypsin from trypsin.

Heu (1995) used gelfiltration chromatography with a Sephadex G-75 column (2.6 × 75 cm) to first isolate chymotrypsin by molecular size. The crude chymotrypsin solution was then further purified using ion chromatography with a column packed with diethylaminoethyl (DEAE) cellulose, designed to interact with negatively charged

proteins. Ryan (1965) used a similar column to purify chymotrypsin from chicken. Raae and Walther (1989) used both ion chromatography with a DEAE-Sepharose column and gel filtration with a PBA (Phenyl-Butyl-Amine)-Sepharose column to purify chymotrypsin and eluted with 35-40% ethleneglycolin. Ionic chromatography with CM-cellulose was used in the double purification of chymotrypsin and chymotrypsinogen from turtle and fish by equilibrating with 0.01M sodium succinate and 0.001M EDTA and eluting with salt gradients (Möckel and Barnard, 1969 a; Möckel and Barnard, 1969 b). Yang et al. (2009) used ionic chromatography with DEAE-Sephacel eluted with NaCl at a concentration of 0.2M to purify chymotrypsin from crucian carp (*Carassius auratus*) and detected two peaks: the unretained portion was cationic chymotrypsin (B) and the retained portion was anionic chymotrypsin (A). After gel-filtration on Sephacryl S-200, the two active portions were respectively subjected to hydrophobic interaction chromatography using Phenyl-Sepharose and SP-Sepharose for further purification. Most of the contaminated proteins were removed by washing with ammonium sulfate with concentrations ranging from 0 to 1M. The unretained ionic chymotrypsin B was further purified with a SP-Sepharose cationic exchange column. The results of purification are shown in Figure 3.8.

Affinity chromatography has been used by Fujiwara et al. (1974), Branchini and Ziolkowski (1979), Nishikata (1983) and Ahn and Chung (1985). In affinity chromatography, a column contains cross-linked insoluble polymer or gel attached to a competitive chymotrypsin inhibitor or ligand (Cuatrecasas, 1970 and Ahn and Chung, 1984). During the chromatography, all other unwanted proteins pass through the column but chymotrypsin is absorbed and bonded through extended hydrocarbon chains which make the ligand (an ion or molecule that binds to a central metal atom to form coordination complex) at different distances from the gel matrix backbone. The whole process is pH dependent because pH affects the ionization of functional group. Chymotrypsin can be recovered by elution with 0.1 M acetic acid. Fujiwara et al. (1974) used carbobrnzoxyl-L-phenylalanyl-triethylenetetraminyl- Sepharose (Z-L-

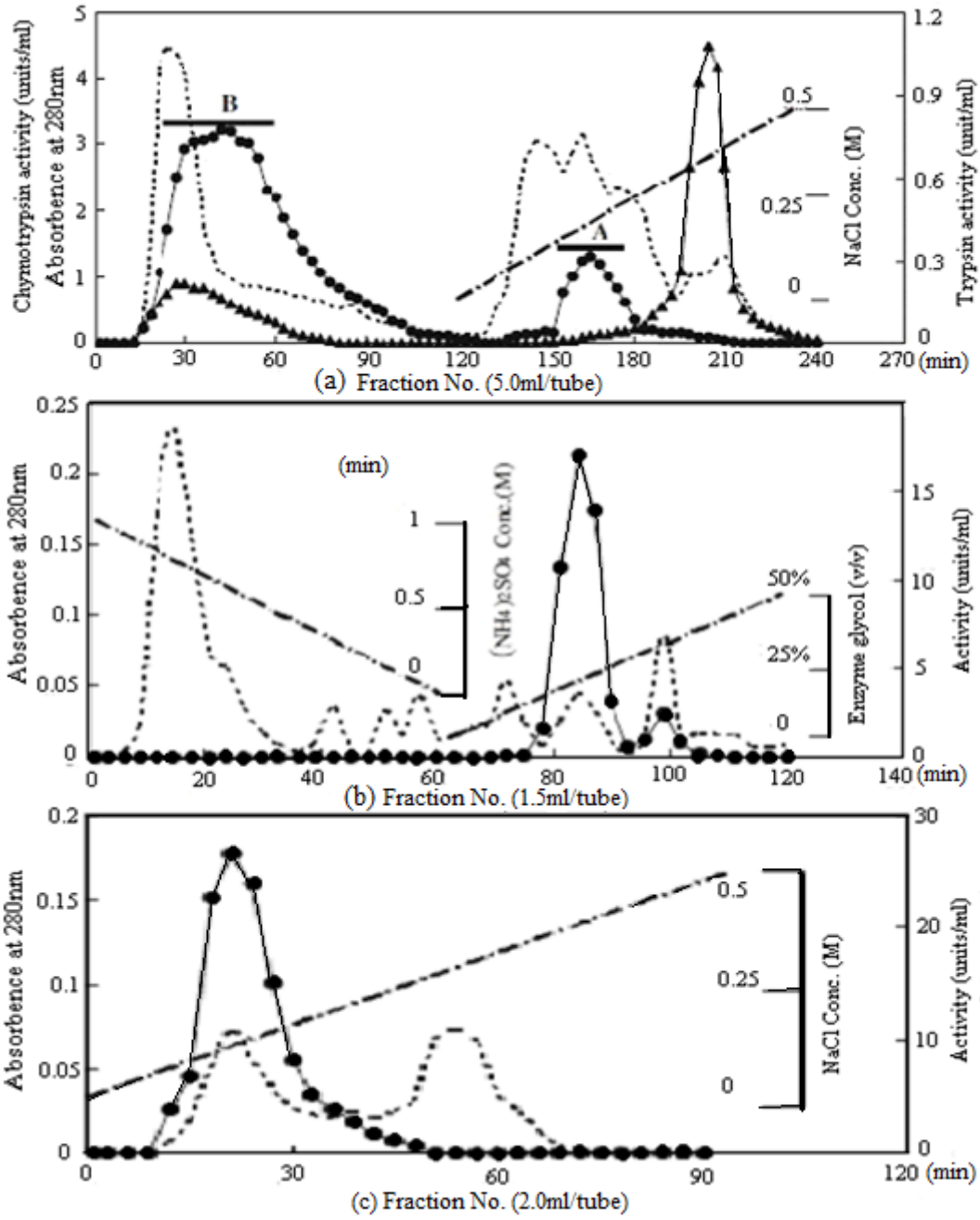

Figure 3.8. Chromatographic purification of crucian carp chymotrypsins. (a) DEAE-Sepharose chromatography. (b) Phenyl-Sepharose chromatography. (c)SP-Sepharose chromatography. (●): Suc-Leu-Leu-Val-Tyr-AMC hydrolyzing activity. (▲): Boc-Phe-Ser-Arg-MAC hydrolyzing activity. (Yang et al., 2009).

Phe-T-Sepharose). Cuatrecasas (1970) used Sepharose-bound D-tryptophan methyl ester. Nishikata (1983) used sepharose with chymostatin analogue (Gly-Gly-L-Leu-L-phenylalaninal). Ahn and Chung (1984) used 4-phenylbutylamine as a ligand in chymotrypsin purification.

3.8 Activation of Chymotrypsinogen

Chymotrypsinogen is an inactive form of chymotrypsin existing in the pancreas. The extraction and purification processes result in pure chymotrypsinogen. Although chymotrypsinogen has enzymatic activity, the low level of activity makes it hard to detect in normal conditions. In order to evaluate the properties of chymotrypsin, chymotrypsinogen activation is required (Blow, 1976). Trypsin and other bacterial proteases excreted by *Bacillus subtilis* or *Penicillium* can be applied as a natural activator of chymotrypsin (Sakota, 1954; Dreyer and Neurrat 1955; Prokuryakov, 1970). However, the optimal pHs of activators are different. For instance, the optimum pH for trypsin is 7.5 while the optimal pH for *Aspergillus oryzae* is 3.0-5.0. The optimal pH of *Kaufman* and *Erlanger* ranges from 3.2 to 3.4 (Prokuryakov, 1970).

Since chymotrypsinogen is a single polypeptide chain made up of 245 amino acids, different forms of chymotrypsin can be activated by controlling the reaction condition (Boeris et al., 2009). In the whole process, trypsin only hydrolyzes one peptide bond between Arg 15 and Lie Ile 16 and forms π-chymotrypsin (Appel, 1986). The remainder of the N-terminal peptide plays an important role in protecting chymotrypsinogen from other non-specific activators by using disulfide bonds, which are connected to the left molecule (Spilliaert and Gudmundsdottir, 2000). Then, π-chymotrypsin autocatalyzes itself to form chymotrypsin and three new disulphide bonds to link the polypeptide chain as shown in Figure 3.9. A further reaction will produce 2 types of chymotrypsin (δ-chymotrypsin and γ- chymotrypsin). Additionally, in the production of δ-chymotrypsin, it will lose the dipeptide Ser14-Arg15 bonds. The relative activity of trypsin actived and

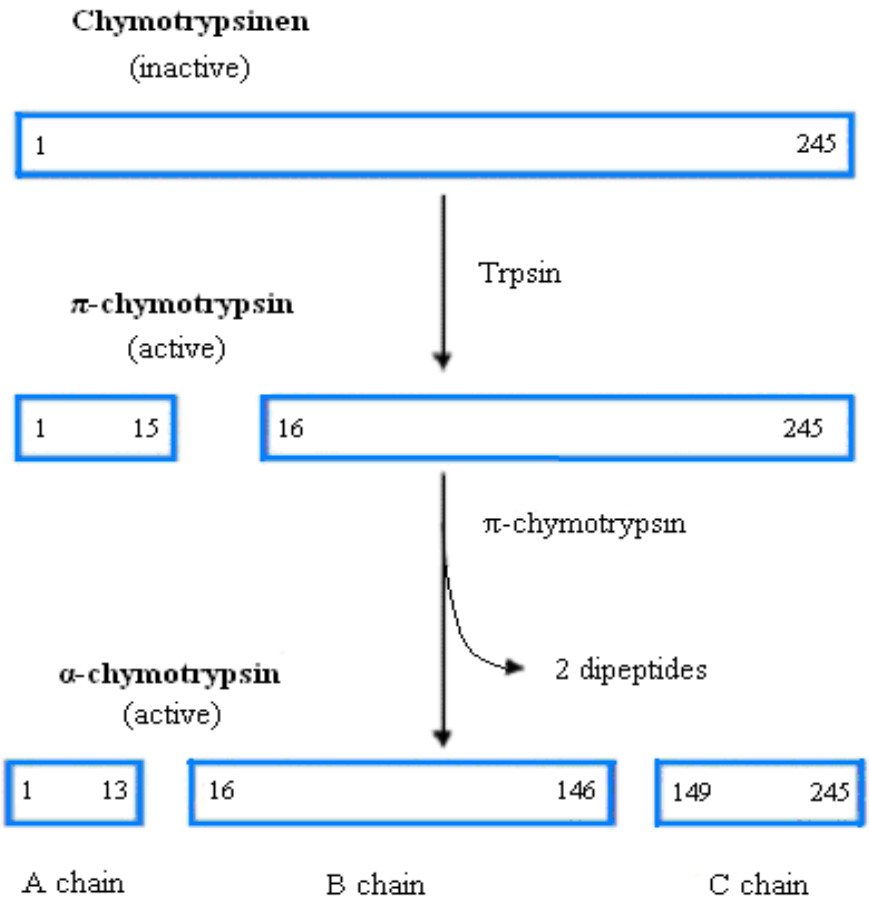

Figure 3.9. Activation of chymotrypsinogen, peptide bonds split by trypsin and chymotrypsin in an autocatalytic process leading to defined chains and peptides (Berg et al., 2007).

non-trypsin actived chymotrypsinogen depends on incubation time as shown in Figure 3.10. Several factors contribute to chymotrypsinogen activation rates including the concentration of trypsin, incubation time, temperature and pH.

3.8.1 Trypsin Concentration

Trypsin is an activator of chymotrypsinogen which can dramatically accelerate the speed of zymogen activation even at a very low concentration (0.01 mg/g of homogenate). The relative activity of chymotrypsin during the zymogen activation process in the presence or absence of trypsin is shown in Figure 3.10 (Boeris et al., 2009). Glazer and Steer (1977) found that an increase in trypsin concentations at low concentations (0.5%-1.0%) increased the rate of activation but trypsin concentrations above 10% showed little increse in activation (Figure 3.11). Thus, properly controlled concentrations of trypsin in industry have commercial benefits.

Trypsin not only plays a significant role in chymotrypsinogen activation but also contributes to different types of final products. With the process of large amounts of trypsin, π-chymotrypsin is rapidly formed and becomes the predominate product in the process and it further loses its dipeptide Ser 14-Arg 15 bonds. These bonds are autocatalytically produced by δ-chymotrypsin, which is known as rapid activation of chymotrypsinogen (Bettelheim and Neurath, 1954; Appel, 1986). Slow activation occurs in the presence of low concentrations of trypsin. The production of α-chymotrypsin is the major pathway and γ-chymotrypsin will be formed only slowly (Appel, 1986).

3.8.2 Incubation Time

The incubation time required for the chymotrypsinogen activation process depends on the activation method applied. Usually, there are two types: classical activation (pH 7.5, 5ºC, trypsin free, 48 incubation time) and rapid activation (activated by trypsin with incubation times varying from 1 to 48 hr) (Bettelheim and Neurath, 1954; Miller et al.,

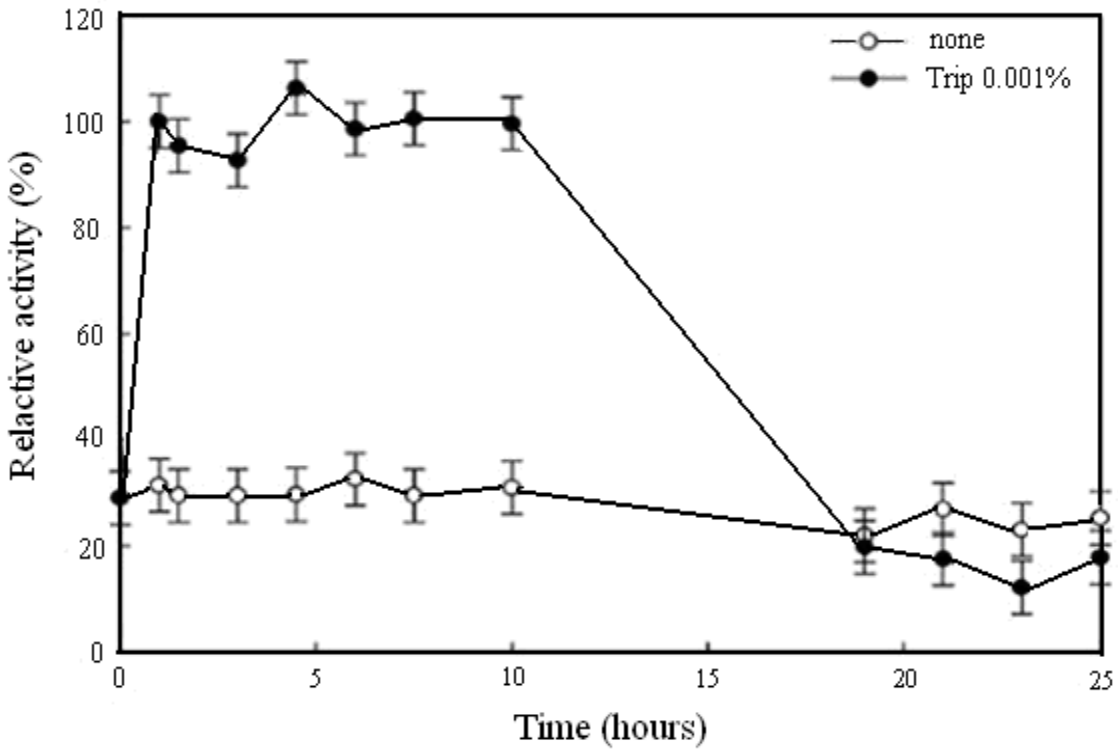

Figure 3.10. Chymotrypsin activity from bovine pancreas homogenate in a medium of sodium citrate-Tris-HCl 50 mM at a pH of 8.2 (Boeris et al., 2009).

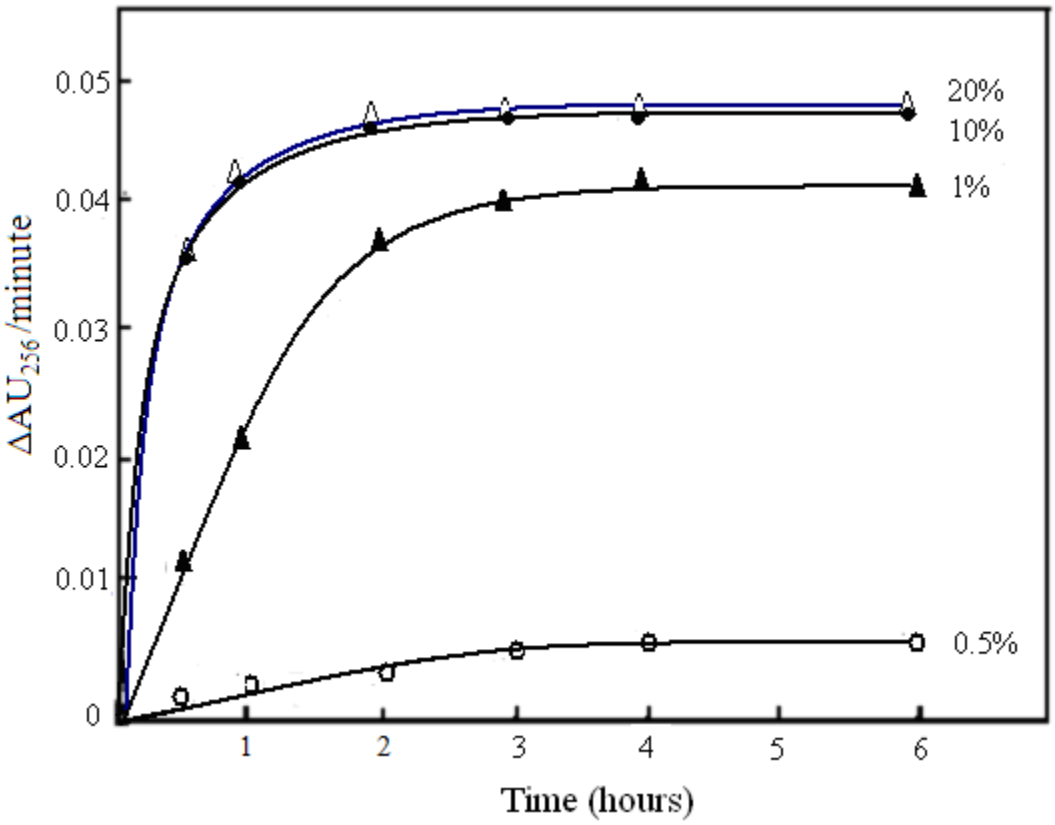

Figure 3.11. Trypsin requirement for chymotrypsinogen activation (Glazer and Steer, 1977).

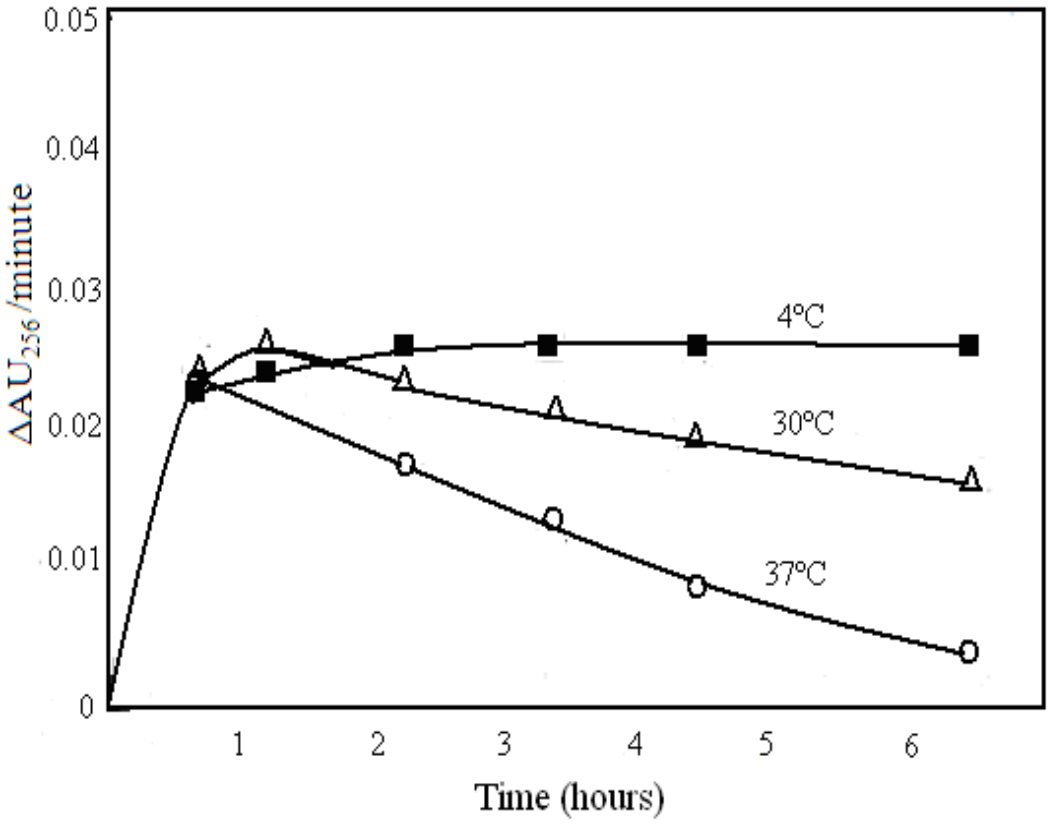

Figure 3.12. The temperature dependence of chymotrypsinogen (Glazer and Steer, 1977).

1971). With the addition of trypsin, the enzymogen activation significantly increases in one hour with the decrease of the homogenate solution viscosity. Sakota (1954) reported that the activity of chymotrypsin increases at a constant rate in the first 10 hours followed by an observed decrease in catalytic activity after 24 hr. The possible reason contributing to the decreased chymotrypsin activity could be the non-active form of trypsin being self-generating in the solution and destroying the structure of chymotrypsinogen (Sakota, 1954; Boeris et al., 2009). Engel and Alexander (1966) reported that activation of chymotrypsinogen was completed within 30 min. Dreyer and Neurath (1955), Miller et al. (1971) and Glazer and Steer (1977) reported that the activity of chymotrypsin was stable at 4ºC and observed decreasing activity under high incubation temperatures.

3.8.3 pH

Guyonnet et al. (1999) reported that the optimal pH for chymotrypsinogen activation was 7.5 (Figure 3.13) and that the catalytic activity of chymotrypsin decreased with a decrease in pH. Similar results were reported by Engel and Alexander (1966), Bettelheim and Neurath (1954), Glazer and Steer (1977) and Guyonnet et al. (1999).

3.9 Assaying of Chymotrypsin

3.9.1 Enzyme Concentration

Three main methods are commonly used to evaluate chymotrypsin concentration: (a) absorbance at 280 nm, (b) the Bradford method and (c) the Lowry method.

Protein in solution has a maximum absorbance of ultraviolet light at 280nm. When measuring enzyme concentration, the wavelength must be adjusted to 280 nm and the system calibrated to zero with buffer solution. The absorbance of protein solution is then measured and the concentration is calculated by the following equations (Layne 1957; Stoscheck 1990).

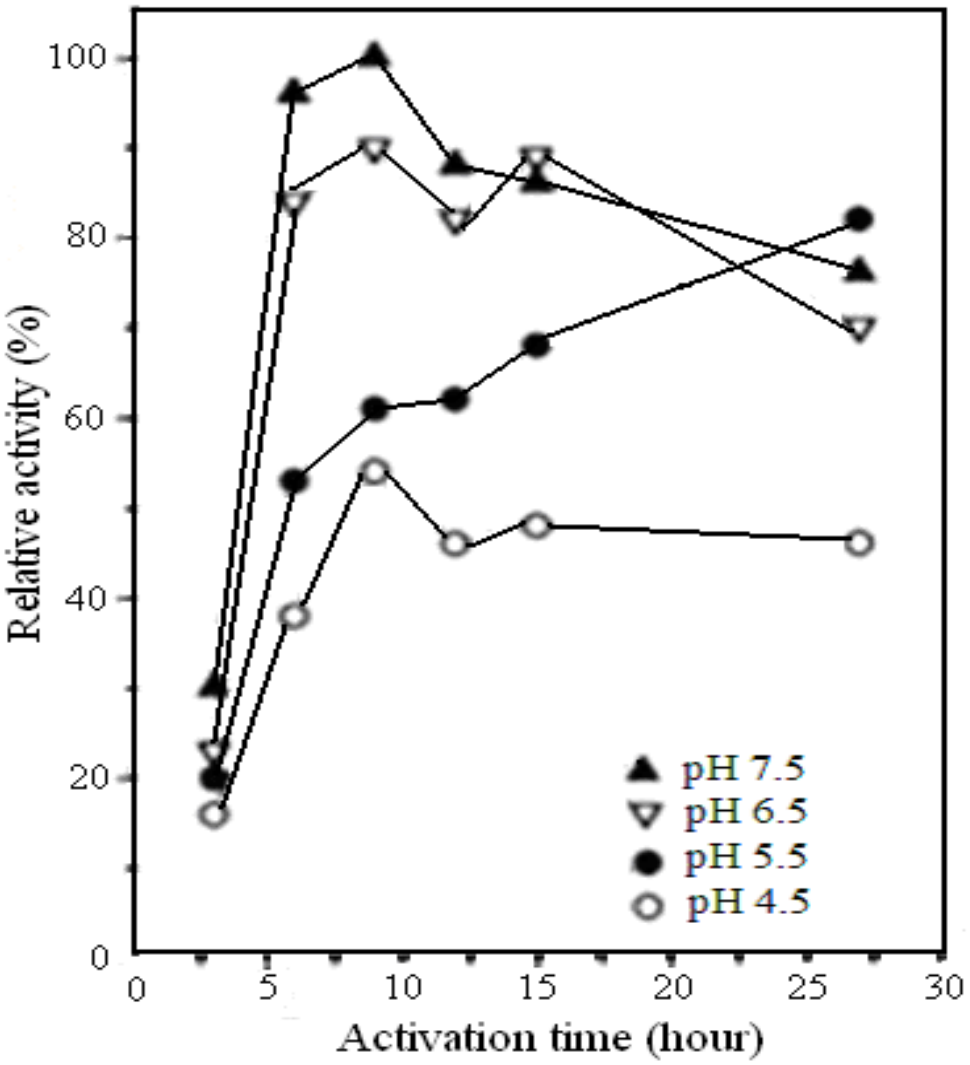

Figure 3.13. The effects of pH on the activation of chymotrypsinogen (Guyonnet et al. 1999).

(a) For protein mixtures:

$$\text{Concentration (mg/ml)} = \Delta AU_{280}/\text{path length (cm)} \quad (2)$$

(b) For protein mixtures with possible nucleic acid contamination:

$$\text{Concentration (mg/ml)} = 1.55\times\Delta AU_{280} - 0.76\times\Delta AU_{260} \quad (3)$$

In the Bradford method, Coomassie Blue reagent is mixed with the enzyme solution and the absorbance is read at 595 nm after incubation for 15 minutes. Concentrations are determined relative to a standard curve based on bovine serum albumin (BSA).

The method developed by Lowry (1951) is a relatively sensitive method but more complicated and time consuming compared to the Bradford Method. The enzyme solution is treated with Folin-Ciocaltea reagent to create a blue compound and allowed to incubate for 10 minutes. Absorbance is read at 660 nm and a standard curve using BSA is required. There are many substrates that have been reported to affect the results obtained from this method including Tris, EDTA, sulfhydryl compounds, potassium compounds, disdulfide compounds, carbohydrates, glycerol, Tricine, detergents, most phenols, uric acid, guanine, magnesium and calcium (Olson and Markwell, 2007).

3.9.2 Enzyme Activity

A number of substrates are used to assay chymotrypsin activity including N-acetyl-L-tyrosine ethyl ester (ATEE), benzoyl-tyrosine ethyl ester (BTEE) and N-Suc-Ala-Ala-Pro-Phe-p-nitroanilide (SAAPPNA) (Hummel, 1959; Erlanger et al., 1961; Ramakrishna et al., 1987; Sabapathy and Teo, 1995; Heu et al., 1995; Chong et al., 2002; Chakrabarti et al., 2006; Li et al., 2005; Sveinsdóttir et al., 2006). The activity of chymotrypsin is determined as the change in absorbance of chymotrypsin used in the assay per mg protein per min (Chakrabarti et al., 2006). A standard curve is required.

When ATEE is used as substrate, one unit of enzyme activity is defined as the decrease in absorbance of 0.0075 per min measured at 237 nm of at 25 °C. Enzyme is mixed with ATEE solution in potassiium phosphate buffer and the absorbance is measured every half minute for 5 min. The activity is calculated using the following equation.

$$\text{Activity} = \frac{\Delta AU_{237}/\text{min} \times \text{dilution}}{0.0075 \times 0.2 \times \text{mg enzyme/ml original solution}} \quad (4)$$

When BTEE is used as substrate, one unit of enzyme activity is defined as one unit of enzyme hydrolyzing one micromole of BTEE per minute at pH 7.5 and 25ºC. Enzyme is added to BTEE solution, dissolved in Tris-HCl buffer (10 mM CaCl2 with a pH of 7.5) at room temperature and the absorbance is measured at 256 nm every half minutes for 5 min. The activity is calculated using the following equations (Hummel, 1959; Sabapathy and Teo, 1995; Li et al., 2005; Parra et al., 2007; Tubio et al., 2009; Boeris et al., 2009).

$$\text{Activity} = \frac{\Delta AU_{256}/\text{min} \times 1000}{964 \times \text{mg enzyme/mlin the reaction mixture}} \quad (5)$$

When SAAPNA is used as a substrate, the enzyme activity is defined as one unit of enzyme activity hydrolyzing SAAPNA and releasing one micromole of p-nitroaniline at pH 7.5 and 25ºC. The p-nitroaniline molar extinction coefficient is 8800 $M^{-1}cm^{-1}$. The enzyme is mixed with SAAPNA, dissolved in 50 mM Tris-HCl buffer (10 mM CaCl2 with a pH of 7.5), and absorbance is measured at 410 nm every half minute for 5 minutes at room temperature. The activity is calculated using the following equation (Hummel, 1959; Chong et al., 2002; Sveinsdóttir et al., 2006; Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009).

$$\text{Activity} = \frac{\Delta AU_{410}/\text{min} \times 1000 \times \text{volume of reaction mixture}}{8800 \times \text{mg protein in the assay}} \quad (6)$$

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) has been widely used to determine molecular weight of chymotrypsin after extraction using zymograms as substrate (Garcia-Carreno et al., 1993; Heu et al., 1995; Chong et al., 2002). The SDS-PAGE consisted of 12% separating gel and 5% stacking gel. The extracted chymotrypsin was mixed with Tris-HCl buffer (pH 6.8) to make a proteinase sample (Chong et al., 2002). A five-microliter mixture of the chymotrypsin sample was loaded into the SDS-PAGE gel and the gel is dipped in a Tris-HCl buffer with 3% casein (pH 7.5) for 30 min at 5 °C in order to allow the casein to enter the gel. After incubating the gel at 25°C for 60 min, the gel is washed, stained with Coomassie Brilliant Blue for 2 hours and destained. Clear bands on the gel indicating enzyme activities are compared with molecule weight markers to indicate the molecule weight of chymotrypsin (Chong et al., 2002); Chakrabarti, 2006). Additionally, Sephacryl S-100 column (0.9 × 55 cm) gel filtration can also be used to determine the molecule weight of chymotrypsin (Heu et al., 1995).

3.9.4 Effect of Inhibitors

To study the effect of inhibitors on chymotrypsin activity, purified enzyme was incubated with several specific protease inhibitors such as ethylenediaminetetraacetic acid (EDTA), chymotrypsin specific inhibitors tosyl-phenylalanine chloromethyl-ketone (TPCK), serine protease inhibitors soybean trypsiin inhibitor (SBTI) and phenyl-methyl-sulphonyl-fluoride (PMSF), benzamidine, 4-(2-aminoethyl)-benzenesulfonyl fluoreide (AEBSF), leupeptin, benzamidine, ela-statinal or tosyl-L-lysine chloromethyl ketone (TLCK) (Lam et al., 1999; Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009; Yang et al., 2009). After incubation, substrate solutions was added and the residual activity was measured. The percentage activity was calculated using the activity of the blank as 100% (Lam et al., 1999; Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009; Yang et al., 2009).

3.9.5 Isoelectric Point

The isoelectric point (pI) of chymotrypsin is always measured by analytical electrofocusing in thin-layer polyacrylamide flat gel (LKB ampholyne PAG plate) containing ampholyne in the pH range of 3.5-9.5 (Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009). Purified protein is stained by Coomassie Brilliant Blue. The result is compared with isoelectric focusing calibration kits with 11 pI known proteins (Gildberg et al., 1990; Castillo-Yañez et al., 2006; Castillo-Yañez et al., 2009).

CHAPTER 4. EXPERIMENT DESIGN

The experimental work was divided into 3 parts as shown in Figure 4.1. In the first part, the optimization of the reverse micelles method was carried out by investigating the effects of AOT concentration in the forward extraction, pH in both forward and backward extractions and the salt (KCl) concentration in the backward extraction on the yield, purity and activity of enzyme as shown in Figures 4.2 and Tables 4.1 to 4.3. In the second part, the effects of alcohol in the backward extraction on enzyme activity, extracted protein concentration, specific activity, purification fold and recovery yield were studied. In the third part, the extraction and purification of chymotrypsin was carried out using the ammonium sulphate method. The fourth part of the experiment was devoted to comparing the reverse micelles method with the ammonium sulphate extraction method in terms of enzyme activity, extracted protein concentration, specific activity, purification fold and the recovery yield (Figure 4.2).

The selected levels of AOT were from 1 mM to 35 mM. The levels of pH for forward the extraction (pH-1) were 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5; the selected pH levels for the backward extraction (pH-2) were 6.5, 7.0, 7.5, 8.0 and 8.5. The levels of KCl concentration in the buffer of the backward extraction step were 0.5, 1, 1.5 and 2 M. In the optimization of the forward step, pH 8.0 and KCl concentration 1.5M was applied in the backward extraction; in the optimization of the backward extraction, the optimum conditions determined from the first step were applied in the forward extraction. The effect of alcohol on chymotrypsin recovery yield was studied by comparing samples with the addition of 15% (v/v) alcohol or the same volume of distilled water in the backward extraction step. Both the reverse micelles method and the ammonium sulphate extraction were compared based on enzyme activity, specific activity purification fold and recovery yield.

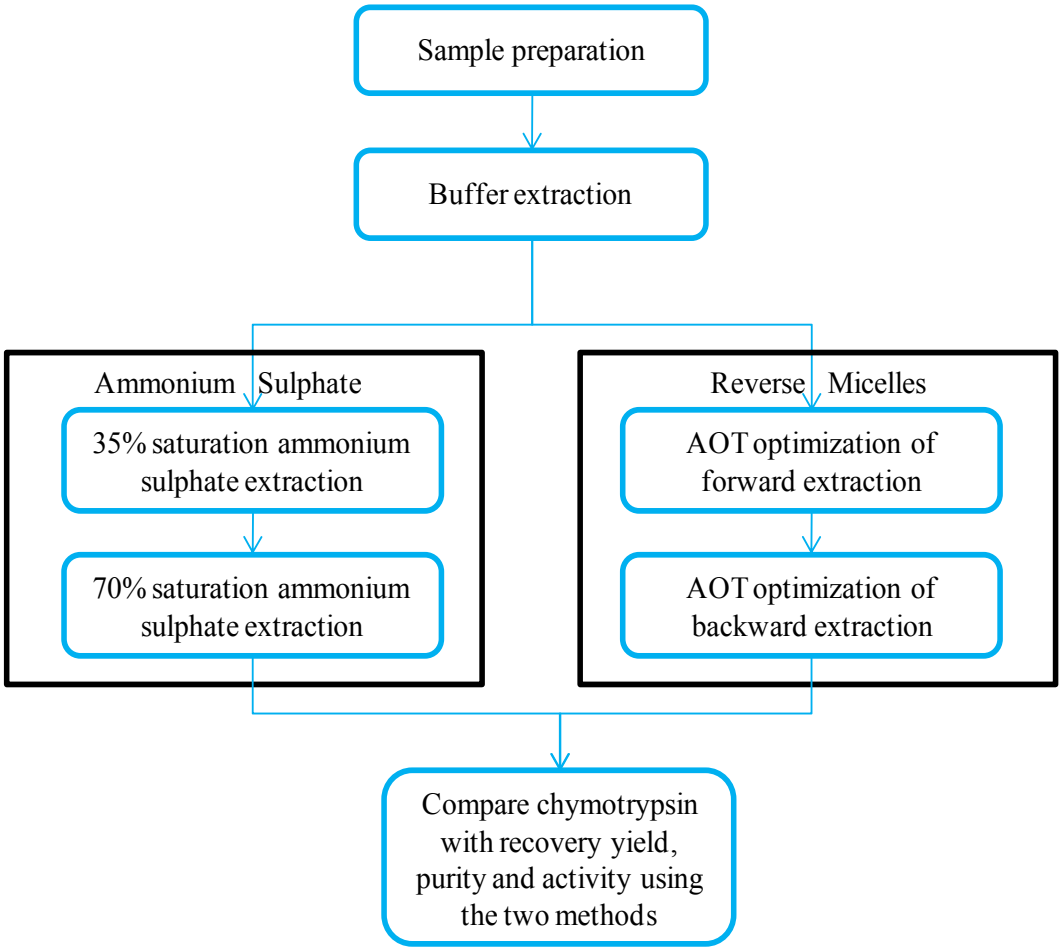

Figure 4.1. Schematic of the experimental plan for the proposed research.

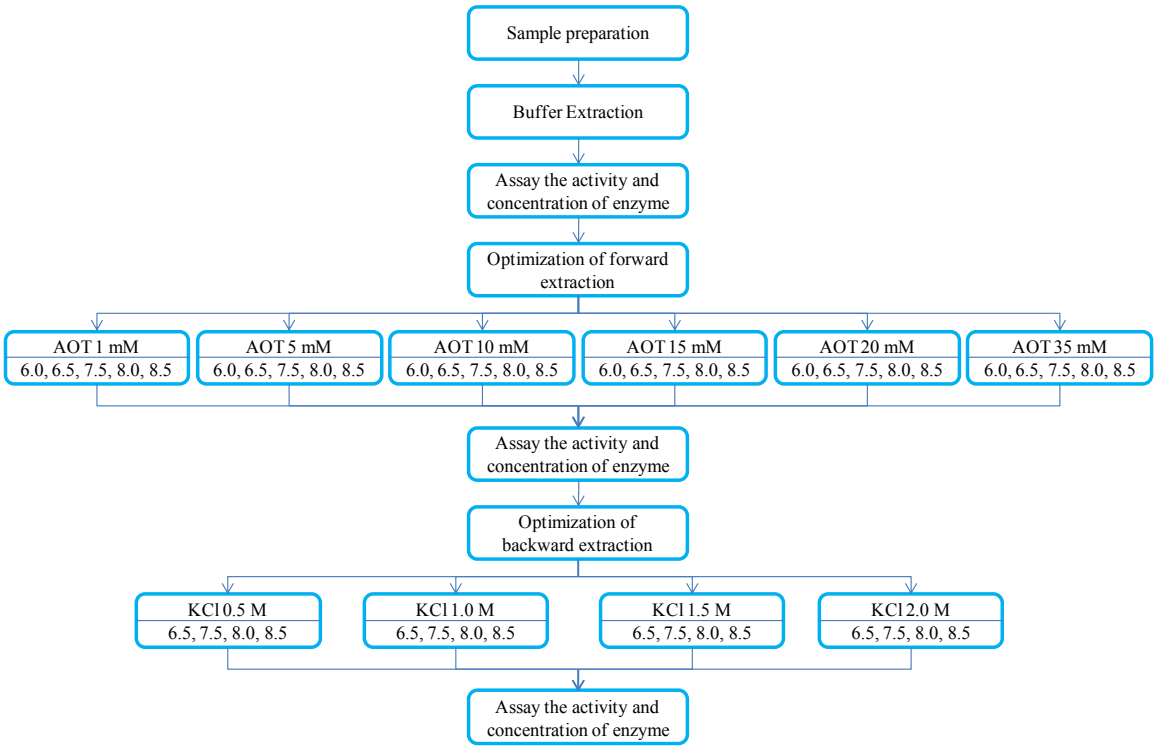

Figure 4.2. Schematic of the experimental plan for the optimization of the reverse micelles method.

Table 4.1. Optimization of the forward extraction (changing AOT concentration and pH-1).

Factors	Parameters
AOT	1 mM, 5 mM, 10 mM, 15 mM, 20 mM, 35 mM
pH-1	6.0, 6.5, 7.0, 7.5, 8.0, 8.5
pH-2	8.0
Temperature	4°C
Salt Concentration	1.5 M
Time-1	30 min
Time-2	1 hr

No. of replicates = 3
No. of runs = 108

Table 4.2. Optimization of the backward extraction (changing pH-2 and KCl concentration).

Factors	Parameters
AOT	Optimum from FE
pH-1	Optimum from FE
pH-2	6.5, 7.0, 7.5, 8.0, 8.5
Temperature	4°C
Salt concentration	0.5, 1.0, 1.5 and 2 M
Time-1	30 min
Time-2	1 hr

No. of replicates = 3
No. of runs = 60

Table 4.3. Effect of alcohol on the backward extraction.

Factors	Parameters
AOT	Optimum from FE
pH-1	Optimum from FE
pH-2	Optimum from BE
Temperature	4°C
Salt concentration	Optimum from BE
Alcohol	15% v/v alcohol; 15% v/v distilled water
Time-1	30 min
Time-2	1 hr

No. of replicates=3
Total no. of runs= 6

CHAPTER 5. EXPERIMENTAL MATERIALS

5.1. Glassware

The glassware used in the experiment included test tubes, 500 ml beakers, conical flasks, pipettes and separating funnels. All glassware was washed with soap, tap water and distilled water.

5.2. Chemicals

Tris, HCl, $CaCl_2$, NaCl, ammonium sulphate, benzoyl-tyrosine ethyl ester (BTEE), methyl alcohol and n-butyl alcohol were obtained from Sigma-Aldrich, Oakville, Ontario, Canada. AOT, isooctane (2,2,4-trimethylpentane), chymotrypsin, isobuty alcohol and BSA (bovine serum albumin) were obtained from Fisher scientific, Ottawa, Ontario, Canada.

5.3. Reagents

Reagents used included 0.05M Tris-HCl buffer (0.01M $CaCl_2$, pH7.5), 10% v/v isobutyl alcohol, 10 μM BTEE and stopping agent (methyl alcohol: n-butyl alcohol: distilled water = 35:30:35). Tris-HCl buffer was made by first determining the number of moles of Tris base required by multiplying the desired molar concentration of buffer by the volume of buffer being made. The Tris base was dissolved in deionized water approximately 1/3 the volume of buffer to be made. A pH meter was used when Tris was titrated with 1M of HCl until the desired pH was reached. In a volumetric flask, deionized water was added to the Tris-HCl solution to reach the desired volume.

5.4. Equipment

Equipment used in the experiment were: Hewlett Packard 8455 UV/VIS Spectrophotometer (G1103A, Hp, Santa Clara, California, USA), centrifuge (MP4R, International Equipment Company, Needham, Massachusetts, USA), pH meter (Accumet model 15, Fisher Scientific, Toronto, Ontario, Canada), homogenizer (Polytron PT1035, Brinkmann Instruments, Toronto, Ontario, Canada) and incubator shaker (Classic C24, New Brunswick Scientific Company, Edison, New Jersey, USA).

5.5. Fish Sample

The fish, red perch (*Sebastes marinus*), used in the experiment were collected from Clearwater Seafoods Ltd., Halifax, Nova Scotia, Canada. Samples were collected in sealed plastic bags and transported to the food science laboratory for the removal of the gut.

CHAPTER 6. EXPERIMENTAL PROCEDURES

6.1. Sample Preparation

Fish gut from red perch was used as raw materials. The intestines were separated from fish, washed with cold water and isotonic saline solution to remove the blood in the tissue according to the procedure described by Chong et al. (2001) and Boeris et al. (2009). The fish gut was chopped into small pieces (1 cm^3), weighed, marked and stored at -20°C for later use.

6.2. Crude Enzyme Extraction

The extraction procedure described by Heu et al. (1995) and Castillo-Yaneza et al. (2006) was followed. The samples were thawed at 4 °C overnight before extraction. A 50 g (wet basis) sample of fish gut was mixed with 150 ml isotonic saline solution and homogenized using a laboratory homogenizer (Polytron PT1035, Brinkmann Instruments, Toronto, Ontario, Canada) for 5 min then incubated for 8 hr at 4°C in the cold storage room to activate the chymotrypsinogen in the samples. After incubation, the sample was centrifuged at 20 000 g at 4°C for 30 min (MP4R, International Equipment Company, Needham, Massachusetts), then filtered and defatted with 50 ml CCl_4. The supernatant was considered a crude enzyme extract. The volume at each step was measured and the activity and concentration of crude enzyme were determined.

6.3. Ammonium Sulphate Precipitation

The process of extracting chymotrypsin from fish waste using the ammonium sulphate method is shown in Figure 6.1. The procedure described by Kunitz (1948) was followed.

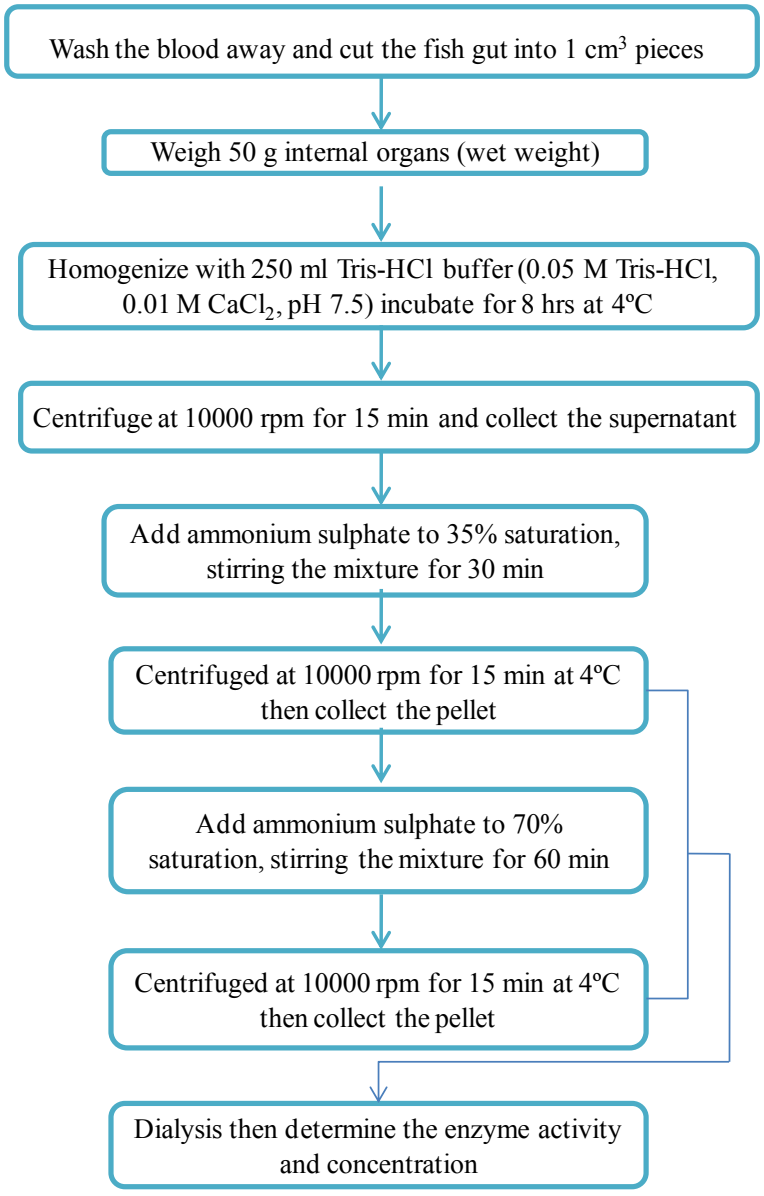

Figure 6.1. Extraction of chymotrypsin from fish waste using the ammonium sulphate method.

The crude extract prepared from gut contents was fractionated by ammonium sulphate precipitation by adding solid ammonium sulphate powder to the crude extract over 5 min to reach 35% saturation with continuous stirring. The mixture was stirred for a further 30 min and then centrifuged at 10 000g for 15 min (MP4R, International Equipment Company, Needham, Massachusetts). The supernatant was collected and the saturation was adjusted to 70% by addition of ammonium sulphate and after 30 min the suspension was centrifuged at 20 000g for 15 min. The fraction collected between 35% and 70% contained precipitated enzymes. The enzyme concentration, activity and yield were determined. The amounts of ammonium sulphate required at a given saturation are shown in Table 6.1.

6.4. Reverse Micelles Extraction

The process of extracting chymotrypsin from fish waste by the reverse micelles method is shown in Figure 6.2. In this procedure, both the forward and backward extractions were optimized and the effect of alcohol was also investigated.

6.4.1. Optimization of the Forward Extraction

The AOT-DOLPA reverse micelles were prepared by dissolving AOT in reagent-grade isooctane and adjusting the AOT concentration to 1, 5, 10, 15, 20 and 35 mM. Crude protein sample solution was prepared by mixing the same volume of crude extract with buffer B (0.1M Tris-HCl) and adjusting the pH to 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5. Equal volumes (5 ml) of the aqueous protein solution and organic solution were mixed and shaked vigorously at 300 rpm in an incubator shaker (Classic C24, New Brunswick Scientific Companny, Edison, New Jersey, USA) in a test tube for 30 min to reach equilibrium. The mixture was then centrifuged for 15 min at 4000 rpm (MP4R, International Equipment Company, Needham, Massachusetts) in order to separate the two phases as described by Kinugasa et al. (2002) and Hentsch et al. (1992).

Table 6.1. Amount of ammonium sulphate required to produce one liter of ammonium sulphate solution at a given saturation.

Desired Percent Saturation	Weight $(NH_4)_2SO_4$ Required (g)
20	106
25	134
30	164
35	194
40	226
45	258
50	291
55	326
60	361
65	398
70	436
75	476
80	516
85	559
90	603
95	650
100	697

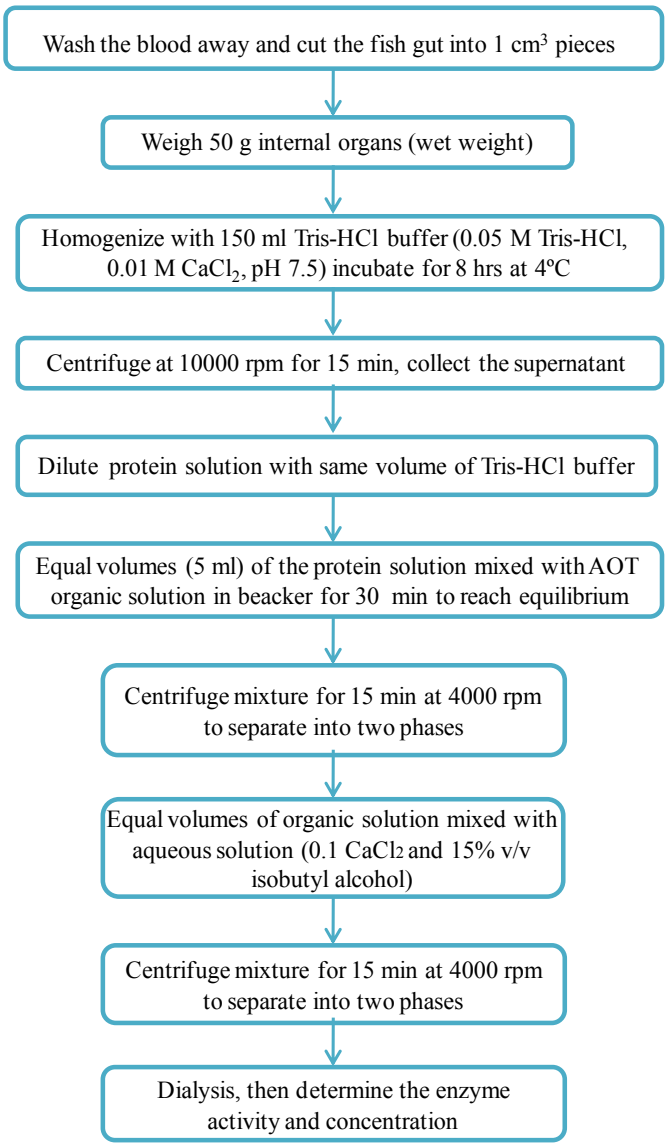

Figure 6.2. Extraction of chymotrypsin from fish waste by using reverse micelles method.

6.4.2. Optimize Backward Transfer

Aqueous solutions containing different KCl concentrations (0.5, 1, 1.5, 2M) were prepared. Tris buffer was used as a stripping solution in the backward transfer and the pH of the solution was adjusted to 6.5, 7.0, 7.5, 8.0 and 8.5 as recommended by Hu and Gulari (1995). Equal volumes of aqueous solution and organic solution (which was obtained from the forward extraction) were mixed in the tube and 15% v/v isobutyl alcohol was added to the reversed micelles phase. Then the two phase systems were mixed for one hr in a beaker placed on a magnetic stirrer (Canlab, NO. S8290, Atlanta GA, Georgia, USA). The mixture was then centrifuged for 15 min at 4000 rpm (MP4R, International Equipment Company, Needham, Massachusetts) in order to separate into two phases (Kinugasa et al., 2002, Goto et al., 1998, Hu and Gulari, 1995).

6.4.3 Effect of Alcohol

The optimal conditions for both the forward extraction and backward extractions were determined from the previous steps. Distilled water was used as a control. After the forward extraction step, the same volumes (1.5 ml) of distilled water and isobutyl alcohol were added to the aqueous phase during backward extraction.

6.5 pH

Raw of material (50 g) were mixed with 150 ml cold distilled water, then homogenized in an ice bath. The pH was measured with a pH meter (Fisher Accumet®, Model 805 MP, Fisher Scientific, Hampton, New Hampshire) while the mixture was agitated in a beaker with a magnetic stirrer (Canlab, NO. S8290, Atlanta GA, Georgia, USA) for 8 hours and then centrifuged at 20,000 g (MP4R, International Equipment Company, Needham, Massachusetts). The samples for forward extraction were prepared by mixing the same volumes of centrifuged crude extract and the Tris buffer (0.1M Tris, 0.2 M KCl, pH adjusted to 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) and the pH was measured with a pH meter.

6.6 Protein Concentration

The Bradford method was used for the determination of protein concentration according to the procedures described by Yang et al. (2009) and Castillo-Yaneza et al. (2006). Two standard curves were developed using a series of concentrations of Bovine Serum Albumin (BSA): the standard standard curve (assay range 10-150 μg/ml) and the micro standard curve (assay range 1-10 μg/ml). The following solutions were prepared.

- 0.1 g of BSA was dissolved in 10 ml of Tris-HCl buffer at room temperature.
- The stock BSA solution was diluted to span the 100-1,500 μg/ml range in the Table 6.2.
- BSA solution in the range of 100-900 μg/ml was diluted ten times more for the micro standard curve.
- 10 μL of each standard was mixed with 5 ml of Bradford reagent. This was repeated twice for each concentration allowing three measurements to be made for each concentration of standard. Each sample was allowed to incubate at room temperature for 10 minutes and no longer than a half hour before being measured.
- The absorbance of each standard was measured at 595 nm against a blank that was composed of 10 μL of buffer and 5 ml of Bradford reagent. 0.1 ml diluted sample (concentration between 5 to 100μg/L) was mixed with 5 ml Bradford reagent and incubated for 5 min and then the absorbance was measured at 595 nm (Olson and Markwell, 2007). The result was compared with the standard curve to determine the sample protein concentration.

The results of absorbance measured using Bradford method (at wave length of 595 nm) of different protein concentrations are shown in Table 6.3. In Figure 6.3, the plots show the linear relationship between absorbance and protein concentration.

Table 6.2. The concentration of BSA used to make standard curves.

[BSA] µg/ml	Volume (µL) of 10 mg/ml BSA Stock	Volume (µL) of Tris-HCl Buffer
100	5	495
200	10	490
400	20	480
600	30	470
900	45	455
1,200	60	440
1,500	75	425

Table 6.3. The absorbance of protein concentrations measured (at 595 nm).

	Concentration (µg/ml)									
Samples	2	4	6	8	10	20	40	60	80	100
1	0.003	0.012	0.026	0.046	0.046	0.160	0.324	0.437	0.584	0.729
2	0.004	0.012	0.030	0.049	0.060	0.165	0.313	0.431	0.559	0.728
3	0.005	0.012	0.029	0.050	0.051	0.169	0.328	0.467	0.593	0.735
Average	0.004	0.012	0.028	0.048	0.052	0.165	0.322	0.445	0.579	0.731

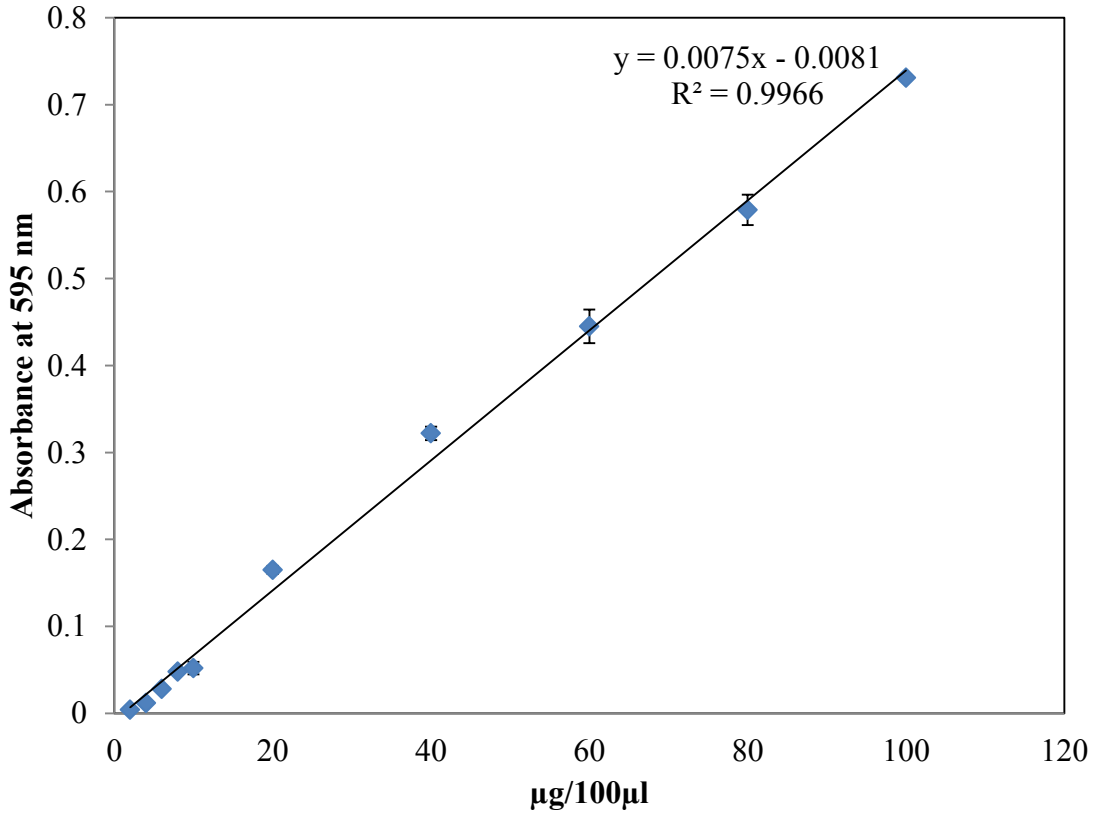

Figure 6.3. The standard curve for protein concentration (mean±std, n=3).

6.7 Enzyme Activity

The activity of chymotrypsin was determined as the change of absorbance measured at 256 nm in one minute caused by the addition 1 ml chymotrypsin protein solution used in the assay (Chakrabarti et al., 2005). The substrate used in the experiment was benzoyl-tyrosine ethyl ester (BTEE). The release of p-nitroaniline was followed by recording the increasing absorbance value every min for 5 min at 256 nm (Chong et al., 2001; Sveinsdóttir et al., 2006). The spectrophotometer was adjusted to 256 nm. 1.5 ml Tris-HCl buffer (0.08M tris, pH 7.8, 0.1M $CaCl_2$), 1.4 ml of 0.00107 M BTEE and 0.1 ml test enzyme solution were placed into cuvettes. The enzyme activity was calculated as follows.

$$\text{Units/ml Enzyme} = \frac{\Delta\, U_{256} \times (3) \times (\text{Df})}{(0.964) \times (0.10)} \qquad (7)$$

Where:

ΔU_{256}: The change of the absorbance at the wave length 256 nm per minute.

3: Volume (in milliliters) of reaction mixture

Df: Dilution factor

0.964: Millimolar extinction coefficient of BTEE at 256 nm

0.10: Volume (in millilitres) of test enzyme solution used in assay

6.8 Total Activity

The increase of absorbance value per min is considered as 0.1 ml enzyme solution activity and the total activity is defined as the change in the absorbance value per min from the total chymotrypsin extract from 100 g of red perch intestine. The total activity was calculated as follows.

$$\text{Total activity} = \frac{\Delta\, U_{256} \times (3) \times (\text{Df})}{(0.964) \times (0.10)} \times \text{Total volume of crude solution} \quad (8)$$

6.9 Specific Activity

Specific activity is defined as the ability of 1 mg enzyme to hydrolysis BTEE in one min at a pH of 7.5 and temperature of 25°C. 0.1 ml enzyme solution was added into cuvettes and the absorbance change was assayed in the same way described above for 5 mins. Then the specific activity was calculated using the following equation.

$$\text{Units/mg Protein} = \frac{\text{Units/ml Enzyme}}{\text{mg Protein/ml Enzyme}} \quad (9)$$

The enzyme solution concentration was determined using the Bradford method and then divided by 30 (dilution times) to be considered as the protein concentration in the activity reaction mixture.

6.10 Purification Fold

Purification fold is used to evaluate the increase in purity of the enzyme after the purification step. It can be calculated by the following equation:

$$\text{Purification Fold} = \frac{\text{Units/mg Purified protein}}{\text{Units/mg Crude protein}} \quad (10)$$

6.11 Relative Activity (Recovery Yield)

Relative activity is defined as the ratio of total refined enzyme activity and total crude enzyme activity. Relative activity represents the chymotrypsin activity remaining

in the purification process. When combined with specific activity it can show the effectiveness of a purification method.

Recovery yield

$$= \frac{\text{Units/ml Purified protein solution}}{\text{Units/ml Crude protein solution}} \times 100\% \qquad (11)$$

6.12 Statistical Analysis

The data for solution volume protein concentration and activity were collected and total activity, recovery yield, specific activity and standard errors were calculated. The α-level was chosen as 0.05. All the statistical analysis of data was conducted using Minitab statistics software (Ver 15.1.10, Minitab Inc) to examine the coefficient data with a two-way analysis of variance (ANOVA) to determine the significant effects of single and two parameters on the results. Also, all the statistical analysis of data was conducted using Minitab statistics software with a Duncan multiple test to determine the significance between levels of parameters chosen.

CHAPTER 7. RESULTS

7.1 Crude Extraction

Crude protein was extracted from the intestine (50 g) of red perch. The total volume (TV) was measured after homogenization, centrifugation and dilution. The activity of enzyme (A_E), total activity (TA), specific activity (SA), protein concentration (Cp), purification fold (PF) and recovery yield (RY) were determined (Table 7.1). After centrifugation, the total volume decreased from 183 to 144 ml (21.32%). The total activity decreased from 14.60 to 13.53 U (7.33%). The concentration decreased from 4486.2 to 1975.8 μg/ml (55.96%). The enzyme activity increased from 0.080 to 0.094 U/ml (17.50%). The specific activity increased from 0.178 to 0.479 U/mg (169.1%). The purification fold of the centrifugation step was 2.69 and the recovery yield was 92.7%.

7.2 Reverse Micelles Forward Extraction

During the optimization of the forward extraction, two parameters were studied: AOT and pH-1. Six levels of AOT (1, 5, 10, 15, 20 and 35 mM) and six levels of pH (6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) were investigated. The volumes of phase 1 (water phase) and phase 2 (organic phase) and the total volume (TV) were measured. The activity of enzyme (A_E), total activity (TA), specific activity (SA), protein concentration (Cp), purification fold (PF) and recovery yield (RY) were determined (Tables 7.2-7.7).

7.2.1 Total Volume (TV)

The effects of pH on the TV at different AOT concentrations are shown in Figure 7.1. All the TVs slightly increased with increased pH. When the pH was increased from 6.0 to 8.5 (41.67%), the TV increased from 9.84 to 9.91 ml (1.01%), from 9.81 to 9.88 ml (0.99%), from 9.72 to 9.82 ml (0.99%), from 9.58 to 9.69 ml (1.15%), from 9.65 to 9.74

Table 7.1. The results of crude extraction.

Extraction step	TV	A_E	TA	Cp	SA	PF	RY
After Homogenizing*	183	0.80	146.40	4486.2	0.178	-	-
After Centrifuging	144	0.94	135.36	1975.8	0.479	2.69	92.7
After Dilution and pH adjustment	288	0.47	135.36	987.9	0.479	-	100.0

*Sample size: 50 g
TV: total volume (ml)
A_E: activity of enzyme (Unit/ml)
TA: total activity (Unit)
Cp: protein concentration (μg/ml)
SA: specific activity (Unit/mg)
PF: purification folds (-)
RY: Recovery Yield (%)

Table 7.2. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 1mM).

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.0	4.93±0.21	4.91±0.11	9.84±0.02	1.02±0.01	0.11±0.01	14.18±0.27	7.57±0.95	15.81±1.99	22.63±2.53
6.5	4.93±0.02	4.92±0.07	9.85±0.03	1.00±0.00	0.13±0.03	16.01±0.52	8.27±2.04	17.27±4.27	27.85±6.39
7.0	4.93±0.02	4.94±0.04	9.87±0.01	1.00±0.00	0.20±0.01	19.38±1.22	10.29±1.26	21.48±2.64	41.90±2.64
7.5	4.94±0.05	4.94±0.02	9.88±0.03	1.00±0.00	0.13±0.02	14.35±1.45	8.90±1.08	18.57±2.26	27.01±5.06
8.0	4.94±0.07	4.95±0.02	9.89±0.03	1.00±0.00	0.11±0.02	14.29±0.34	8.30±0.91	17.32±1.89	22.63±3.35
8.5	4.94±0.04	4.97±0.04	9.91±0.03	0.99±0.00	0.11±0.02	14.34±0.18	7.48±1.15	15.62±2.40	22.63±3.35

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 min
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (µg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

Table 7.3. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 5mM)

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.0	4.92±0.05	4.89±0.04	9.79±0.03	1.00±0.013	0.20±0.01	19.22±1.73	10.49±0.57	21.90±1.19	42.44±1.41
6.5	4.91±0.06	4.89±0.05	9.75±0.06	1.00±0.00	0.20±0.01	19.08±0.17	10.54±0.34	22.00±0.71	42.44±1.41
7.0	4.92±0.06	4.92±0.03	9.79±0.03	1.01±0.01	0.26±0.01	22.30±0.45	11.79±0.63	24.61±1.31	55.49±2.83
7.5	4.92±0.08	4.93±0.04	9.86±0.03	1.00±0.01	0.22±0.01	21.22±1.18	10.56±1.22	22.05±0.25	47.33±2.83
8.0	4.92±0.08	4.93±0.12	9.84±0.02	1.00±0.00	0.17±0.03	18.95±0.24	8.98±1.82	18.76±3.80	35.91±7.07
8.5	4.93±0.04	4.95±0.01	9.88±0.01	1.00±0.00	0.17±0.03	18.85±1.04	8.88±1.86	18.53±3.88	35.09±5.65

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 min
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (μg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

Table 7.4. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 10mM)

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	C_P	SA	PF	RY
6.0	4.88±0.11	4.85±0.09	9.72±0.10	1.01±0.01	0.25±0.04	22.76±2.38	10.86±0.62	22.66±1.29	52.37±8.25
6.5	4.88±0.08	4.86±0.11	9.75±0.03	1.00±0.01	0.29±0.03	24.84±0.45	11.63±1.28	24.28±2.67	60.93±5.95
7.0	4.89±0.12	4.88±0.07	9.83±0.04	0.99±0.02	0.32±0.02	25.09±0.70	12.60±1.10	26.30±2.30	66.65±4.36
7.5	4.91±0.07	4.89±0.11	9.75±0.10	1.01±0.00	0.30±0.02	24.18±1.22	12.49±0.27	26.08±0.57	63.79±4.36
8.0	4.91±0.09	4.92±0.13	9.83±0.04	1.00±0.00	0.22±0.041	19.97±1.38	10.78±1.29	22.51±2.70	45.7±8.57
8.5	4.91±0.02	4.93±0.05	9.82±0.02	1.00±0.00	0.16±0.02	16.48±0.39	9.57±0.71	19.98±1.48	33.32±3.30

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 in
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (μg/ml
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

Table 7.5. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 15mM)

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.0	4.82±0.12	4.76±0.08	9.40±0.15	1.01±0.01	0.25±0.01	24.73±0.35	10.26±0.39	21.42±0.82	53.57±2.14
6.5	4.84±0.06	4.79±0.06	9.55±0.04	1.01±0.00	0.29±0.02	25.01±1.08	11.63±0.18	24.28±0.37	61.43±3.27
7.0	4.84±0.11	4.81±0.14	9.67±0.03	1.00±0.01	0.34±0.01	26.64±0.35	12.70±0.42	26.51±0.88	71.43±2.86
7.5	4.85±0.06	4.85±0.04	9.60±0.11	1.00±0.00	0.32±0.01	26.33±0.29	12.06±0.30	25.18±0.63	67.06±2.40
8.0	4.85±0.04	4.86±0.12	9.72±0.04	1.00±0.00	0.25±0.02	21.93±0.29	11.31±0.79	23.61±1.66	52.38±4.36
8.5	4.87±0.04	4.89±0.04	9.69±0.02	1.00±0.00	0.21±0.02	20.09±0.68	10.55±0.59	22.03±1.22	44.76±3.30

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 min
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (μg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

Table 7.6. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 20mM)

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.0	4.77±0.11	4.73±0.13	9.65±0.01	1.01±0.00	0.28±0.02	26.85±0.38	10.30±0.67	21.50±1.39	58.40±4.38
6.5	4.79±0.07	4.76±0.11	9.63±0.04	1.01±0.00	0.30±0.04	27.57±0.80	10.71±1.19	22.36±2.47	62.42±8.27
7.0	4.80±0.09	4.79±0.06	9.63±0.04	1.00±0.01	0.42±0.02	29.59±0.39	14.19±0.45	29.63±0.94	88.70±3.96
7.5	4.82±0.17	4.82±0.07	9.69±0.03	1.01±0.00	0.38±0.04	29.53±0.44	13.01±1.41	27.16±2.94	81.03±7.84
8.0	4.83±0.12	4.84±0.09	9.72±0.2	1.00±0.00	0.35±0.001	28.96±0.29	11.93±0.24	24.91±0.49	72.95±1.72
8.5	4.83±0.07	4.85±0.005	9.74±0.02	1.00±0.00	0.30±0.04	28.81±0.61	10.36±1.08	21.63±2.26	62.58±7.40

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 min
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (μg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

Table 7.7. Effect of forward extraction pH (pH-1) of reverse micelles on the purification of chymotrypsin (AOT 35mM)

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.0	4.43±0.11	4.40±0.12	8.84±0.5	1.01±0.01	0.18±0.03	19.43±1.05	9.95±1.52	20.78±3.17	40.88±7.04
6.5	4.47±0.09	4.44±0.08	8.94±0.03	1.01±0.01	0.20±0.04	21.07±1.2	10.33±1.59	21.56±3.33	46.14±9.45
7.0	4.52±0.16	4.48±0.12	9.04±0.07	1.01±0.01	0.22±0.04	21.27±1.54	11.74±1.95	24.52±4.07	50.37±9.55
7.5	4.53±0.13	4.50±0.10	9.05±0.1	1.00±0.00	0.17±0.01	16.33±0.42	11.01±0.85	22.98±1.77	37.96±3.35
8.0	4.56±0.13	4.57±0.11	9.14±0.03	1.00±0.01	0.14±0.01	15.97±0.34	9.52±0.80	19.87±1.67	32.12±3.35
8.5	4.58±0.20	4.60±0.018	9.18±0.04	1.00±0.00	0.11±0.01	14.68±1.17	8.26±0.29	17.24±0.61	25.55±1.26

Backward extraction: KCl 1.5 M, pH 8.0
Phase 1: Water phase after stirring for 30 min
Phase 2: Organic phase after stirring for 30 min
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (μg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds (-)
RY: Recovery Yield (%)

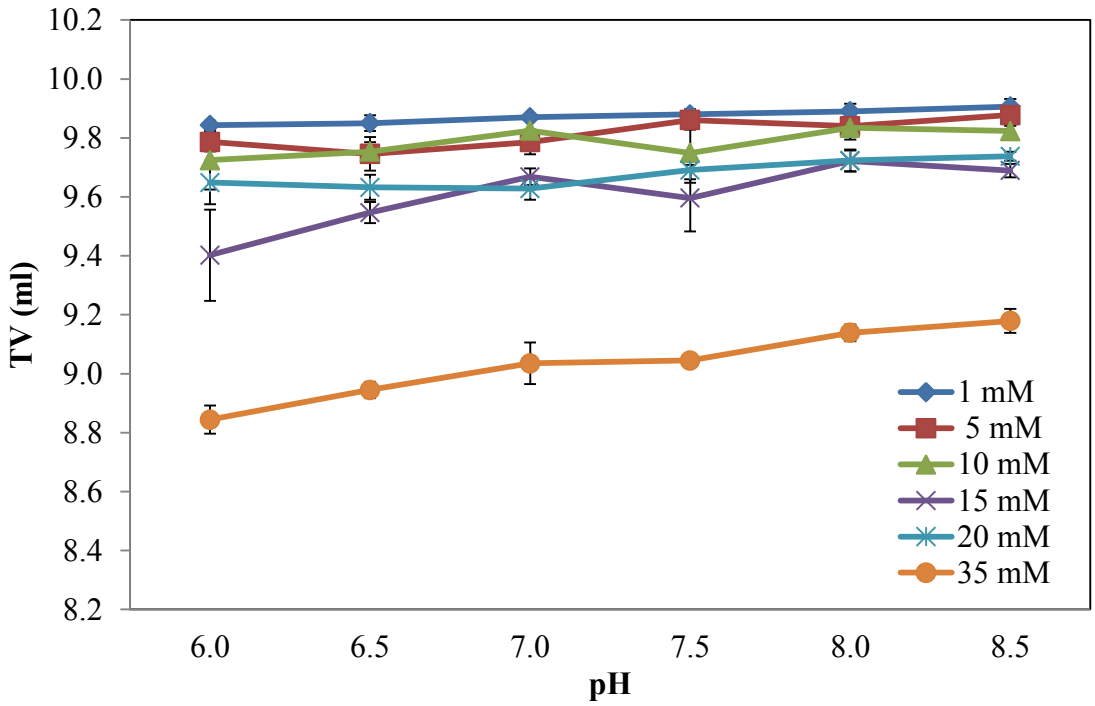

Figure 7.1. Effect of pH-1 on total volume at various AOT concentrations (mean ± std, n=3).

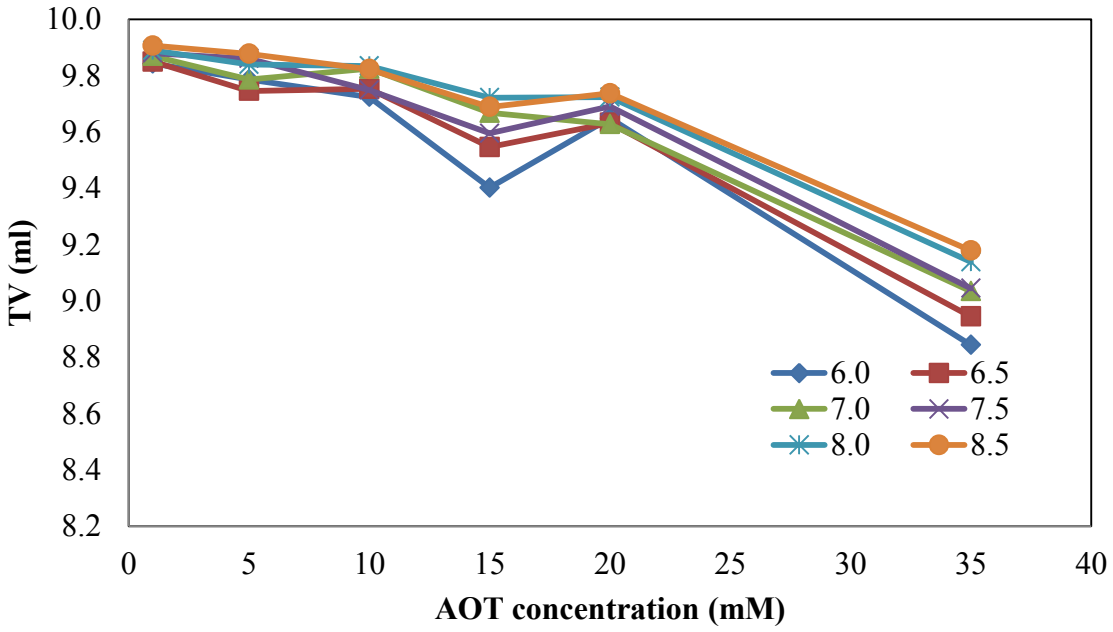

Figure 7.2. Effect of AOT concentrations on total volume at various pHs (mean ± std, n=3).

ml (0.93%) and from 8.84 to 9.18 ml (0.96%) for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM, respectively.

The effects of AOT concentration on the TV at various pHs are shown in Figure 7.2. All the TVs steadily decreased with increased AOT concentration. When the AOT concentration was increased from 1 to 35 mM (3500%) the TVs steadily decreased from 9.84 to 8.84 ml (11.44%), from 9.85 to 8.94 ml (10.55%), from 9.87 to 9.04 ml (9.67%), from 9.88 to 9.05 ml (9.41%), from 9.89 to 9.14 ml (8.32%) and from 9.91 to 9.18 ml (8.28%) for the pHs of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the VT data and the results are shown in Tables 7.8 and 7.9. The effects of pH and AOT concentration were highly significant at the 0.0001 level. The results also show a significant interaction between pH and AOT concentration at 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that the six pH levels were not significantly different from each other at the 0.05 level. The AOT concentration 35 mM was significantly different from the other AOT concentrations at 0.05 level. The AOT concentrations of 1, 5 and 10 mM and the AOT concentrations of 15 and 20 were not significantly different from each other. The highest VT was achieved at the pH of 6.0 and the AOT concentration of 1 mM.

7.2.2 Volume Ratio (VR)

The effects of pH on the VR at various AOT concentrations are shown Figure 7.3. All the VRs decreased slightly with increased pH but the change were not significant. When the pH was increased from 6.0 to 8.5, the RV decreased from 1.02 to 0.99 (1%); from 1.00 to 1.00 (0%); from 1.01 to 1.00 (1%), from 1.01 to 1.00 (1%); from 1.01 to 1.00 (1%) and from 1.01 to 1.00 (1%) for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM, respectively. The effects of AOT concentration on the VR at various pHs are shown in Figure 7.4. AOT concentration did not have a significant effect on VRs.

Table 7.8. Analysis of variance of total volume in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	9.37634			
pH	5	0.35124	0.07025	25.94	0.0001
AOT	5	8.62874	1.72575	637.18	0.0001
pH*AOT	25	0.20135	0.00805	2.97	0.0001
Error	72	0.19501	0.00271		

DF: Degree of freedom,
SS: Sum of square
MS: Mean of square
R^2: 97.92%

Table 7.9. Effects of pH-1 and AOT concentration on total volume in the forward extraction.

Factor	Level	Number of Observations	Mean (ml)	Duncan Grouping
pH	6.0	18	9.702	A
	6.5	18	9.692	A
	7.0	18	9.617	A
	7.5	18	9.635	A
	8.0	18	9.579	A
	8.5	18	9.542	A
AOT Concentration (mM)	1	18	9.873	A
	5	18	9.816	AB
	10	18	9.785	B
	15	18	9.677	C
	20	18	9.604	C
	35	18	9.031	D

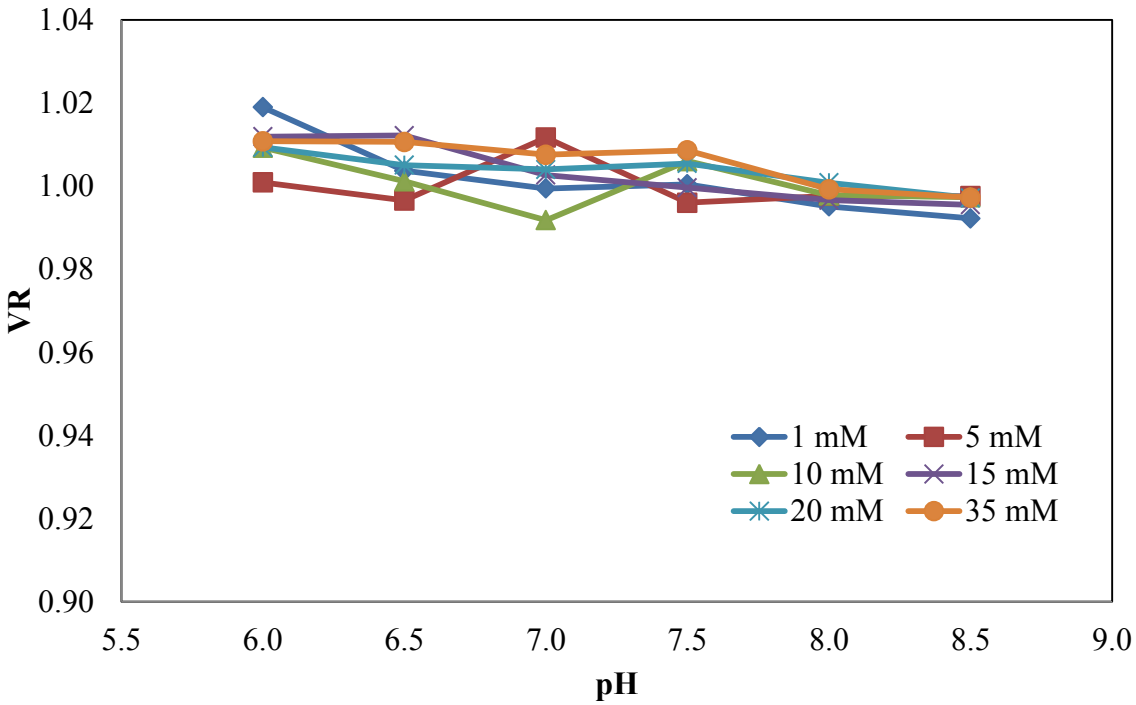

Figure 7.3. Effect of pH-1 on VR at various of AOT concentrations (mean ± std, n=3).

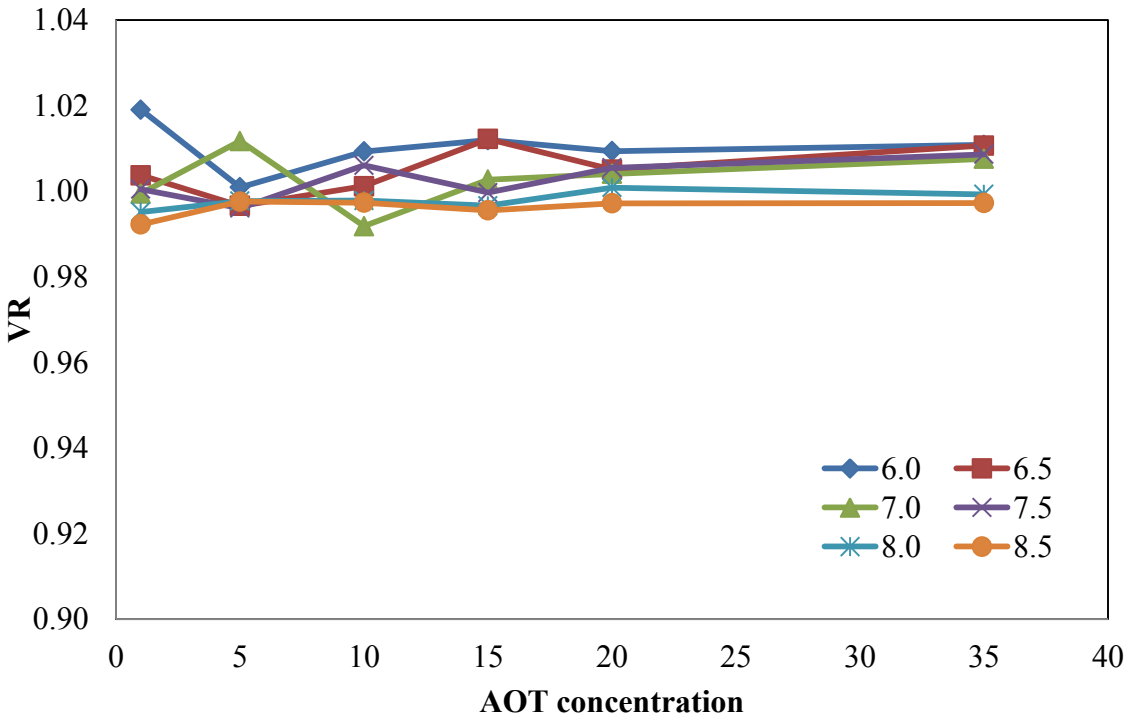

Figure 7.4. Effects of AOT concentration on volume ratio at various pHs (mean ± std, n=3).

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the VR data and the results are shown in Tables 7.10 and 7.11. The effects of pH were highly significant at the 0.0001 level while the effect of AOT concentration was not. There was not a significant interaction between pH and AOT concentration. The results obtained from Duncan Multiple Range Test indicated that six pH levels were not significantly different from each other at the 0.05 level. The highest VR was achieved at pH 6.0 and AOT concentration of 15 mM.

7.2.3 Enzyme Activity (A_E)

The effects of pH on the A_E at various AOT concentrations are shown in Figure 7.5. All A_E values increased when the pH was increased from 6.0 to 7.0 and then decreased when the pH was further increased from 7.0 to 8.5. When the pH was increased from 6.0 to 7.0, the A_E for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM were increased from 0.11 to 0.20 U/ml (81.8%), from 0.20 to 0.26 U/ml (20.0%), from 0.25 to 0.32 U/ml (28.0%), from 0.25 to 0.34 U/ml (36.0%), from 0.28 to 0.42 U/ml (50.0%) and from 0.18 to 0.22 U/ml (22.2%), respectively. When the pH was further increased from 7.0 to 8.5, the A_E for the AOT concentration of 1, 5, 10, 15, 20 and 35 mM were decreased from 0.20 to 0.11 U/ml (45.0%), from 0.26 to 0.17 U/ml (34.6%), from 0.32 to 0.16 U/ml (50.0%), from 0.34 to 0.21 U/ml (38.2%), from 0.42 to 0.30 U/ml (28.6%) and 0.22 to 0.11 U/ml (50.0%), respectively.

The effects of AOT concentration on the A_E at different pH levels are shown in Figure 7.6. When the AOT concentration was increased from 1 to 20 mM, the A_Es for the pHs of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 increased from 0.11 to 0.28 U/ml (154.5%), from 0.13 to 0.30 U/ml (172.7%), from 0.20 to 0.42 U/ml (110.0%), from 0.13 to 0.38 U/ml (192.3%), from 0.11 to 0.35 U/ml (218.2%) and from 0.11 to 0.30 U/ml (172.7%),respectively. When the AOT concentration was further increased from 20 to 35 mM, A_Es decreased from 0.28 to 0.18 U/ml (35.7%), from 0.30 to 0.20 U/ml (33.3%), from 0.42 to 0.22 U/ml (47.6%), from 0.38 to 0.17 U/ml (55.3%), from 0.35 to 0.14 U/ml

Table 7.10. Analysis of variance of volume ratio in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	0.00771			
pH	5	0.00228	0.000456	10.13	0.0001
AOT	5	0.0004	0.00008	1.77	0.1290
pH*AOT	25	0.00179	0.000071	1.59	0.0660
Error	72	0.00324	0.000045		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 57.97%

Table 7.11. Effects of pH-1 and AOT concentration on volume ratio in the forward extraction.

Factor	Level	Number of Observations	Mean	Duncan Grouping
pH	6.0	18	0.21109	BC
	6.5	18	0.2352	ABC
	7.0	18	0.29286	A
	7.5	18	0.2538	AB
	8.0	18	0.20479	BC
	8.5	18	0.17535	C
AOT Concentration (mM)	1	18	0.12998	D
	5	18	0.20422	C
	10	18	0.2548	B
	15	18	0.27679	B
	20	18	0.33635	A
	35	18	0.17095	CD

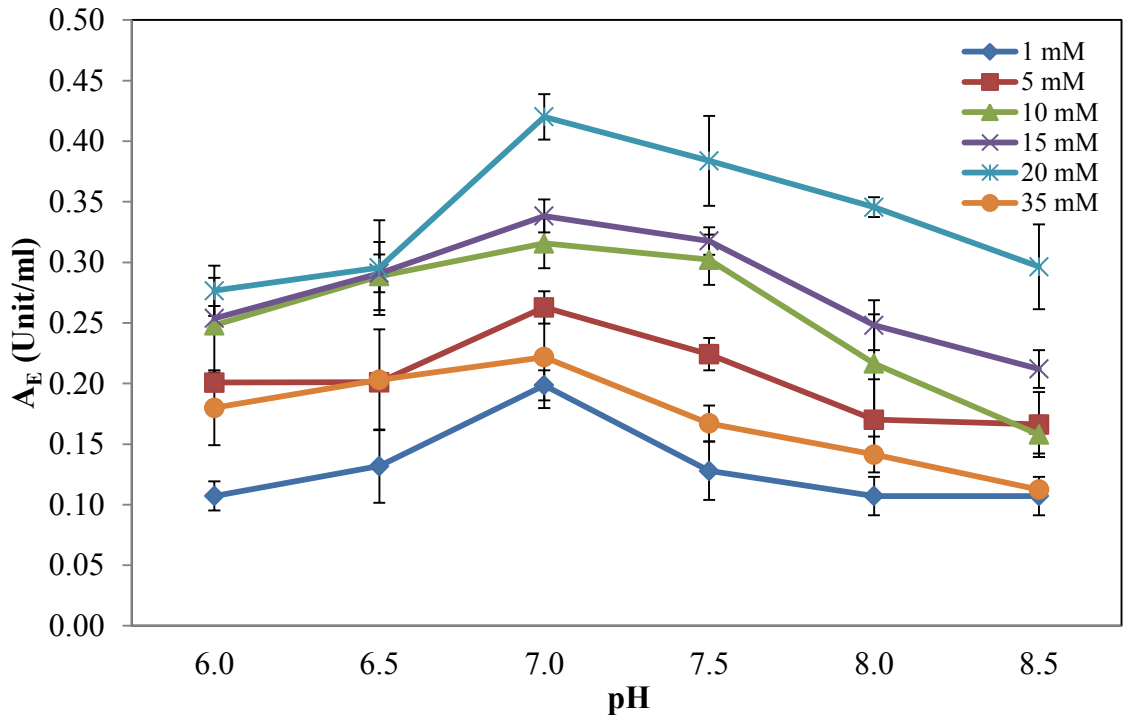

Figure7.5. Effect of pH-1 on A_E at various of AOT concentrations (mean ± std, n=3).

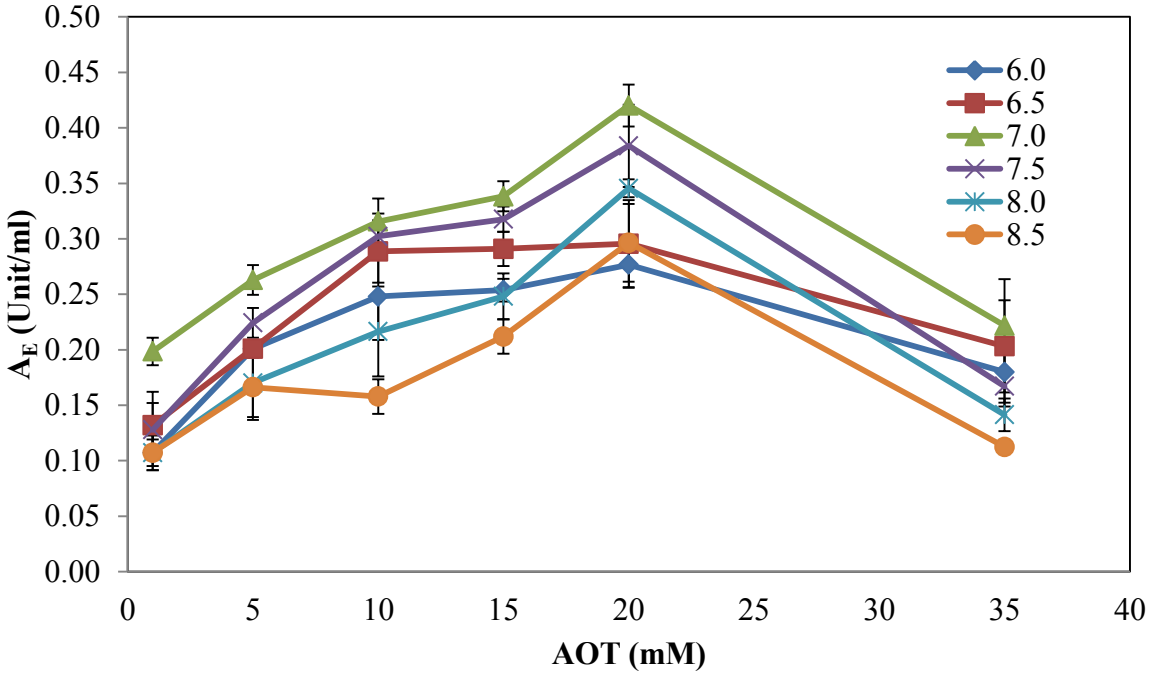

Figure 7.6. Effects of AOT concentration on A_E at various pHs (mean ± std, n=3).

(60%), from 0.30 to 0.11 U/ml (63.3%) for the pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the A_E data and the results are shown in Tables 7.12 and 7.13. The effects of pH and AOT concentration were highly significant at the 0.0001 level. There also appears to be a significant interaction between pH and AOT concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that the pH 7.0 is significantly different from the pH 8.5 at the 0.05 level. The AOT concentrations of 1, 5, 10, 15 and 20 mM were significantly different from the each other at 0.05 level. The highest A_E was achieved at the pH 7.0 and the AOT concentration of 20 mM.

7.2.4 Protein Concentration (Cp)

The effects of pH on the Cp at various AOT concentrations are shown in Figure 7.7. For all AOT, the Cp increased when the pH was increased from 6.0 to 7.0 and then decreased when the pH was further increased from 7.0 to 8.5. When pH increased from 6.0 to 7.0, the Cp increased from 14.18 to 19.38 μg/ml (36.67%), from 19.92 to 22.30 μg/ml (11.95%), from 22.76 to 25.09 μg/ml (10.24%), from 24.73 to 26.64 μg/ml (7.72%), from 26.85 to 29.59 μg/ml (10.20%) and from 19.43 to 21.27 μg/ml (9.47%), for the AOT concentration 1, 5, 10, 15, 20 and 35 mM, respectively. When pH was further increased from 7.0 to 8.5, the Cp was decreased from 19.38 to 14.34 μg/ml (26.01%), from 22.30 to 18.85 μg/ml (36.30%), from 25.09 to 16.48 μg/ml (34.32%), from 26.64 to 20.19 μg/ml (24.21%), from 29.59 to 28.81 μg/ml (2.63%) and from 21.27 to 14.68 μg/ml (30.98%) for the AOT concentration of 1, 5, 10, 15, 20 and 35 mM, respectively.

The effects of AOT concentration on the Cp at various pHs are shown in Figure 7.8. For all pHs, the Cp increased when the AOT concentration was increased from 1 to 20 mM and then decreased when the AOT concentration was further increased from 20 to 35

Table 7.12. Analysis of variance of enzyme activity in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	0.748821			
pH	5	0.153302	0.030660	52.87	0.0001
AOT	5	0.508719	0.101744	175.43	0.0001
pH*AOT	25	0.045042	0.001802	3.11	0.0001
Error	72	0.041758	0.00058		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 94.42%

Table 7.13. Effects of pH-1 and AOT concentration on enzyme activity in the forward extraction.

Factor	Level	Number of Observations	Mean (Unit/ml)	Duncan Grouping
pH	6.0	18	0.21109	BC
	6.5	18	0.2352	ABC
	7.0	18	0.29286	A
	7.5	18	0.2538	AB
	8.0	18	0.20479	BC
	8.5	18	0.17535	C
AOT Concentration (mM)	1	18	0.12998	D
	5	18	0.20422	C
	10	18	0.2548	B
	15	18	0.27679	B
	20	18	0.33635	A
	35	18	0.17095	CD

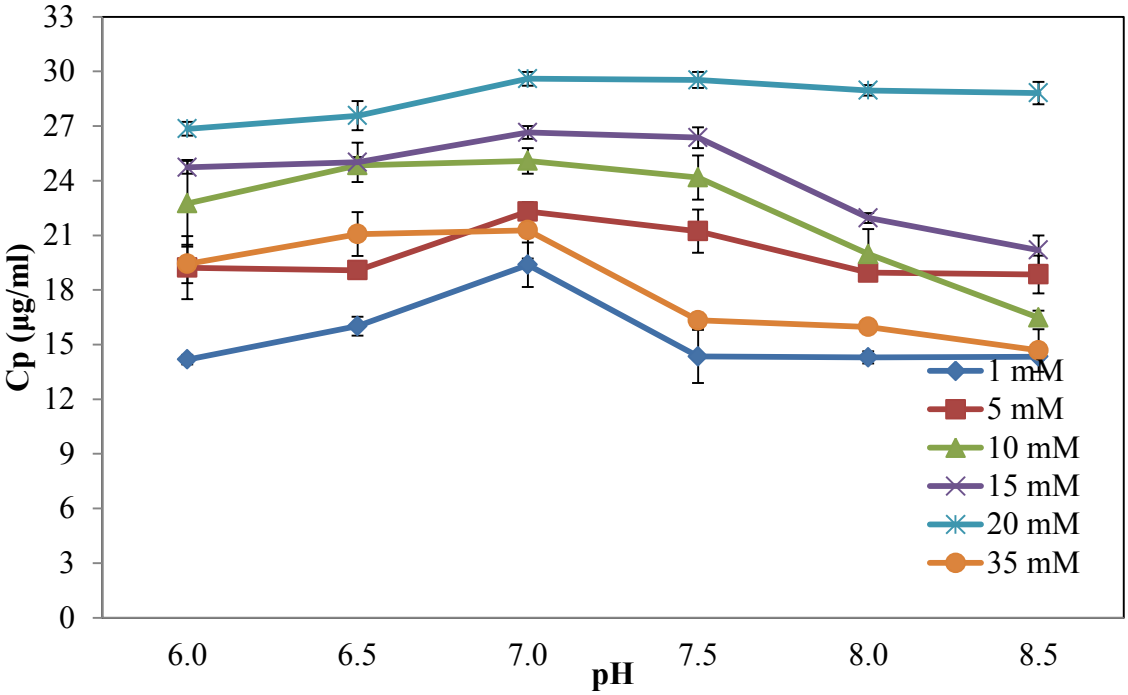

Figure 7.7. Effect of pH-1 on protein concentration at various AOT concentrations (mean ± std, n=3).

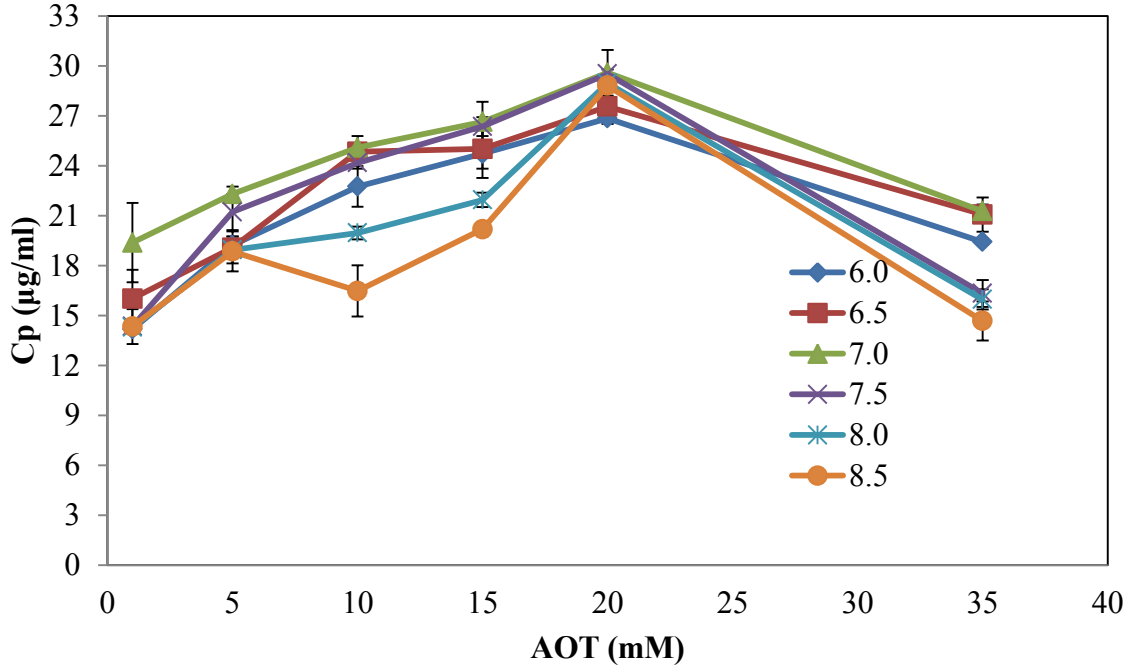

Figure 7.8. Effect of AOT concentration on protein concentration at various pHs (mean ± std, n=3).

mM. When the AOT concentration increased from 1 to 20 mM, the Cp increased from 14.18 to 21.50 μg/ml (51.6%), from 16.01 to 27.57 μg/ml (72.3%), from 19.38 to 29.59 μg/ml (52.7%), from 14.35 to 29.53 μg/ml (105.8%), from 14.29 to 28.96 μg/ml (102.7%) and from 14.34 to 28.81 μg/ml (100.9%) for the pHs of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively. When the AOT was increased from 20 to 35mM the Cps then decreased from 26.85 to 19.43 μg/ml (27.6%), from 27.57 to 21.07 μg/ml (23.6%), from 29.59 to 21.27 μg/ml (28.1%), from 28.96 to 15.97 μg/ml (44.9%) and from 28.81 to 14.68 μg/ml (49.0%), for the pHs of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the Cp data and the results are shown in Tables 7.14 and 7.15. The effects of pH and AOT concentration were highly significant at the 0.0001 level. There also appears to be a significant interaction between the pH and AOT concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that all the pH levels were not significantly different from each other at the 0.05 level. The AOT concentrations 1, 5, 35, 10, 15 and 20 mM were significantly different from each other at 0.05 level. The highest Cp was achieved at pH 7.0 and the AOT concentration of 20 mM.

7.2.5 Specific Activity (SA)

Figure 7.9 shows the effects of pH on the SA at various AOT concentrations. All SAs increased when the pH was increased from 6.0 to 7.0 and then decreased with further increases in the pH. The SA values for the AOT concentration 1, 5, 10, 15, 20 and 35 mM increased from 7.57 to 10.29 U/mg (35.9%), 10.49 to 11.79 U/mg (12.4%), 10.86 to 12.60 U/mg (16.0%), 10.26 to 12.70 U/mg (23.8%), 10.30 to 14.19 U/mg (37.8%) and 9.95 to 11.74 U/mg (18.0%) when the pH was increased from 6.0 to 7.0 and then decreased from 10.29 to 7.48 U/mg (27.3%), 11.79 to 8.88 U/mg (24.7%), 12.60 to 9.57 U/mg (24.1%), 12.70 to 10.55 U/mg (16.9%), 14.19 to 10.36 U/mg (27.0%) and 11.74 to 8.26 U/mg (29.6%) when pH increased from 7.0 to 8.5, respectively.

Table 7.14. Analysis of variance of protein concentration in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	2496.22			
pH	5	299.76	59.952	72.25	0.0001
AOT	5	1930.26	386.051	465.26	0.0001
pH*AOT	25	206.46	8.258	9.95	0.0001
Error	72	59.74	0.83		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 97.61%

Table 7.15. Effects of pH-1 and AOT concentration on protein concentration in the forward extraction.

Factor	Factor	Number of Observations	Mean (μg/ml)	Duncan Grouping
pH	6.0	18	21.195	AB
	6.5	18	22.263	AB
	7.0	18	24.048	A
	7.5	18	21.989	AB
	8.0	18	20.011	AB
	8.5	18	18.835	B
AOT Concentration (mM)	1	18	15.425	D
	5	18	19.938	C
	10	18	22.218	B
	15	18	24.122	B
	20	18	28.513	A
	35	18	18.125	C

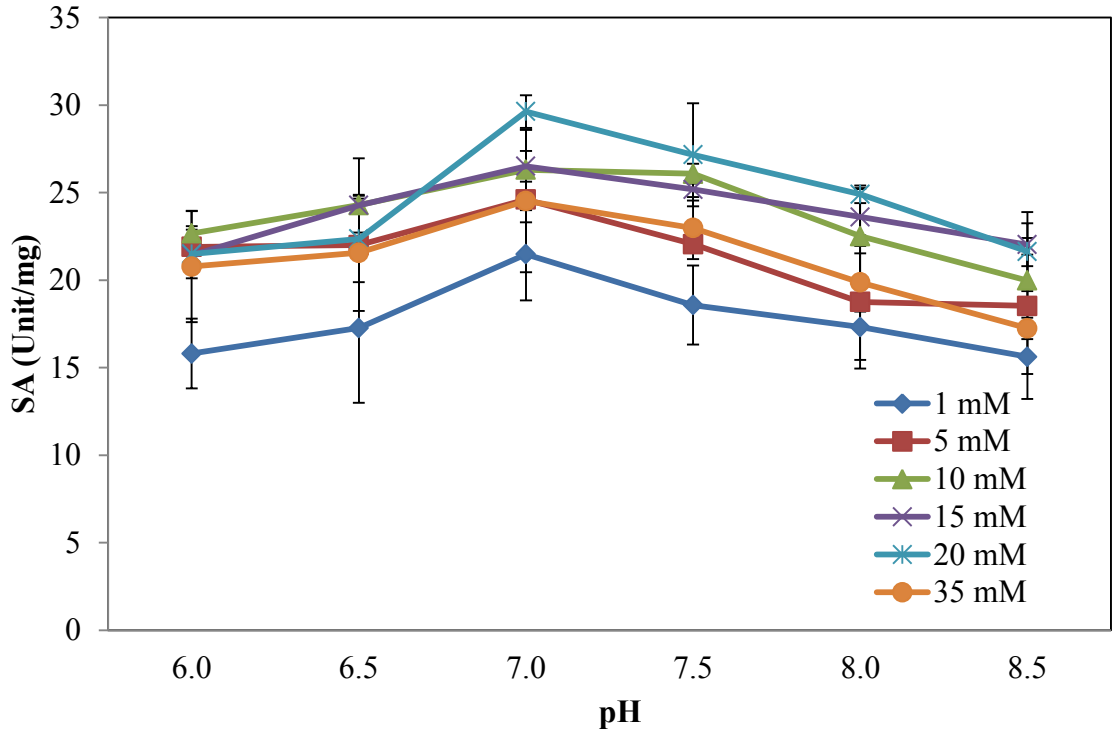

Figure 7.9. Effect of pH-1 on specific activity at various AOT concentrations (mean ± std, n=3).

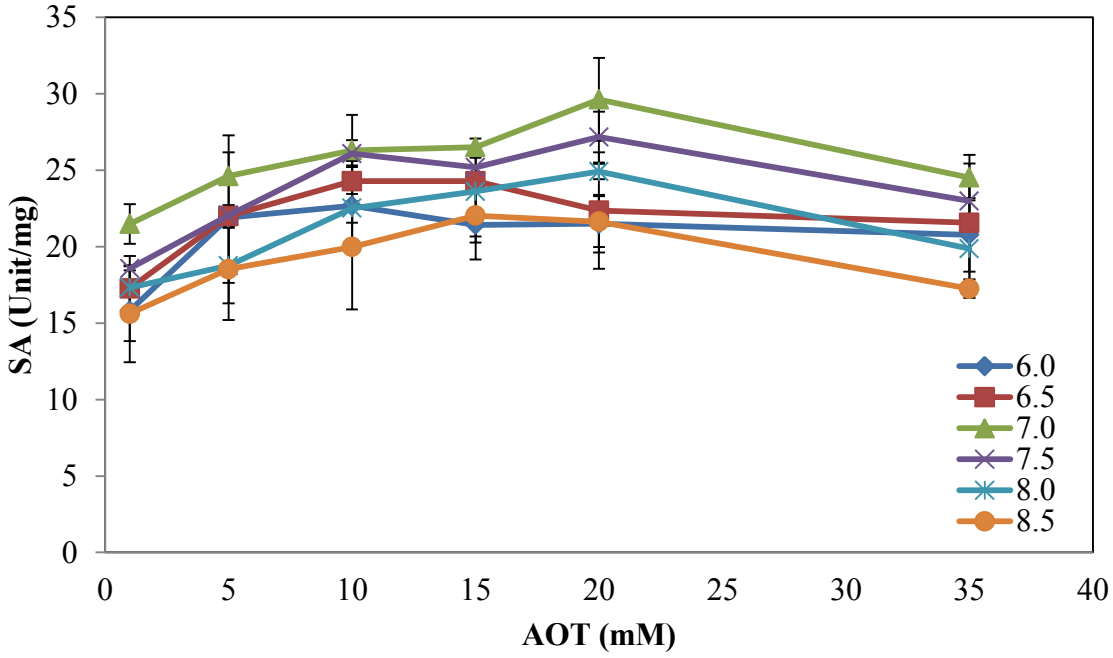

Figure 7.10. Effect of AOT concentration on specific activity at various pHs (mean ± std, n=3).

The effects of the AOT concentration on the SA are shown in Figure 7.10. When the AOT concentration was increased from 1 to 20 mM, the SA increased from 7.57 to 10.30 U/mg (36.1%), from 8.27 to 10.71 U/mg (29.5%), from 10.29 to 14.19 U/mg (37.9%), from 8.90 to 13.01 U/mg (46.2%), from 8.30 to 11.93 U/mg (43.7%) and from 7.48 to 10.36 U/mg (38.5%) for pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively. When the AOT concentration was further increased from 20 to 35 mM, SA decreased from 10.30 to 9.95 U/mg (3.4%), from 10.71 to 10.33 U/mg (3.5%), from 14.19 to 11.74 U/mg (17.3%), from 13.01 to 11.01 U/mg (15.4%), from 11.93 to 9.52 U/mg (20.2%) and from 10.36 to 8.26 U/mg (20.3%) for the pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the SA data and the results are shown in Tables 7.16 and 7.17. The effects of pH and AOT concentration were highly significant at the 0.0001 level but the interaction between the pH and AOT concentration was not significant. The results obtained from Duncan Multiple Range Test indicated that pH 7.0 and 8.5 were significantly different from the other pHs. The AOT concentrations 1, 20 and 35 mM were significantly different from the other AOT concentrations at the 0.05 level. The highest SA was achieved at pH 7.0 and the AOT concentration of 20 mM.

7.2.6 Purification Fold (PF)

The effects of the pH on the PF at various AOT concentrations are shown in Figure 7.11. When the pH was increased from 6.0 to 7.0, the PF for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM increased from 15.81 to 21.48 (35.86%), from 21.90 to24.61 (12.37%), from 22.66 to 26.30 (16.06%), from 21.42 to 26.51 (23.76%), from 21.50 to 29.63 (37.81%) and from 20.78 to 24.52 (18.00%), respectively. When the pH was further increased from 7.0 to 8.5, PF decreased from 21.48 to 15.62 (27.28%), from 24.61 to 18.53 (24.71%), from 26.30 to 19.98 (24.03%), from 26.51 to 22.03 (16.90%), from 29.63 to 21.63 (27.0%) and from 24.52 to 17.24 (29.69%) for the AOT concentrations 1, 5, 10, 15, 20 and 35 mM, respectively.

Table 7.16. Analysis of variance of specific activity in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	340.295			
pH	5	105.513	21.1027	19.04	0.0001
AOT	5	133.499	26.6998	24.09	0.0001
pH*AOT	25	21.482	0.8593	0.78	0.7580
Error	72	79.801	1.1083		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 76.55%

Table 7.17. Effects of pH-1 and AOT concentration on specific activity in the forward extraction.

Factor	Factor	Number of Observations	Mean (Unit/mg)	Duncan Grouping
pH	6.0	18	9.905	CD
	6.5	18	10.517	BC
	7.0	18	12.218	A
	7.5	18	11.338	AB
	8.0	18	10.136	BCD
	8.5	18	9.183	D
AOT Concentration (mM)	1	18	8.467	D
	5	18	10.206	BC
	10	18	11.321	ABC
	15	18	11.418	AB
	20	18	11.75	A
	35	18	10.134	C

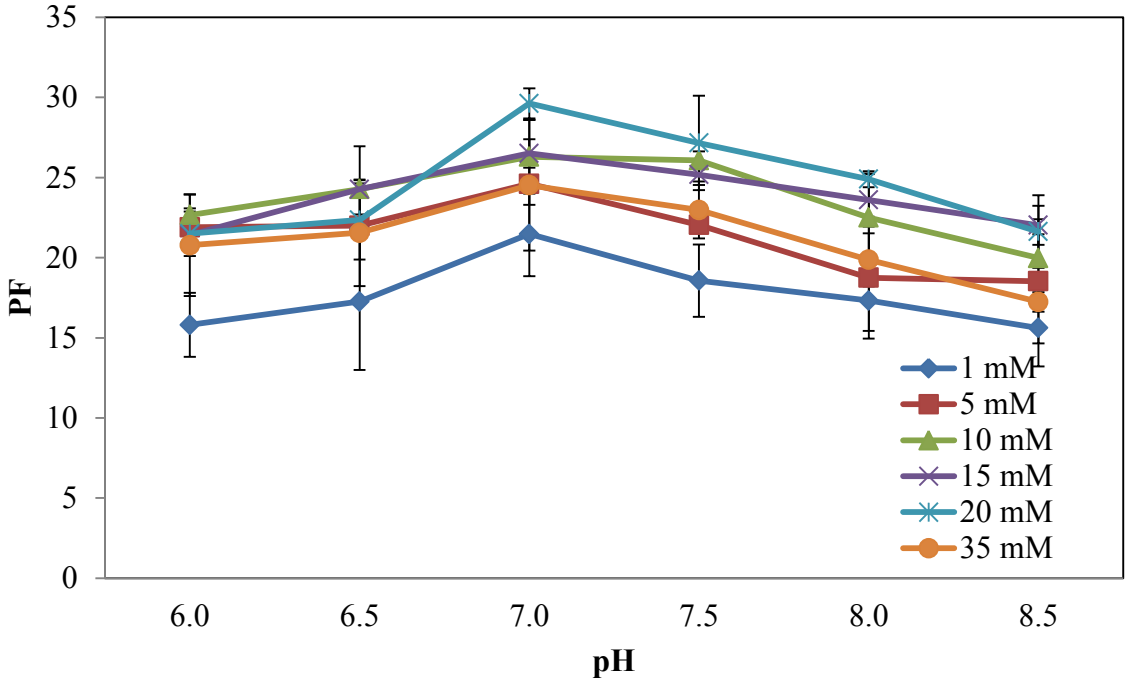

Figure 7.11. The effect of pH-1 on purification folds at various AOT concentrations (mean ± std, n=3).

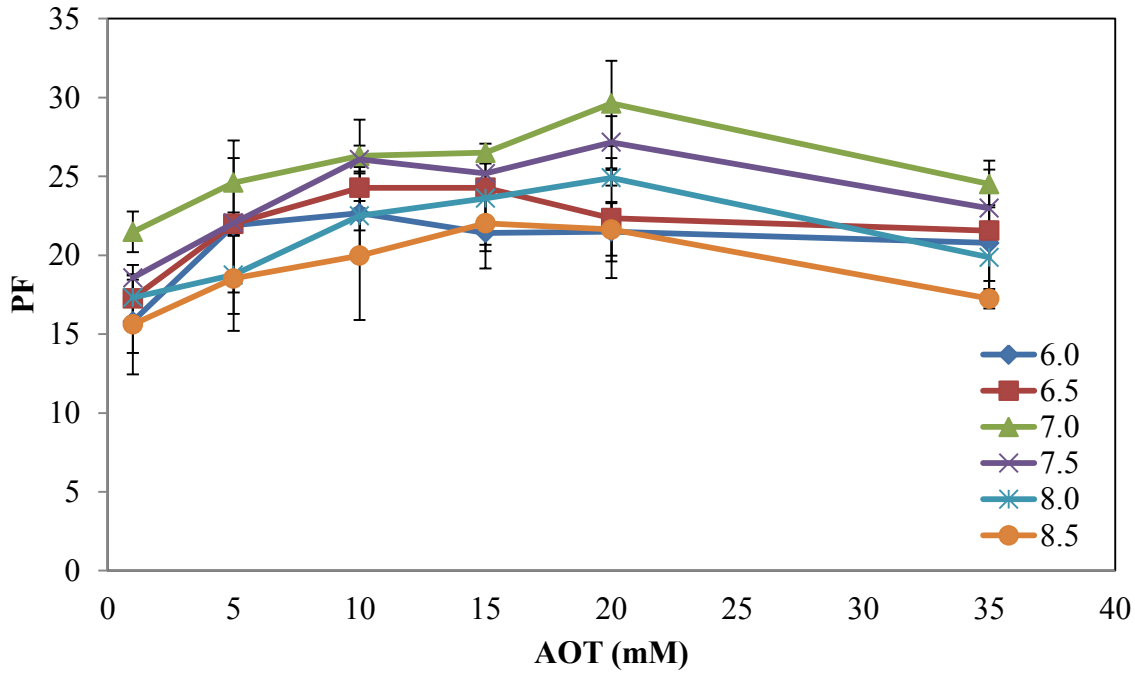

Figure 7.12. Effects of AOT concentrations on purification folds at various pHs (mean ± std, n=3).

The effects of AOT concentration on PF are shown in Figure 7.12. When the AOT concentration was increased from 1 to 20 mM, the PF for the pHs of 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5 increased from 15.81 to 21.50, from 17.27 to 22.36, from 21.48 to 29.63, from 18.57 to 27.16, from 17.32 to 24.91, from 15.62 to 21.63, respectively. When the AOT concentration was further increased from 20 to 35 mM, PF decreased from 21.50 to 20.78, from 22.36 to 21.56, from 29.63 to 24.52, from 27.16 to 22.98, from 24.91 to 19.87, from 21.63 to 17.24 for the pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the PF data and the results are shown in Tables 7.18 and 7.19. The effects of pH and AOT concentration were highly significant at the 0.0001 level but the interaction between the pH and AOT concentration were not significant. The results obtained from Duncan Multiple Range Test indicated that pH 7.0 was significantly different from the pH 8.5 at the 0.05 level. The AOT concentrations 1, 20 and 35 mM were significantly different from other AOT concentrations at the 0.05 level. The highest PF was achieved at pH 7.0 and AOT concentration of 20 mM.

7.2.7 Recovery Yield (RY)

Figure 7.13 shows the effects of pH on the RY at different AOT concentrations. For all AOT concentrations, the RY first increased with increases in the pH up to pH of 7.0 and then decreased. When the pH was increased from 6.0 to 7.0, RY increased from 22.63 to 41.90% (19.27%) from 42.40 to 55.49% (13.09%), from 52.37 to 66.65% (14.18%), from 53.57 to 71.43% (17.86%), from 58.40 to 88.70% (30.30%) and from 40.88 to 50.37% (9.49%) for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM, respectively. When the pH further increased from 7.0 to 8.5, RY decreased from 41.90 to 22.63% (19.27%) from 55.49 to 35.09% (20.4%), from 66.65 to 33.32% (33.33%), from 71.43 to 44.76% (26.67%), from 88.70 to 62.58% (26.12%) and from 50.37 to 25.55% (24.82%) for the AOT concentrations of 1, 5, 10, 15, 20 and 35 mM, respectively.

Table 7.18. Analysis of variance of purification folds in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	1483.15			
pH	5	459.87	91.974	19.04	0.0001
AOT	5	581.84	116.369	24.09	0.0001
pH*AOT	25	93.63	3.745	0.78	0.7580
Error	72	347.8	4.831		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 76.55%

Table 7.19. Effects of pH-1 and AOT concentration on purification folds in the forward extraction.

Factor	Level	Number of Observations	Mean	Duncan Grouping
pH	6	18	20.678	CD
	6.5	18	21.956	BC
	7	18	25.508	A
	7.5	18	23.67	AB
	8	18	21.161	BCD
	8.5	18	19.171	D
AOT Concentration (mM)	1	18	17.677	D
	5	18	21.307	BC
	10	18	23.635	ABC
	15	18	23.836	AB
	20	18	24.531	A
	35	18	21.157	C

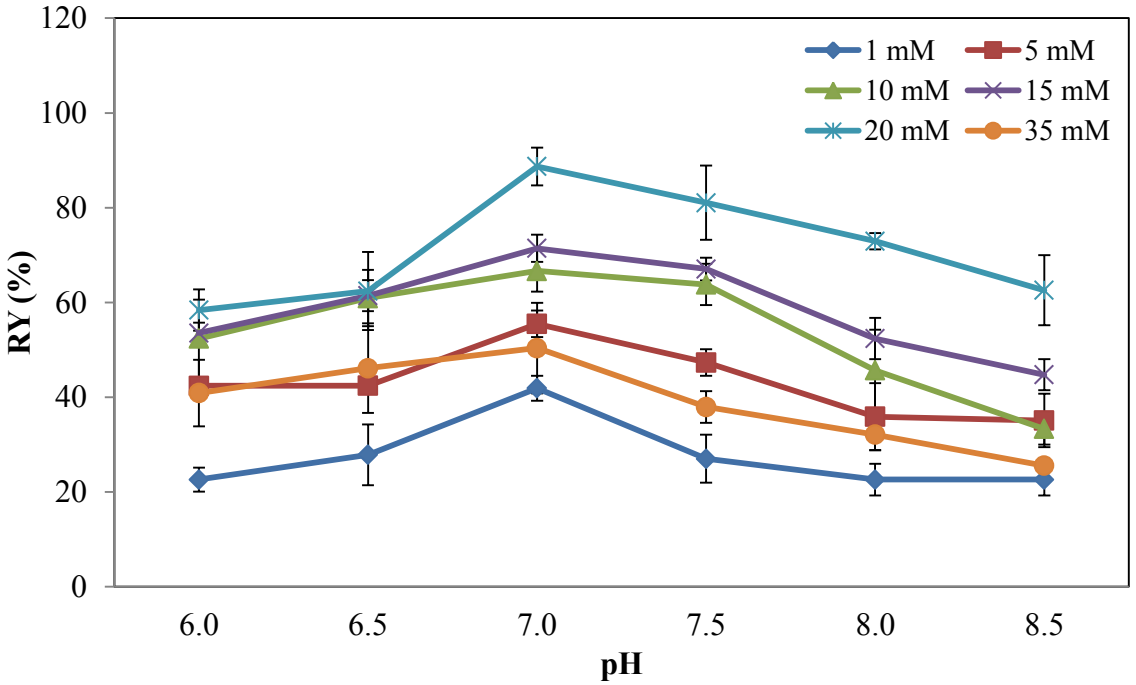

Figure 7.13. Effect of pH-1 on recovery yields at various of AOT concentrations (mean ± std, n=3).

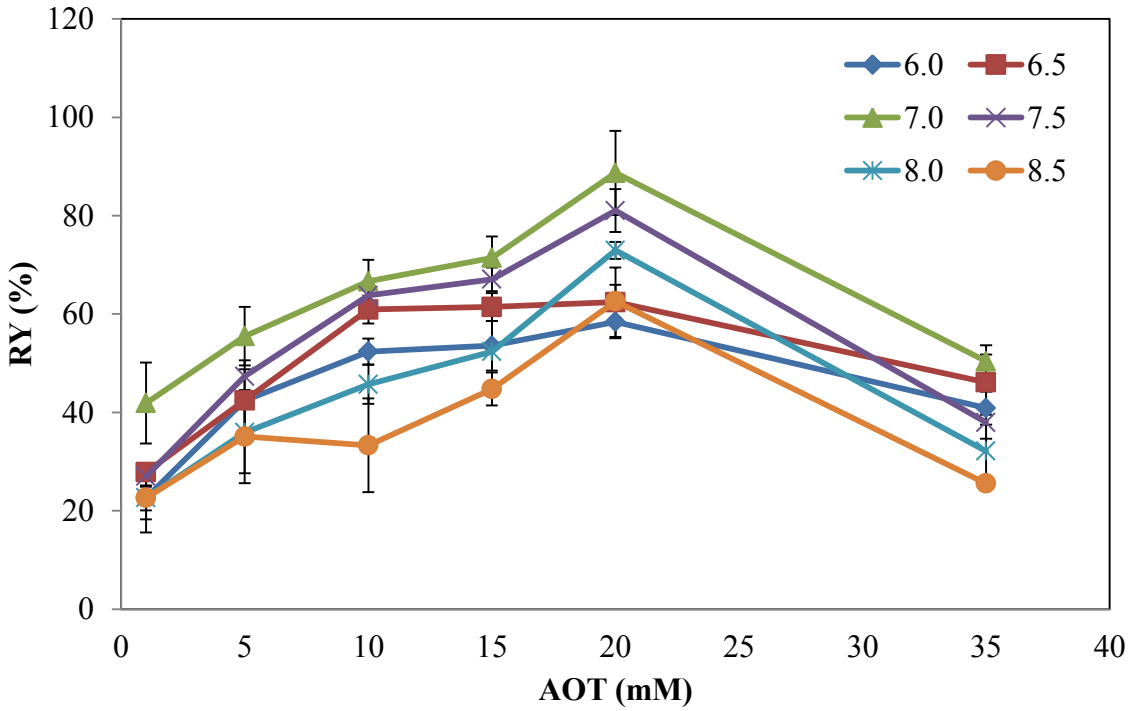

Figure 7.14. Effect of AOT concentrations on RY at various pH levels (mean ± std, n=3).

The effects of KCl concentration on RY at different pH are shown Figure 7.14. When the AOT concentration was increased from 1 to 20 mM, RY increased from 22.63 to 58.40% (35.77%), from 22.75 to 62.42% (39.67%), from 41.90 to 88.70% (46.8%), from 27.01 to 81.03% (54.02%) and from 22.63 to 72.95% (50.32%) and from 22.63 to 62.58% (39.95%) for the pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively. When the AOT concentration was further increased from 20 to 35 mM, RY decreased from 58.40 to 40.88% (17.52%), from 62.42 to 46.14% (16.28%), from 88.70 to 50.37% (38.33%), from 81.03 to 37.96% (43.07%), from 72.95 to 32.12% (40.83%) and from 62.58 to 25.55% (37.03%) for the pHs 6.0, 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the RY data and the results are shown in Tables 7.20 and 7.21. The effects of pH and AOT concentration were highly significant at the 0.0001 levels. There also appears to be a significant interaction between the pH and AOT concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that RY at pH 7.0 is significantly different from RY at pH of 8.5 at the 0.05 level. The AOT concentrations 1, 5, 10, 20 and 35 mM were significantly different from each other at 0.05 level. The highest RY was achieved at pH 7.0 and AOT concentration of 20 mM.

7.3 Backward Extraction

The optimal conditions for the forward extraction (AOT 20 mM, pH 7.0) were applied in the optimization of the backward extraction step. Two parameters (KCl concentration and pH-2) were changed during the backward extraction step. Four levels of KCl (0.5, 1.0, 1.5 and 2.0 M) and five levels of pH-2 (6.5, 7.0, 7.5, 8.0 and 8.5) were investigated. The results are shown in Tables 7.22-7.7.25.

Table 7.20. Analysis of variance of recovery yield in the forward extraction.

Source	DF	SS	MS	F	P
Total	107	32520.9			
pH	5	6977.5	1395.51	52.04	0.0001
AOT	5	21580.4	4316.07	160.96	0.0001
pH*AOT	25	2032.5	81.3	3.03	0.0001
Error	72	1930.6	26.81		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 94.06%

Table 7.21. Effects of pH-1 and AOT concentration on recovery yield in the forward extraction.

Factor	Factor	Number of Observations	Mean (%)	Duncan Grouping
pH	6.0	18	45.05	BC
	6.5	18	50.2	ABC
	7.0	18	62.42	A
	7.5	18	54.03	AB
	8.0	18	43.61	BC
	8.5	18	37.32	C
AOT Concentration (mM)	1	18	27.44	D
	5	18	43.12	C
	10	18	53.79	B
	15	18	58.44	B
	20	18	71.01	A
	35	18	38.84	C

Table 7.22. Effect of backward extraction pH (pH-2) on the purification of chymotrypsin (KCl 0.5 M).

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.165±0.005	15.37±0.44	10.77±0.39	22.49±0.81	35.21±1.09
7.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.211±0.006	19.59±0.59	10.78±0.43	22.51±0.90	44.90±1.26
7.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.246±0.007	19.981±0.38	12.30±0.10	25.69±0.21	52.31±1.41
8.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.216±0.005	18.26±0.13	11.85±0.16	24.74±0.34	46.04±0.95
8.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.186±0.005	17.30±0.20	10.74±0.30	22.42±0.63	39.53±1.04

Forward extraction: AOT 20 mM, pH 7.0
Phase 1: Water phase after BE
Phase 2: Organic phase after BE
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (µg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds
RY: Recovery Yield (%)

Table 7.23. Effect of backward extraction pH (pH-2) on the purification of chymotrypsin (KCl 1.0 M).

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.405±0.010	29.70±0.44	13.64±0.16	28.47±0.33	86.18±2.10
7.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.446±0.011	31.50±0.93	14.17±0.59	29.57±1.24	94.88±2.33
7.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.481±0.006	31.13±0.93	15.44±0.55	32.24±1.15	102.24±1.35
8.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.382±0.009	30.21±1.00	12.65±0.40	26.40±0.84	81.24±1.97
8.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.342±0.011	29.64±1.02	11.55±0.32	24.11±0.66	72.83±2.37

Forward extraction: AOT 20 mM, pH 7.0
Phase 1: Water phase after BE
Phase 2: Organic phase after BE
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (µg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds
RY: Recovery Yield (%)

Table 7.24. Effect of backward extraction pH (pH-2) on the purification of chymotrypsin (KCl 1.5 M).

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	C_P	SA	PF	RY
6.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.381±0.008	20.31±1.54	18.84±1.70	39.34±3.55	81.06±1.60
7.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.432±0.007	20.76±0.77	20.84±1.02	43.50±2.13	91.91±1.51
7.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.468±0.011	21.64±1.54	21.67±1.11	45.23±2.32	93.43±2.37
8.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.335±0.006	17.31±0.77	19.41±1.01	40.52±2.10	71.38±1.29
8.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.332±0.011	17.37±0.60	19.13±0.18	39.94±0.39	70.68±2.37

Forward extraction: AOT 20 mM, pH 7.0
Phase 1: Water phase after BE
Phase 2: Organic phase after BE
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (µg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds
RY: Recovery Yield (%)

Table 7.25. Effect of backward extraction pH (pH-2) on the purification of chymotrypsin (KCl 2.0 M).

	Volume (ml)								
pH	Phase 1	Phase 2	Total	VR	A_E	Cp	SA	PF	RY
6.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.189±0.004	16.97±1.02	11.16±0.86	23.31±1.80	40.18±0.78
7.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.241±0.007	18.09±0.85	13.37±0.82	27.90±1.71	51.35±1.42
7.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.256±0.006	18.09±0.77	14.15±0.84	29.58±1.75	54.44±1.27
8.0	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.235±0.006	17.31±0.77	13.62±0.93	28.43±1.95	50.05±1.30
8.5	5.0±0.1	6.5±0.1	11.5±0.1	0.77	0.207±0.010	17.20±1.33	12.07±0.76	25.20±1.58	44.14±2.21

Forward extraction: AOT 20 mM, pH 7.0
Phase 1: Water phase after BE
Phase 2: Organic phase after BE
VR: Volume ratio (phase 1/ phase 2)
A_E: Activity of Enzyme (Unit/ml enzyme)
Cp: Concentration of protein (µg/ml)
SA: Specific activity (Unit/mg)
PF: Purification folds
RY: Recovery Yield (%)

7.3.1 Total Volume (TV)

During the back extraction step, the volumes for phase 1 and phase 2 remained constant regardless of the pH and KCl concentration used. The total volume for each of the five pH-2 levels (6.5, 7.0, 7.5, 8.0 and 8.5) and the four KCl concentration (0.5, 1.0, 1.5 and 2.0 M) was 11.5 ml. pH or KCl concentration did not have an effect on the total volume or the volumes of phases 1 and 2.

7.3.2 Volume Ratio (VR)

The VR of the two phases remained constant at 0.77 regardless of the pH and KCl concentration used.

7.3.3 Enzyme Activity (A_E)

The effects of pH on the A_E at various KCl concentrations are shown in Figure 7.15. All A_E values increased when the pH was increased from 6.5 to 7.5 and then decreased when the pH was further increased from 7.5 to 8.5. When the pH was increased from 6.5 to 7.5, the A_E for KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M were increased from 0.17 to 0.25 Unit/ml (47.06%), from 0.41 to 0.48 Unit/ml (11.9%), from 0.38 to 0.47 Unit/ml (26.3%) and from 0.19 to 0.26 Unit/ml (36.8%), respectively. When the pH was further increased from 7.5 to 8.5, the A_E values decreased from 0.25 to 0.19 Unit/ml (24.0%), from 0.48 to 0.34 Unit/ml (29.2%), from 0.47 to 0.33 Unit/ml (24.4%) and from 0.26 to 0.21 Unit/ml (19.2%) for KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M, respectively.

The effects of KCl concentration on the A_E at various pH levels are shown in Figure 7.16. A_E increased from 0.17 to 0.41 Unit/ml (192.9%), from 0.21 to 0.45 Unit/ml (114.3%), from 0.25 to 0.48 Unit/ml (92.0%), from 0.22 to 0.38 Unit/ml (72.7%) and from 0.19 to 0.34 Unit/ml (78.9%) when KCl concentration increased from 0.5 to 1.0 M and then decreased from 0.41 to 0.19 Unit/ml (53.7%), from 0.45 to 0.24 Unit/ml (46.7%), from 0.48 to 0.26 Unit/ml (45.8%), from 0.38 to 0.24 Unit/ml (36.8%) and from

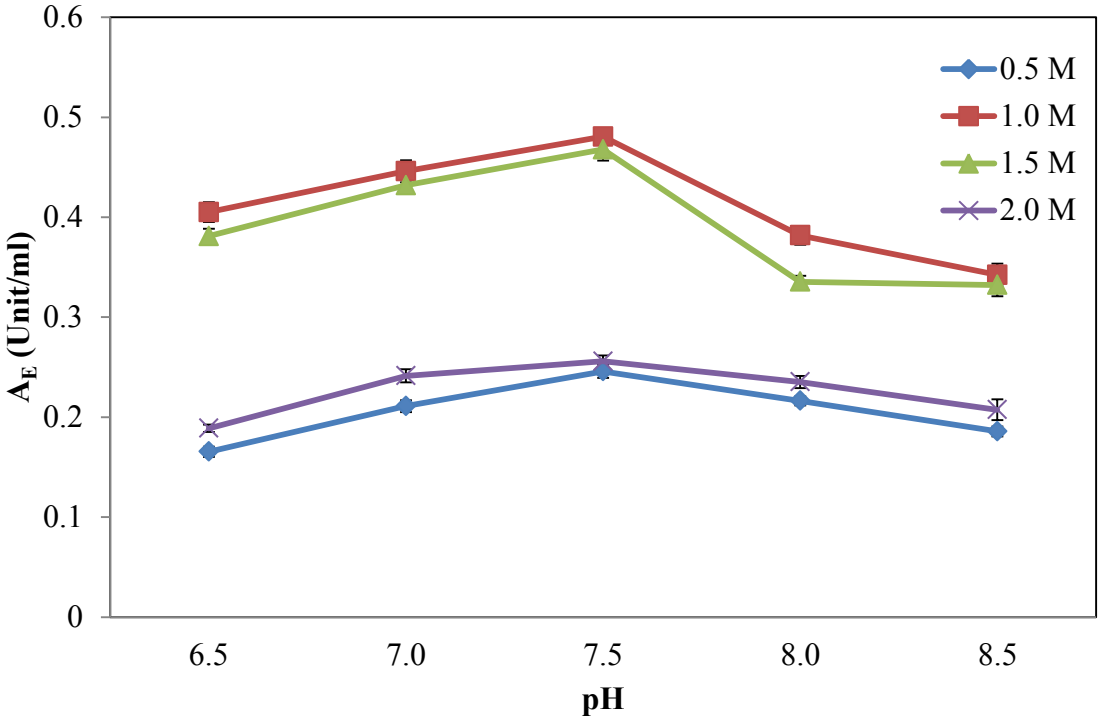

Figure 7.15. The effect of pH-2 on EA at various KCl concentrations (mean ± std, n=3).

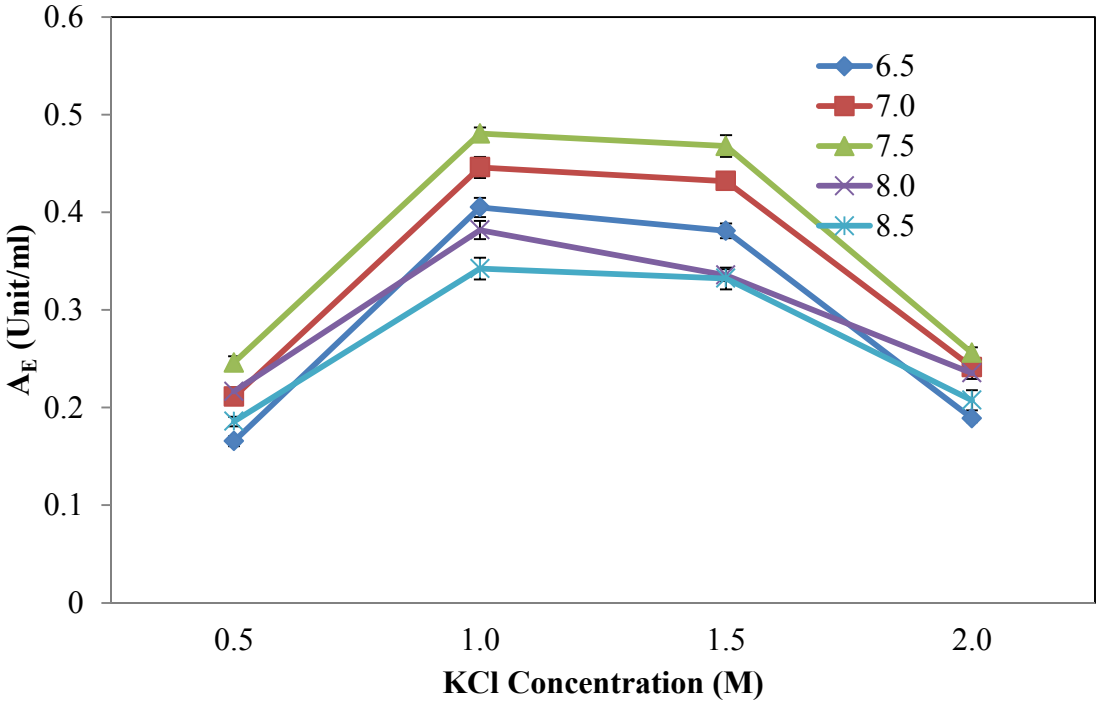

Figure 7.16. Effects of KCl concentrations on EA at various pH-2 levels (mean ± std, n=3).

0.34 to 0.21 Unit/ml (38.2%) when the KCl concentration further increased from 1.0 to 2.0 M for pH-2 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the A_E data and the results are shown in Tables 7.26 and 7.27. The effects of pH and KCl concentration were highly significant at the 0.0001 level. There also appears to be a significant interaction between the pH and KCl concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that all the pH levels were not significantly different from each other at 0.05 level. The KCl concentrations 0.5, 1.0, 1.5 and 2.0 mM were significantly different from each other at the 0.05 level. The highest A_E was achieved at pH 7.5 and KCl concentration of 1.0 M.

7.3.4 Protein Concentration (Cp)

The effects of pH on the Cp at various KCl concentrations are shown in Figure 7.17. When the pH was increased from 6.5 to 7.5, the Cp for the KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M increased from 15.37 to 19.98 μg/ml (30.0%), from 29.70 to 31.13 μg/ml (4.8%), from 20.31 to 21.64 μg/ml (6.5%) and from 16.97 to 18.09 μg/ml (6.6%), respectively. When the KCl concentration was further increased from 1.0 to 2.0 M, the Cp decreased from 19.98 to 17.30 μg/ml (13.4%), from 31.13 to 29.64 μg/ml (4.9%), from 21.64 to 17.37 μg/ml (19.7%) and from 18.09 to 17.20 μg/ml (4.9%) for the KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M, respectively.

Figure 7.18 shows the effects of KCl concentration on the Cp at various pHs. The Cp increased when KCl concentration was increased from 0.5 to 1.0 M and then decreased when the Cp was further increased. For pHs 6.5, 7.0, 7.5, 8.0 and 8.5, the Cp was increased from 15.37 to 29.70 μg/ml (93.2%), from 19.59 to 31.50 μg/ml (60.8%), from 19.98 to 31.13 μg/ml (55.8%), from 18.26 to 30.21 μg/ml (65.4%), from 17.30 to 29.64 μg/ml (71.3%) respectively. When the KCl concentration was further increased from 1.0 to 2.0 M, the Cp decreased from 29.70 to 16.97 μg/ml (42.9%), from 31.50 to 18.09

Table 7.26. Analysis of variance of enzyme activity in the backward extraction.

Source	DF	SS	MS	F	P
Total	59	0.620682			
pH	4	0.072411	0.018103	291.02	0.0001
KCl	3	0.520497	0.173499	2789.23	0.0001
pH*KCl	12	0.024925	0.002077	33.88	0.0001
Error	40	0.029511	0.000738		

DF: Degree of freedom,
SS: Sum of square
MS: Mean of square
R^2: 99.60%

Table 7.27. Effects of pH-2 and KCl concentration on enzyme activity in the backward extraction.

Factor	Level	Number of Observations	Mean (Unit/ml)	Duncan Grouping
pH	6.5	12	0.28509	A
	7.0	12	0.33258	A
	7.5	12	0.36251	A
	8.0	12	0.29224	A
	8.5	12	0.26694	A
KCl Concentration (M)	0.5	15	0.20491	B
	1.0	15	0.41113	A
	1.5	15	0.38969	A
	2.0	15	0.22576	B

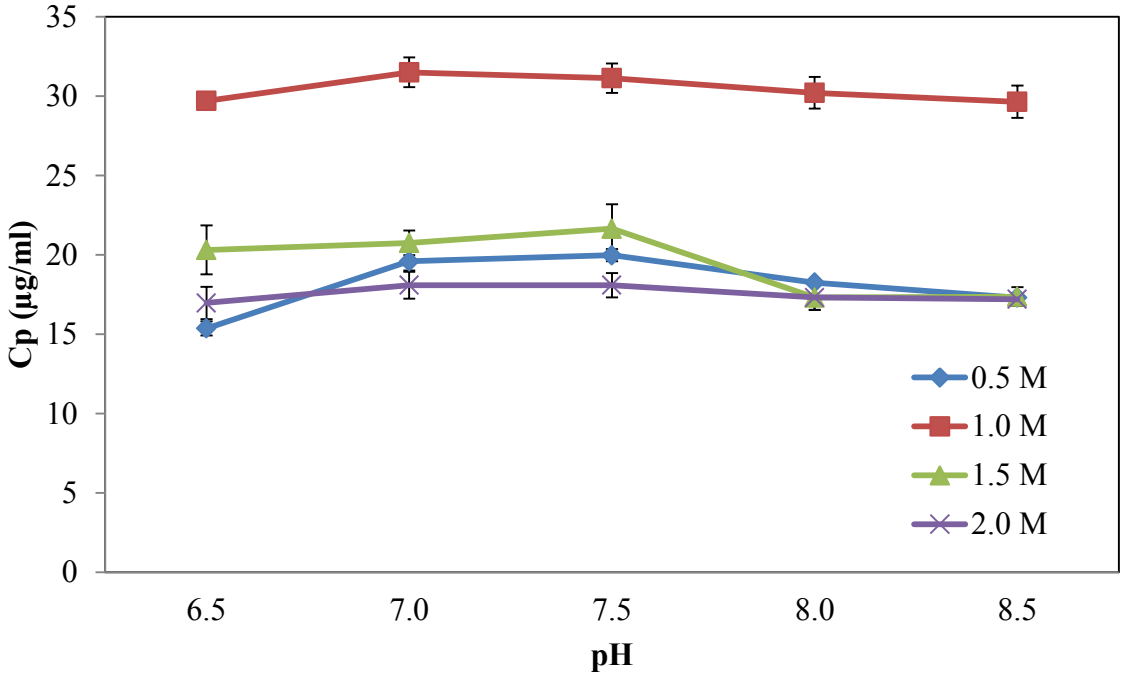

Figure 7.17. The effect of pH-2 on Cp at various KCl concentrations (mean ± std, n=3).

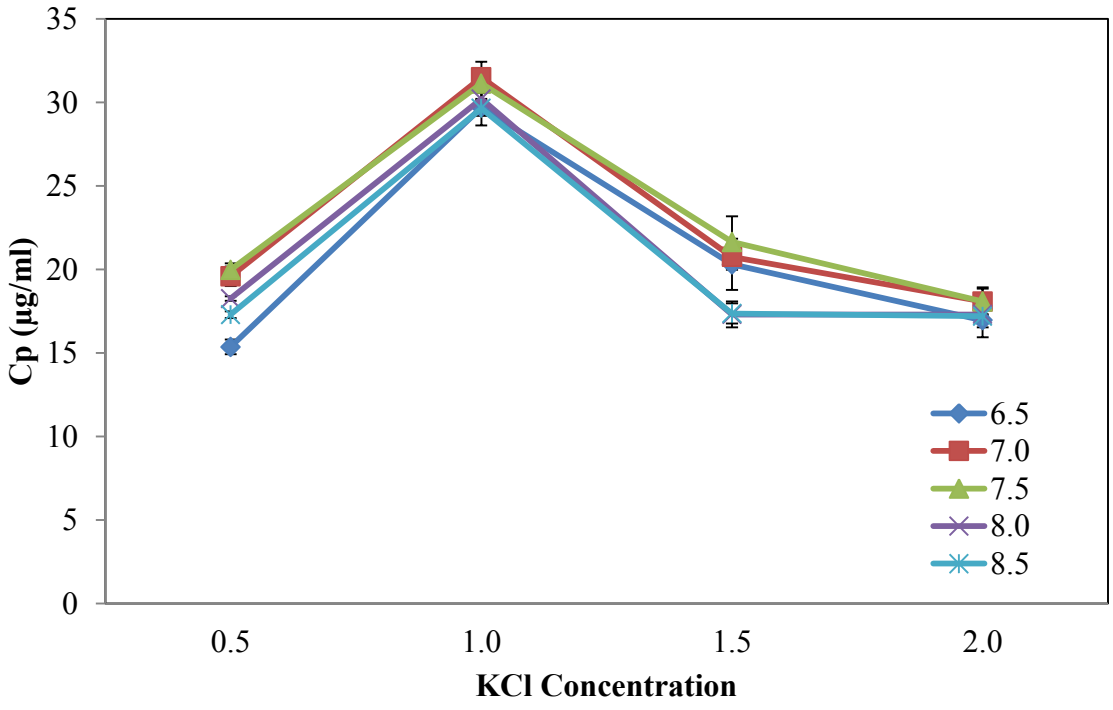

Figure 7.18. Effects of KCl concentration on Cp at various pH-2 levels (mean ± std, n=3).

μg/ml (42.6%), from 31.13 to 18.09 μg/ml (41.9%), from 30.21 to 17.31 μg/ml (42.7%) and from 29.64 to 17.20 μg/ml (42.0%) for the pHs of 6.5, 7.0, 7.5, 8.0 and 8.5 respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the Cp data and the results are shown in Tables 7.28 and 7.29. The effects of pH and AOT concentration were highly significant at the 0.0001 level. There was a significant interaction between pH and KCl concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that all the pH levels were not significantly different from each other at the 0.05 level. The KCl concentrations 1, 1.5 and 0.5/2.0 mM were significantly different from each other at 0.05 level. The highest Cp was achieved at pH 7.5 and KCl concentration of 1.0 M.

7.3.5 Specific Activity (SA)

The effects of pH on the SA at various KCl concentrations are shown in Figure 7.19. SA increased when the pH was increased from 6.5 to 7.5 and then decreased when the pH was further increased. SA increased from 10.77 to 12.30 Unit/mg (14.2%), from 13.64 to 15.44 Unit/mg (13.2%), from 18.84 to 21.67 Unit/mg (15.0%) and from 11.16 to 14.17 Unit/mg (27.0%) when pH increased from 6.5 to 7.5 and then decreased from 12.30 to 10.74 Unit/mg (12.7%), from 15.44 to 11.55 Unit/mg (25.2%), from 21.57 to 19.13 Unit/mg (11.3%) and from 14.17 to 12.07 Unit/mg (14.8%) and when the pH was further increased to 8.5 for KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M, respectively.

Figure 7.20 shows the effects of KCl concentration on SA at various pH levels. The SA for pHs of 6.5, 7.0, 7.5, 8.0 and 8.5 increased from 10.77 to 18.84 Unit/mg (74.9%), from 10.78 to 20.84 Unit/mg (93.3%), from 12.30 to 21.67 Unit/mg (76.2%), from 11.85 to 19.41 Unit/mg (63.8%) and from 10.74 to 19.13 Unit/mg (78.1%) respectively. When KCl was further increased from 1.5 to 2.0 M, SA decreased from 18.84 to 11.16 Unit/mg (40.8%), from 20.84 to 13.37 Unit/mg (35.8%), from 21.67 to 14.17 Unit/mg (34.6%),

Table 7.28. Analysis of variance of protein concentration in the backward extraction.

Source	DF	SS	MS	F	P
Total	59	1798.69			
pH	4	59.95	14.987	21.33	0.0001
KCl	3	1668.61	556.203	791.79	0.0001
pH*KCl	12	42.03	3.503	4.99	0.0001
Error	40	28.1	0.702		

DF: Degree of freedom,
SS: Sum of square
MS: Mean of square
R^2:98.44%

Table 7.29. Effects of pH-2 and KCl concentration on protein concentration in the backward extraction.

Factor	Level	Number of Observations	Mean (µg/ml)	Duncan Grouping
pH	6.5	12	20.586	A
	7.0	12	22.484	A
	7.5	12	22.711	A
	8.0	12	22.771	A
	8.5	12	20.378	A
KCl Concentration (M)	0.5	15	18.098	C
	1.0	15	30.437	A
	1.5	15	19.478	B
	2.0	15	17.531	C

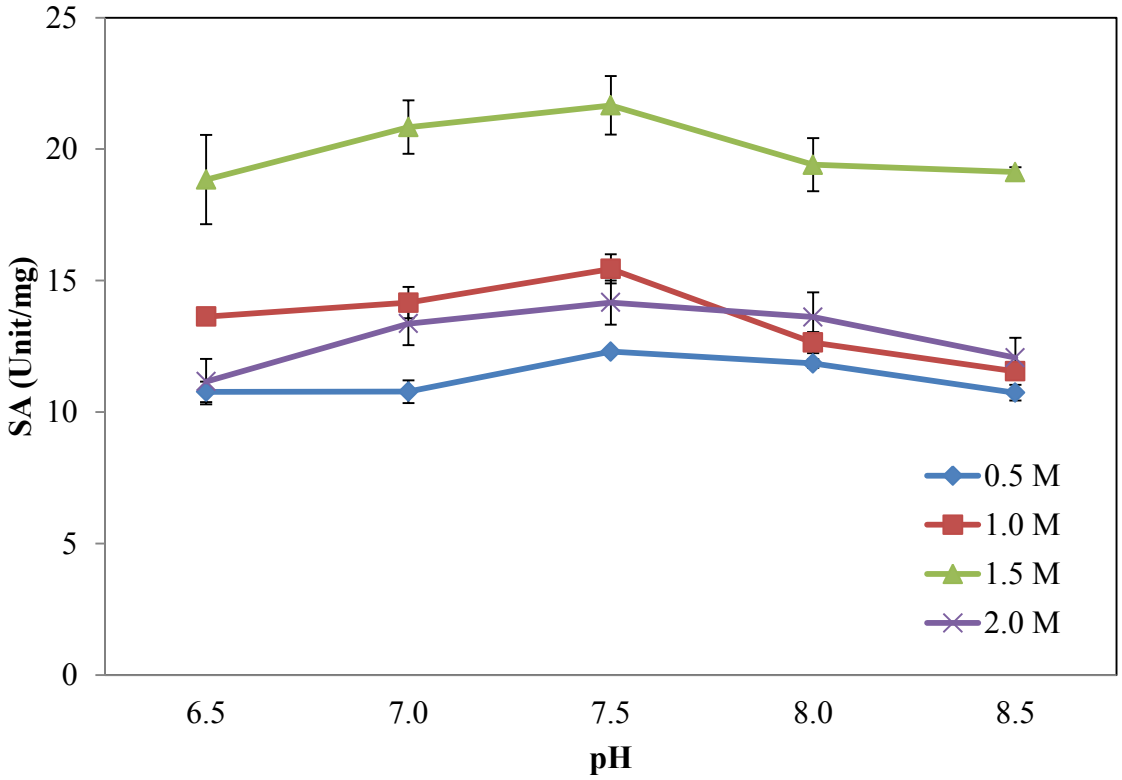

Figure 7.19. Effects of pH-2 on SA at various KCl concentrations (mean ± std, n=3).

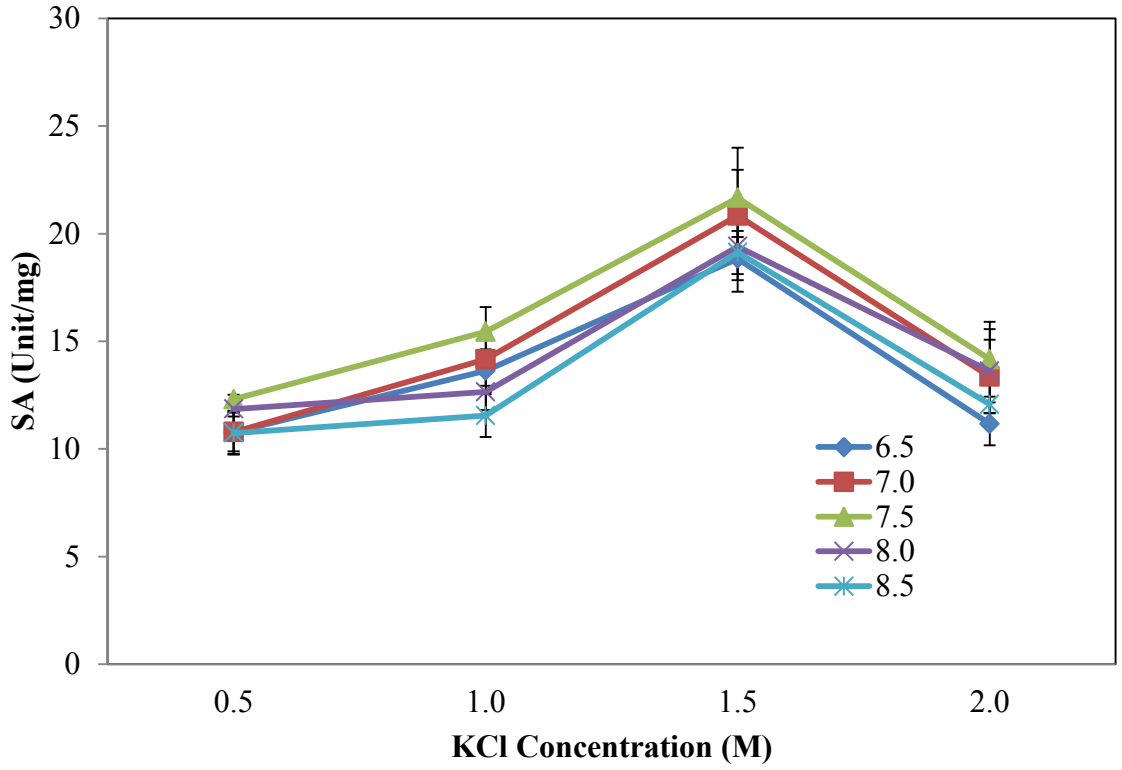

Figure 7.20. Effects of KCl concentrations on SA at various pH-2 levels (mean ± std, n=3).

from 19.41 to 13.62 Unit/mg (29.8%) and from 19.13 to 12.07 Unit/mg (36.9%) for the pHs 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the SA data and the results are shown in Tables 7.30 and 7.31. The effects of pH and KCl concentration were highly significant at the 0.0001 level. There also appears to be a significant interaction between pH and KCl concentration at the 0.05 level. The results obtained from Duncan Multiple Range Test indicated that all pH levels were not significantly different from each other at the 0.05 level. KCl concentrations 0.5, 1.0/2.0 and 1.5 M were significantly different from each other at the 0.05 level. The highest SA was achieved at pH 7.5 and KCl concentration of 1.5 M.

7.3.6 Purification Fold (PF)

The effects of pH on the PF at various KCl concentrations are shown in Figure 7.21. When the pH was increased from 6.5 to 7.5, PF increased from 22.49 to 25.69 (14.2%), from 28.47 to 32.24 (13.2%), from 39.34 to 45.23 (15.0%), from 23.31 to 29.58 (26.9%) for KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M, respectively. When the pH was further increased to 8.5, PF decreased from 25.69 to 22.42 (12.7%), from 32.24 to 24.12 (25.2%), from 45.23 to 39.94 (11.7%) and from 29.58 to 25.20 (14.8%) for KCl concentrations of 0.5, 1.0, 1.5 and 2.0, respectively.

Figure 7.22 shows the effects of KCl concentration on PF at various pH levels. When KCl was increased from 0.5 to 1.5 M, PF increased from 22.49 to 39.34 (74.9%), from 22.51 to 43.50 (93.2%), from 25.69 to 45.23 (76.1%), from 24.74 to 40.52 (63.8%) and from 22.42 to 39.94 (78.1%) and when KCl was further increased from 1.5 to 2.0, PF decreased from 39.34 to 23.31(40.7%), from 43.50 to 27.90 (35.9%), from 45.23 to 29.58 (34.6%), from 40.52 to 28.43 (29.8%) and from 39.94 to 25.20 (36.9%) for the pHs 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Table 7.30. Analysis of variance of specific activity in the backward extraction.

Source	DF	SS	MS	F	P
Total	59	749.826			
pH	4	48.911	12.228	21.89	0.0001
KCl	3	658.776	219.592	393.15	0.0001
pH*KCl	12	19.797	1.65	2.95	0.0050
Error	40	22.342	0.559		

DF: Degree of freedom,

SS: Sum of square

MS: Mean of square

R^2: 97.02%

Table 7.31. Effects of pH-2 and KCl concentration on specific activity in the backward extraction.

Factor	Level	Number of Observations	Mean (Unit/mg)	Duncan Grouping
pH	6.5	12	13.604	A
	7.0	12	14.787	A
	7.5	12	15.895	A
	8.0	12	14.381	A
	8.5	12	13.372	A
KCl Concentration (M)	0.5	15	11.29	C
	1.0	15	13.489	B
	1.5	15	19.976	A
	2.0	15	12.877	B

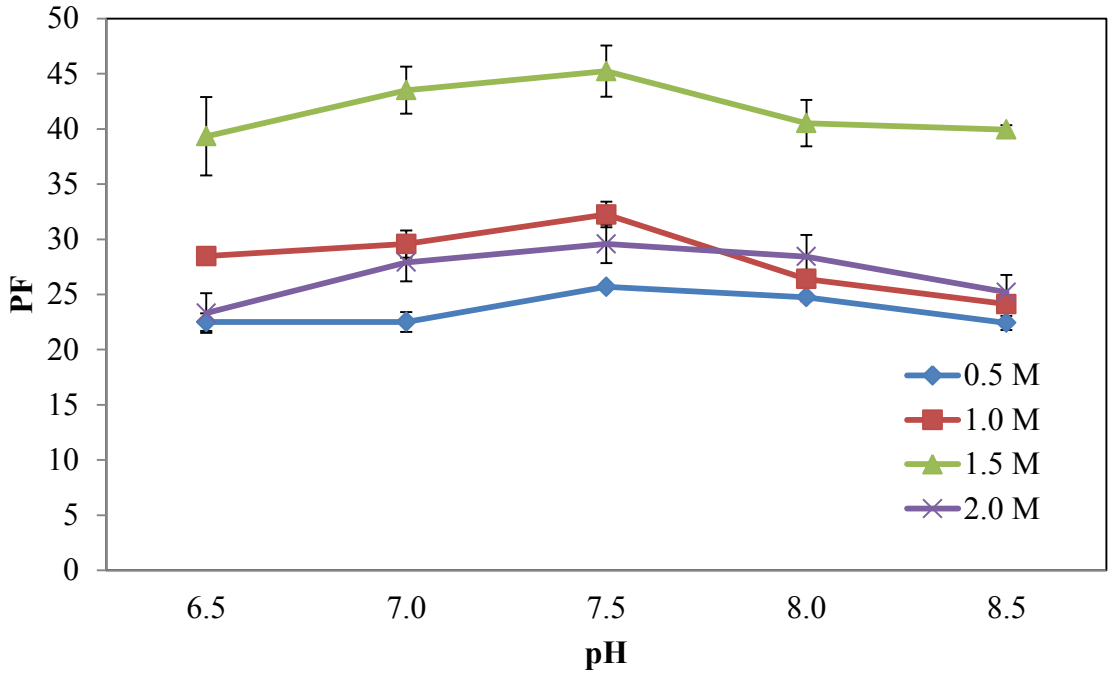

Figure 7.21. The effect of pH-2 on PF at various KCl concentrations (mean ± std, n=3).

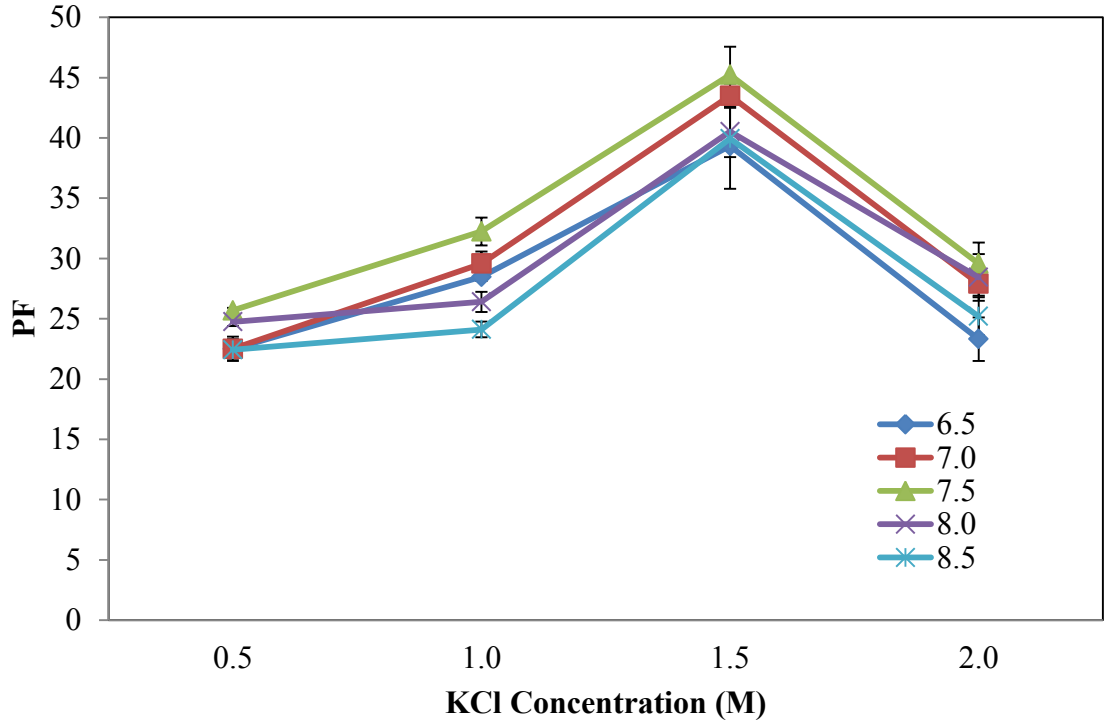

Figure 7.22. Effects of KCl concentrations on PF at various pH-2 levels (mean ± std, n=3).

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the PF data and the results are shown in Tables 8.32 and 8.33. The effects of pH and KCl concentration were highly significant at the 0.0001 levels. There also appears to be a significant interaction between pH and KCl concentration at the 0.005 level. The results obtained from Duncan Multiple Range Test indicated that all pH levels were not significant different from each other at the 0.001 level. KCl concentrations 0.5, 1.0/2.0 and 1.5 M were significantly different from each other at the 0.05 level. The highest PF was achieved at pH 7.5 and KCl concentration of 1.5 M.

7.3.7 Recovery Yield (RY)

Figure 7.23 shows the effects of pH on the RY at different KCl concentrations. When the pH was increased from 6.5 to 7.5, RY increased from 35.21 to 52.31% (17.1%), from 86.18 to 102.24% (16.06%), from 81.06 to 99.53% (18.47%) and from 40.18 to 54.44 % (14.26%) then decreased from 52.31 to 39.53% (12.78%), from 102.24 to 72.83% (29.41%), from 99.53 to 70.86% (28.67%) and from 54.44 to 44.14 % (10.30%) when the pH was further increased to 8.5 for KCl concentrations of 0.5, 1.0, 1.5 and 2.0 M, respectively.

The effects of KCl concentration on RY at various pH-2 levels are shown in Figure 7.24. RY increased from 35.21 to 86.18 % (50.97%), from 44.90 to 94.88 % (49.98%), from 52.31 to 102.24% (49.93%), from 46.04 to 81.24% (35.20%) and from 39.53 to 72.83% (33.30%) when KCl concentration increased from 0.5 to 1.0 M and then decreased to 40.18% (46.00%), 51.35% (43.53%), 54.44% (47.80%), 50.05% (31.19%) and 44.14% (28.69%) when the KCl concentration increased from 1.0 to 2.0 M for the pHs 6.5, 7.0, 7.5, 8.0 and 8.5, respectively.

Analysis of variance (ANOVA) and Duncan Multiple Range Test were performed on the RY data and the results are shown in Tables 8.34 and 8.35. The effects of pH and KCl

Table 7.32. Analysis of variance of purification fold in the backward extraction.

Source	DF	SS	MS	F	P
Total	59	3268.06			
pH	4	213.18	53.294	21.89	0.0001
KCl	3	2871.22	957.074	393.15	0.0001
pH*KCl	12	86.28	7.19	2.95	0.0050
Error	40	97.38	2.434		

DF: Degree of freedom,
SS: Sum of square
MS: Mean of square
R^2: 97.02%

Table 7.33. Effects of pH-2 and KCl concentration on purification fold in the backward extraction.

Factor	Factor	Number of Observations	Mean	Duncan Grouping
pH	6.5	12	26.401	A
	7.0	12	30.871	A
	7.5	12	33.185	A
	8.0	12	30.023	A
	8.5	12	27.917	A
KCl Concentration (M)	0.5	15	23.57	C
	1.0	15	28.16	B
	1.5	15	41.704	A
	2.0	15	36.883	B

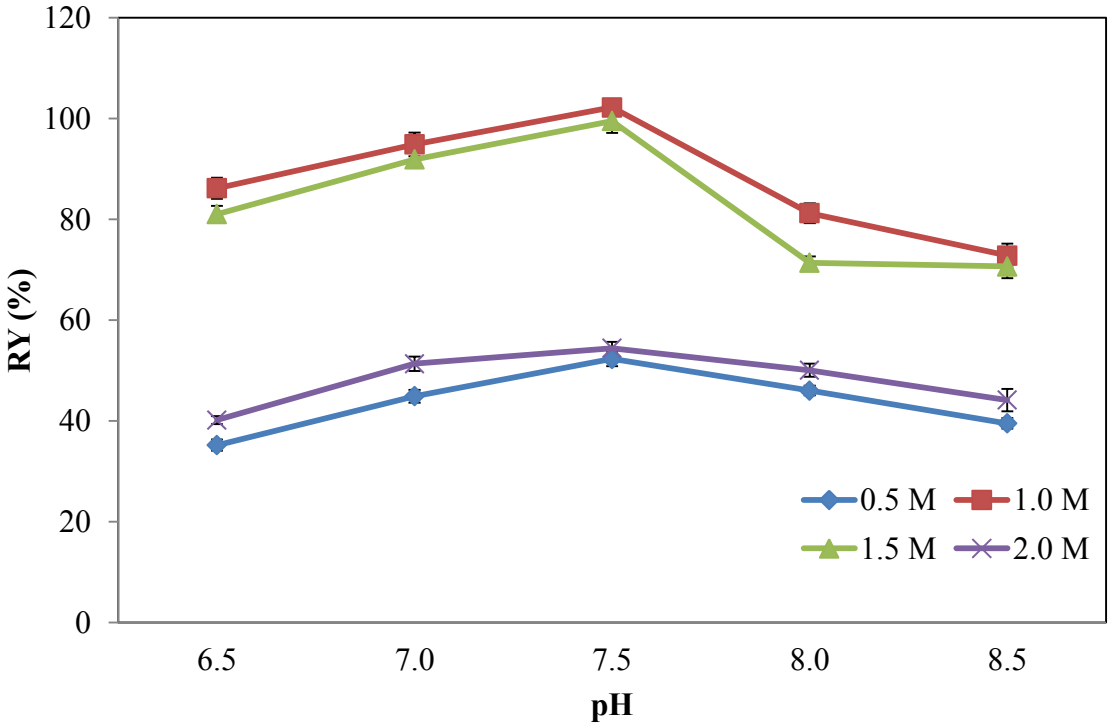

Figure 7.23. Effects of pH-2 on RY at various KCl concentrations (mean ± std, n=3).

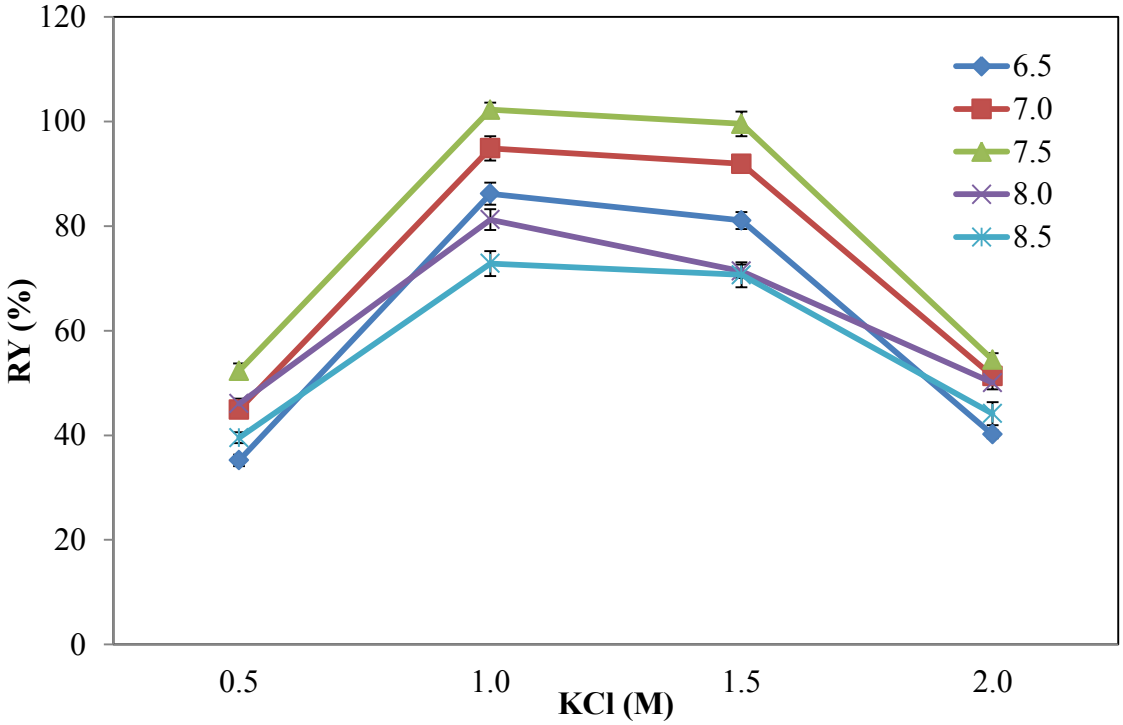

Figure 7.24. Effects of KCl concentration on RY at various pH-2 levels (mean ± std, n=3).

concentration were highly significant at the 0.0001 levels. There also appears to be a significant interaction between pH and KCl concentration at the 0.0001 level. The results obtained from Duncan Multiple Range Test indicated that all pH levels were not significantly different from each other at the 0.05 level. The KCl concentrations 1.0/1.5 and 0.5/2.0 M were significantly different from each other at the 0.05 level. The highest RY was achieved at pH 7.5 and KCl concentration of 1.0 M.

7.4 Alcohol Effect

In order to determine the effects of alcohol in the backward extraction step, an experiment was carried out at the optimal conditions for the forward extraction (AOT 20 mM and pH 7.0) and the backward extraction (KCl 1.0 M and pH 7.5) in which isobutyl alcohol was added in the backward extraction step. Another experiment without alcohol was used as a control. The effects on TV, A_E, TA, Cp, PF and RY for both experiments are shown in Table 7.36.

When alcohol was added in the backward extraction step, TV, A_E, TA, Cp, SA, PF and RY increased from 4.4 to 5.0 ml (13.6%), from 0.11 to 0.48 Unit/ml (336.4%), from 0.54 to 2.39 U (342.6%), from 17.60 to 31.87 μg/ml (81.1%), from 6.08 to 14.98 Unit/mg (146.4%), from 12.70 to 31.27 (146.2%) and from 22.62% to 100.85% (345.8%), respectively. The reason that RY was over 100% could be due to the presence of impurities with chymotrypsin. Analysis of variance (ANOVA) was performed on the TV, A_E, TA, Cp, SA, PF and RY data and the results are shown in Tables 7.37. The effects of alcohol were highly significant at the 0.0001 level for TV, A_E, TA, Cp, SA, PF and RY.

7.5 Ammonium Sulphate Precipitation Method

The crude extract was purified using the ammonium sulphate precipitation method. The enzyme activity (AE), total activity (TA), protein concentration (Cp), specific

Table 7.34. Analysis of variance of recovery yield in the backward extraction.

Source	DF	SS	MS	F	P
Total	59	28097.9			
pH	4	3278	819.49	291.02	0.0001
KCl	3	23562.5	7854.18	2789.23	0.0001
pH*KCl	12	1144.7	95.39	33.88	0.0001
Error	40	112.6	2.82		

DF: Degree of freedom
SS: Sum of square
MS: Mean of square
R^2: 99.60%

Table 7.35. Effects of pH-2 and KCl concentration on recovery yield in the backward extraction.

Factor	Level	Number of Observations	Mean (%)	Duncan Grouping
pH	6.5	12	60.66	A
	7.0	12	70.76	A
	7.5	12	77.13	A
	8.0	12	62.18	A
	8.5	12	56.8	A
KCl Concentration (M)	0.5	15	43.598	B
	1.0	15	87.474	A
	1.5	15	82.913	A
	2.0	15	48.034	B

Table 7.36. Effect of alcohol addition in backward extraction on the purification of chymotrypsin (mean ±std, n=3).

Parameters	TV	A_E	TA	Cp	SA	PF	RY
Without Alcohol	4.4±0.1	0.11±0.01	0.54±0.03	17.60±0.60	6.08±0.37	12.70±0.78	22.62±1.46
With Alcohol	5.0±0.0	0.48±0.01	2.39±0.05	31.87±1.31	14.98±0.49	31.27±1.02	100.85±2.23

Forward extraction: AOT concentration 20 mM, pH 7.0
Backward extraction: KCl concentration 1.0 M, pH 7.5
*Sample size: 5 ml
TV: total volume (ml)
A_E: activity of enzyme (Unit/ml)
TA: total activity (Unit)
Cp: protein concentration (µg/ml)
SA: specific activity (Unit/mg)
PF: purification folds (-)
RY: Recovery Yield (%)

Table 7.37. Analysis of variance of effects of alcohol on the backward extraction.

	Source	DF	SS	MS	F	P	R^2
VT	Alcohol	1	0.5400	0.5400	108	0.0001	0.964
	Error	4	0.0200	0.0050			
	Total	5	0.5600				
A_E	Alcohol	1	0.2054	0.2054	2583	0.0001	0.999
	Error	4	0.0003	0.0001			
	Total	5	0.2057				
TA	Alcohol	1	5.1338	5.1338	2583	0.0001	0.999
	Error	4	0.0080	0.0020			
	Total	5	5.1417				
Cp	Alcohol	1	305.4500	305.4500	294.2	0.0001	0.987
	Error	4	4.1500	1.0400			
	Total	5	309.6000				
SA	Alcohol	1	118.7360	118.7360	629.1	0.0001	0.994
	Error	4	0.7550	0.1890			
	Total	5	119.4910				
PF	Alcohol	1	517.4990	517.4990	629.1	0.0001	0.994
	Error	4	3.2900	0.8230			
	Total	5	520.7900				
RY	Alcohol	1	9178.5200	9178.5200	2583	0.0001	0.999
	Error	4	14.2100	3.5500			
	Total	5	9192.7300				

DF: Degree of freedom
SS: Sum of square
MS: Mean of square

activity (SA), purification folds (PF) and recovery yield (RY) was determined and the results are presented in Table 7.38. A_E, TA and Cp decreased from 0.47 to 0.22 Unit/ml (53.3%), from 2.37 to 1.10 U (53.3%) and from 987.90 to 41.64 μg/ml (95.8%), respectively. SA increased from 0.48 to 5.31 Unit/mg (1012%). PF and RY were 11.1 and 46.72%, respectively.

The reverse micelles procedure was compared to the ammonium sulphate method by performing analysis of variance (ANOVA) on A_E, TA, Cp, SA, PF and RY data and the results are shown in Table 7.39. The effect of method been chosen in purification process was highly significant at the 0.0001 level for TV, A_E, TA, Cp, SA, PF and RY.

Table 7.38. Purification of chymotrypsin from crude extraction using ammonium sulphate method.

Parameters	A_E	TA	Cp	SA	PF	RY
Crude Extraction	0.47±0.03	2.37±0.10	987.9	4.77±0.38	-	-
Ammonium Sulphate	0.22±0.01	1.10±0.05	41.64±5.48	5.31±0.19	11.08±0.39	46.72±2.23

*Sample size: 5 ml
A_E: activity of enzyme (Unit/ml)
TA: total activity (Unit)
Cp: protein concentration (µg/ml)
SA: specific activity (Unit/mg)
PF: purification folds (-)
RY: Recovery Yield (%)

Table 7.39. Analysis of variance of reverse micelles method with alcohol added in backward extraction and ammonium sulphate method.

	Source	DF	SS	MS	F	P	R^2
A_E	Method	1	0.098	0.098	885.62	0.0001	0.0996
	Error	4	0.0004	0.0001			
	Total	5	0.098				
TA	Method	1	2.465	2.465	942.39	0.0001	0.996
	Error	4	0.104	0.0026			
	Total	5	2.476				
Cp	Method	1	143.18	143.18	93.29	0.0001	0.959
	Error	4	6.14	1.53			
	Total	5	149.32				
SA	Method	1	140.301	140.301	1024.9	0.0001	0.996
	Error	4	0.548	0.137			
	Total	5	140.849				
PF	Method	1	611.493	611.493	1024.9	0.0001	0.996
	Error	4	2.387	0.597			
	Total	5	613.879				
RY	Method	1	4393.89	4393.89	885.62	0.0001	0.996
	Error	4	19.85	4.96			
	Total	5	4413.73				

DF: Degree of freedom

SS: Sum of square

MS: Mean of square

CHAPTER 8. DISCUSSION

8.1 Extraction Profiles

After homogenization and centrifugation, the enzyme activity (A_E), total activity (TA), protein concentration (Cp) and recovery yield (RY) decreased while the specific activity and purification fold increased which indicated that a portion of chymotrypsin was lost during the crude extraction process but the remaining portion was concentrated during the extraction.

8.2 Forward Extraction

During the forward extraction step, a certain amount of surfactant (AOT) was required to form the reverse micelles structure in the organic phase. However, high AOT concentrations can cause difficulties in separating protein molecules from the reverse micelles during the backward extraction step (Ono et al., 1996). The pH on the other hand affects the net charge of protein molecules which means that the electrostatic interaction force between chymotrypsin and the surfactant head groups is pH dependent. Changes in pH can, therefore, affect the extraction efficiency (Paradkar and Dordick, 1993; Chang et al., 1994; Hu and Gulari, 1995; Ono et al., 1996). In this study, the effects of pH (6.0, 6.5, 7.0, 7.5, 8.0 and 8.5) and AOT concentration (1, 5, 10, 15, 20 and 35 mM) were investigated in order to determine the optimum conditions for the forward extraction step.

Generally, increasing the AOT concentration resulted in a slight decrease in the TV at all pHs while increasing the pH resulted in a slight increase in the TV. When the AOT concentration increased, a third unclear phase appeared between the organic and aqueous phases due to the formation of reverse micelle structures and the presence of protein molecules. Also, increasing the AOT concentration increased the volume and stability of

the third phase. However, the pH and AOT concentration had no effect on volume ratio (VR).

Wang and Cao (2002) reported a decrease in TV during the extraction of kallikrein using the reverse micelles method. They observed the formation of a stable oil-water mixture between the organic phase and aqueous phase after stirring for half an hour at low temperature. Hentsch et al (1992) reported a third phase (stable white-emulsion) when the AOT concentration was higher than 50 mM. Chen et al (2007) observed a stable third phase which could not be separated by ultra-centrifugation (10,000 rpm) at conditions of low pH (<7.0). Hu and Gulari (1995) reported that the time required for phase separation of AOT was much longer than that required for bis (2-ethylhexyl)phthalate (NaDEHP) and the addition of tributyl phosphate (TBP) was required to inhibit the formation of the third phase between the organic and aqueous phases.

Increasing the pH from 6.0 to 7.0 resulted in initial increases in the A_E, Cp, SA, PF and RY which was then followed by decreases with further increases in the pH (from 7.0 to 8.5). Also, increasing the AOT concentration from 1 to 20 mM resulted in initial increases in these parameters followed by decreases with further increases in the AOT concentration to 35 mM. The primary driving force for extraction of chymotrypsin from the aqueous phase to the reverse micelles water pool is the attractive electrostatic interaction between chymotrypsin molecules and the inner layer charge of the reverse micelles. Hu and Gulari (1995) reported that when the pH of the aqueous phase was lower than the isoelectric point (pI) of chymotrypsin, the positive charge on the chymotrypsin surface interacted with the negative charge on the head structure of the AOT molecule causing the chymotrypsin to transfer from the aqueous phase to the organic phase. Theoretically, the lower the pH of the aqueous phase compared to the pI of chymotrypsin, the stronger the interaction would be and the easier the transfer of chymotrypsin from the aqueous phase to the organic phase. Since the pI of chymotrypsin

is 8.5, increasing the pH from 7.0 to 8.5 weakened the electrostatic interaction between chymotrypsin and AOT which then resulted in dramatic decreases in A_E, Cp, SA, PF and RY.

AOT forms a "microcapsule" structure in the organic phase which acts as a colloidal extractor for chymotrypsin molecules. Thus, the AOT concentration significantly affects the extraction ability of the reverse micelles system. When the AOT concentration was increased from 1 to 20 mM, the "microcapsule" structure increased, resulting in increases in A_E, Cp, SA, PF and RY but when the AOT concentration was further increased to 35 mM, decreases were observed in those parameters likely due to the difficulties in separating chymotrypsin from the reverse micelles structure (Luisi and Smith, 1981; Lang and Jada, 1987; Hentsch et al, 1992).

The crude extraction used in this study contained other proteins so the Cp of the purified solution did not solely represent the chymotrypsin concentration but it can be used to determine the extraction ability of reverse micelles. By using pure commercial chymotrypsin in the extraction process, Hentsch et al (1992) extracted 99% chymotrypsin using AOT reverse micelles and Hu and Gulari (1995) extracted 90% chymotrypsin using NaDEHP.

The SA results obtained in this study are similar to those reported by Hebbar (2008) who found that SA increased, reaching a maximum SA at 49.41 Unit/mg when the pH was increased from 6.0 to 8.0 and then decreased when the pH was further increased from 8.0 to 10.0 while extracting bromelain from pineapple waste. They also found that when the cetyl trimethylammonium bromide (CTAB) concentration was increased from 50 to 150 mM, the SA increased from 22.49 to 40.32 Unit/mg and then decreased to 21.71 Unit/mg when the CTAB concentration was was further increased to 200 mM. Wang and Cao (2007) extracted kallikrein from crude kallikrein with CTAB reverse micelles and obtained a maximum SA of 200 Unit/mg at pH 12.

The PF trends observed in this study are similar to those reported in the literature. Hebbar (2008) found that PF increased to a maximum of 5.3 when the pH increased from 6.0 to 8.0 and then decreased when the pH was further increased from 8.0 to 10.0 during the extraction of bromelain from pineapple waste. They also found that when the CTAB concentration was increased from 50 to 150 mM, PF increased from 2.9 to 5.2 and then decreased to 2.8 when the CTAB concentration was further increased to 200 mM. Wang and Cao (2007) extracted kallikrein from crude kallikrein with CTAB reverse micelles and found the maximum PF to be 7.15.

In this study, 88.9% RY was achieved under the forward extraction condition (pH 7.0, KCl 0.1 M). Hentsch et al (1992) extracted chymotrypsin from a commercial chymotrypsin solution under optimal condition (forward extraction: pH 5.0, KCl 0.2 M; backward extraction: pH 8.0, KCl 1 M) and obtained nearly 100% RY. Goto et al (1998) extracted chymotrypsin with AOT-DOLPA reverse micelles and found the optimal forward extraction condition to be AOT-DOLPA of 20 mM and a pH of 6.8. Chen et al (2007) used AOT reverse micelles to extract matrine and reported a recovery yield of 70.3%. Wang and Cao (2007) reported a recovery yield over 80% when extracting kallikrein using CTAB reverse micelles. Hu and Gulari (1995) reported over 90% recovery yield using NaDEHP reverse micelles to extract α-chymotrypsin from commercial chymotrypsin. Hebbar et al (2008) extracted bromelain from pineapple waste at an optimal pH of 8.0 and obtained an RY of 106%. The reason RY was over 100% could be the presence of impurities with chymotrypsin.

The optimum pH and AOT concentration that give the highest A_E, Cp, SA, PF and RY are shown in Table 8.1. The highest A_E, Cp, SA, PF and RY were reached at the pH 7.0 and the AOT concentration of 20 mM. Therefore, pH 7.0 and the AOT concentration of 20 mM were chosen as the best forward extraction conditions. The optimum pH of the

Table 8.1. Optimum pH-1 and AOT concentration for forward extraction.

Parameters	Optimum Controlled	
	pH	AOT concentration (mM)
A_E	7.0	20
Cp	7.0	20
SA	7.0	20
PF	7.0	20
RY	7.0	20

forward extraction determined in the study (7.0) is within the range reported by other researchers (Goto et al., 1998 and Hu and Gulari, 1995) while the optimum AOT concentration (20 mM) was higher than those repoted by Lang and Jada (1987) and Hentsch et al (1992).

8.3 Backward Extraction

During the backward extraction step, salt was required to break the reverse micelles structure in the organic phase in order to release chymotrypsin into the aqueous phase. However, high salt concentrations would denaturate the protein in the backward extraction step (Ono et al., 1996). The pH on the other hand affects the net charge of protein molecules which affects the electrostatic interaction force between the chymotrypsin and the surfactant and in turn affects the extraction efficiency of the process (Paradkar and Dordick, 1993; Chang et al., 1994; Hu and Gulari, 1995; Ono et al., 1996). In the present study, the effects of pH (6.5, 7.0, 7.5, 8.0 and 8.5) and KCl concentration (0.5, 1.0, 1.5 and 2.0 M) were investigated in order to determine the optimum conditions for the backward extraction step.

During the backward extraction step, the volumes for the organic and aqueous phases remained constant regardless of the pH and KCl concentration used. Thus, the total volume for each of the five pH-2 levels (6.5, 7.0, 7.5, 8.0 and 8.5) and the four KCl concentrations (0.5, 1.0, 1.5 and 2.0 M) was 11.5 ml and the VR of the two phases remained constant at 0.77. Several researchers reported similar results (Paradkar and Dordick, 1993; Hu and Gulari, 1995; Ono et al., 1996; Goto et al., 1998 and Hong et al., 2000).

Increasing the pH from 6.5 to 7.5 initially increased the A_E, Cp, SA, PF and RY but further increases in the pH (from 7.5 to 8.5) resulted in decreases in all these parameters. Also, increasing the KCl concentration from 0.5 to 1.5 M resulted in initial increases in

the A_E, Cp, SA, PF and RY which were then followed by decreases with a further increase in the KCl concentration (to 2.0 M). It has been reported that changes in the structure of proteins and the low rate of backward extraction are the main problems with the backward extraction step and changing the pH and the concentration of salt in the aqueous phase are the most common methods applied to improve A_E, Cp, SA, PF and RY of the backward extraction (Dekker et al., 1990; Nisiki et al., 1993; Nisiki et al., 1995; Hong et al., 2000). When the positive net charge on the chymotrypsin surface decreased, the electrostatic interaction between the protein and the negative AOT molecular head became weaker and chymotrypsins were released into the aqueous phase (Goto et al., 1998). Chang et al. (1994) stated that the KCl concentration has significant effects on the radius of reverse micelles and changing the salt concentration can affect A_E, Cp, SA, PF and RY. They reported that when KCl concentration was increased from 0.2 to 0.8 M, the radius of reverse micelles decreased from 62 to 41 Å which in turn decreased protein solubility and was responsible for releasing protein to the aqueous phase. High salt concentration resulted in unstable reverse micelle structures and led to increases in the A_E, Cp, SA, PF and RY. Goto et al. (1998) reported that during the backward extraction of chymotrypsin using 200 mM AOT concentration, an increase in A_E (from 1 to 6 Unit/ml) was observed when the pH was increased from 4.0 to 7.5 which was followed by a decrease (to 0.5 Unit/ml) with further increases in the pH (to 11.0). Hebbar et al. (2008) reported that during the backward extraction step of extracting bromelain from pineapple waste, the A_E increased when the KBr concentration was increased from 0.25 to 0.50 M and then decreased when the KBr concentration further increased from 0.50 to 0.75 M

Goto et al. (1998) state that when the pH of the aqueous phase approaches the pI of proteins, the Cp increases because of the electrostatic interaction between the protein and AOT reverse micelles becoming weakened and more protein molecules are released from the reverse micelles into the aqueous phase. However, further increases in the pH resulted in decreasing Cp because of protein denaturation. Ono et al. (1996) reported that during the extraction of haemoglobin using DOLPA, when the pH was increased, the backward

extraction rate dramatically increased reaching 90% at a pH of 8.0 and then declined with further increases in the pH. Hatton et al (1989) and Dekker et al. (1991) stated that a high ionic strength in the aqueous phase was not good for protein extraction in the backward extraction step. Hong et al. (2000) extracted BSA, carbon anhydrase and β-lactoglubulin from AOT reverse micelles using a low ionic strength conditions in the aqueous phase (0.1M KCl) and found that high ionic strength could result in protein denaturation. High ionic strength was considered to be a salt concentration >1 M in the literature. Similar results were reported by Chang et al. (1994), Ono et al. (1996) and Hebbar et al. (2008). In this study, SA and PF decreased when KCl concentration increased above 1.5 M, a higher concentration than that reported by others.

High salt concentrations can destabilize reverse micelles and release target protein back to the aqueous phase and as such increase SA. Hebbar et al. (2008) reported that when the aqueous phase pH was increased from 3.9 to 4.2 during the backward extraction of bromelain from pineapple waste, SA and PF increased from 22.6 to 56.15 CDU/mg and from 2.1 to 5.3, respectively. Then, they decreased to 25 CDU/mg and 2.4 when the pH was further increased to 4.5. They also reported that when the KBr concentration increased from 0.25 to 0.50 M, SA increased from 20 to 56 CDU/mg and then decreased to 23 CDU/mg and PF increased from 2.1 to 5.6 and then decreased to 2.4 when the KBr concentration was further increased to 0.75 M.

RY is one of the most important parameters used in evaluating the extraction process. RY was determined in this study at a pH of 7.0. In most other reports, pure enzymes are used to evaluate the extraction efficiency of reverse micelles. RY of the backward extraction is relatively lower than the forward extraction due to the strong interaction between the protein and reverse micelles (Hentsch et al., 1992; Change et al., 1994; Hu and Gulari, 1995; Goto et al., 1998; Ono et al., 1998; Hong et al., 2000; Hebbar et al., 2008). Several researchers reported similar effects of pH in the backward extraction on RY (Chang et al., 1994; Ono et al., 1996; Goto et al., 1998 and Hebbat et al., 2007).

Hebbat et al. (2007) reported that during the extraction of bromelain from pineapple waste, the RY increased from 68 to 100% when the aqueous phase pH was increased from 3.9 to 4.2, which then decreased with further increases in pH. Goto et al. (1998) reported a 100% RY of chymotrypsin using AOT-DOLPA at pH of 7.0. Hu and Gulari (1995) reported a RY of 67.6% during the extraction of α-chymotrypsin using NaDEHP with a $CaCl_2$ concentration of 0.1 M. Hentsh et al. (1992) reported a 100% RY during the backward extraction of chymotrypsin using 1 M KCl at pH of 8.0.

The optimum pH and KCl concentration that gave the highest A_E, Cp, SA, PF and RY for the backward extraction are shown in Table 8.2. The highest A_E, Cp and RY were reached with pH of 7.5 and KCl concentration of 1.0 M. Highest SA and PF were reached with pH of 7.5 and KCl of concentration 1.5 M. RY has been considered as the most important parameter in evaluation of the extraction process. Therefore, pH 7.5 and KCl concentration of 1.0 M were chosen as the best backward extraction conditions.

The optimal pH applied in the backward extractions is always higher than that in forward extraction. Ono et al. (1996) reported pHs 6.5 and 8.0 during the extraction of haemoglobin using DLPA for the forward extraction and backward extraction. Hentsch et al. (1992) used pH 5.0 and 8.0 for forward and backward extraction steps during the extraction of chymotrypsin using AOT. Goto et al. (1998) found the optimal pH conditions for extraction of chymotrypsin using AOT-DOLPA mixed reverse micellar system to be pH 6.8 for forward extraction and pH 7.0 for backward extraction.

8.4 Effect of Alcohol on Backward Extraction

Adding alcohol in the backward extraction step increased VT, A_E, TA, Cp, SA, PF and RY. Paradkar and Dordick (1993), Hu and Gulari (1995), Ono et al (1996), Goto et al (1998) and Hong et al (2000) reported that a clear phase was quickly obtained in the presence of alcohol after stopping the stirring process and with the addition of 10-20%

Table 8.2. Optimum pH and KCl concentration for backward extraction.

Parameters	Optimal Controlled	
	pH	KCl concentration (M)
A_E	7.5	1.0
Cp	7.5	1.0
SA	7.5	1.5
PF	7.5	1.5
RY	7.5	1.0

(v/v) alcohol in the backward extraction step the protein transfer from reverse micelles was 10 times faster than in the absence of alcohol. Paradker and Dordick (1993) added 10% (v/v) ethyl acetate in the backward extraction step and noticed a significant increase in A_E, TA and RY. Goto et al. (1998) reported that without the addition of alcohol, 24 hours were required to obtain equilibrium in the back-extraction step and the extraction time was reduced to less than 2 hours by adding 10% (v/v) isobutyl alcohol. Hong et al (2000) reported that adding 10-15% isopropanol in the backward extraction step resulted in 100% extraction of pepsin and 70% extraction of chymosin. Hu and Gulari (1995) reported that only 10-20% RY were obtained using NaDEHP reverse micelle system without the addition of alcohol in the backward extraction but 98% of active cytochrome-c and 67% of active chymotrypsin were recovered from the aqueous phase in the presence of alcohol.

Goto et al. (1998) studied the effect of alcohol type on the RY and relative activity of recovered chymotrypsin. Their results showed that the RY of the backward extraction with isopropyl alcohol, isobutyl alcohol, isoamyl alcohol, n-hexyl alcohol, n-octyl alcohol, n-decanol and oleyl alcohol were 93.8%, 97.1%, 90.7%, 84.5%, 67.4%, 59.9% and 37.1%respectively. The relative activity (recovered specific activity/original specific activity) for recovered chymotrypsin in the backward extraction using isopropyl alcohol, isobutyl alcohol, isoamyl alcohol, n-hexyl alcohol, n-octyl alcohol, n-decanol and oleyl alcohol were 0.031, 1.00, 0.74, 0.68, 0.24, 0.10 and 0.55 respectively.

Ono et al. (1996) studied the effect of alcohol type and concentration in the backward extraction step using methanol, ethanol, isopropyl alcohol (IPA) and n-propyl alcohol (nPA) at concentrations of 0-30% (v/v). When IPA and nPA was added in the back-extraction step, the hemoglobin recovery rate increased significantly from 0 to 60% when alcohol concentration was increased from 5 to 10% (v/v) and then decreased with further increases in the alcohol concentration. The hemoglobin recovery rate dramatically increased from 0 to 70% (v/v) when the ethanol concentration was increased from 10 to 15% (v/v) and then decreased when the concentration was further increased. The

recovered hemoglobin rate dramatically increased from 0 to 70% (v/v) when the methanol concentration in the back extraction was increased from 20 to 30% (v/v). Alcohol can reduce the interfacial resistance for the reverse micelles because it promotes the fusion/fission of reverse micelles which destabilizes the structure.

8.5 Ammonium Sulphate Method

The results of A_E, TA, SA and PF obtained for purifying chymotrypsin and ammonium sulphate method are comparable to those reported in the literature. Yang et al. (2009) used ammonium sulphate 30-60% saturation to fractionate chymotrypsin from crucian carp (*Carassius auratus*) and reported 85% RY, 4.1 Unit/mg SA and 6.6 PF. Castillo-Yañez used ammonium sulphate 30-70% saturation to fractionate chymotrypsin from the viscera of Monterey sardine (*Sardinops sagax caeruleus*) and reported 57% RY, 10 Unit/ml SA and 1.6 PF. Heu et al. (1995) purified crude extract from the viscera of anchovy (*Engraulis japonica*) using ammonium sulphate at 30-70% saturation and reported 22% RY, 6.62 Unit/mg SA and 8.0 PF. Lam et al. (1999) purified chymotrypsin from midgut of *Locusta migratoria* using ammonium sulphate at 35-70% saturation and reported 101% RY, 3.35 Unit/mg SA and 1.4 PF.

The results obtained with the ammonium sulphate method were compared with those obtained from the reverse micelles as shown in Table 8.3. The results showed that reverse micelles is the best choice in crude chymotrypsin extraction because of the higher RY and SA.

8.6 Industrial Application

The average weight of red perch and the guts, fins, tails and head are shown in Table 8.4. Fish gut is only 5.63% of total weight of fish and 11.82% of the weight of fish waste.

Table 8.3. Comparing reverse micelles method at optimum condition with ammonium sulphate (mean ± std, n=3).

Parameters	Methods	
	Reverse micelles	Ammonium Sulphate
A_E (Unit/ml)	0.48±0.01	0.22±0.01
TA (Unit)	2.39±0.05	1.10±0.05
Cp (µg/ml)	31.87±1.31	41.64±1.16
SA (Unit/mg)	14.89±0.49	5.31±0.19
PF	31.27±1.02	11.10±0.39
RY (%)	100.85±2.23	46.72±2.23

In the fish processing industry, the fish gut should be collected and washed with running water to remove blood to eliminate the effects of enzymatic degradation. The clean guts should then be frozen at -20ºC to maintain the stability of the enzyme untill further extraction and purification of chymotrypsin.

Table 8.4. The weight of fish parts from red perch.

Part	Weight (g)	Pcercent of Whole Fish (%)	Percent of Total Waste (%)
Whole	587.0	-	-
Total Waste	279.5	49.60	-
Head	189.7	32.32	67.90
Tail	42.2	7.18	15.08
Fins	17.5	2.47	5.19
Gut	33.1	5.63	11.83

CHAPTER 9. CONCLUSIONS

The effects of the forward extraction pH-1 (6.0, 6.5, 7.0, 7.5, 7.0 and 8.5) and AOT concentration (1, 5, 10, 15, 20 and 35 mM) and the effects of the backward extraction pH-2 (6.5, 7.0, 7.5, 8.0 and 8.5) and KCl concentration (0.5, 1.0, 1.5 and 2.0 M) on the purification of chymotrypsin from red perch were studied. The following are the conclusions obtained from the study.

1. TV decreased with increases in the AOT concentration and slightly increased with increases in the pH while the VR slightly decreased with increased pH-1.
   (a) Higher amount of surfactant formed a stable oil-water mixture structure at low temperature.
   (b) The effects of pH-1 and AOT concentration on TV were significant and a significant interaction between the pH-1 and AOT concentration was observed at the 0.0001 level.
   (c) The effect of pH-1 on the VR was significant but the effect of AOT concentration was not significant at the 0.05 level and there was no significant interaction between the pH-1 and AOT concentration.
   (d) The highest TV was achieved at pH-1 8.5 with AOT concentration of 1mM.
   (e) The highest V_R was achieved at pH-1 6.0 with AOT concentration of 15 mM.

2. When pH-1 was increased from 6.0 to 7.0, A_E increased by up to 50%, Cp increased by up to 56.7%, SA increased by up to 37.8% and RY increased by up to 30.3% and they all decreased when pH was further increased. Similarly, when the AOT concentration was increased from 1 to 20 mM, A_E increased by up to 218.2%, Cp increased by up to 36.67%, SA increased by up to 37.8% and RY increased by up to 30.3% and they all decreased when the AOT concentration was further increased to 35 mM.

(a) The increases in these parameters were due to the increased electrostatic interaction between the net charge on the surface of protein molecule and reverse micelles inner charge layer caused by increasing pH-1 and the increased reverse micelles structure caused by increased AOT concentration.

(b) The decreases in these parameters were due to the decreased net charge on protein molecules which weakened the electrostatic interaction between chymotrypsin and reverse micelles and the high AOT concentration which increased the difficulties in backward extraction step.

(c) The effects of pH-1 and AOT concentration on A_E were highly significant at the 0.0001 level and the interaction between the pH-1 and concentration were observed.

(d) The effects of pH-1 and AOT concentration on Cp were highly significant at the 0.0001 level and a significant interaction between the pH-1 and concentration was observed.

(e) The effects of pH-1 and AOT concentration on SA were highly significant at the 0.0001 level but there was no interaction between the pH-1 and concentration

(f) The effects of pH-1 and AOT concentration on PF were highly significant at the 0.0001 level but there was no interaction between the pH-1 and concentration was not significant at 0.758 was observed.

(g) The effects of pH-1 and AOT concentration on RY were highly significant at the 0.0001 level and there was an interaction between the pH-1 and concentration

(f) The highest A_E, Cp, SA, PF and RY were achieved with pH-1 7.0 and 20 mM AOT concentration

3. Changing the pH-2 from 6.5 to 8.5 and the KCl concentration from 0.5 to 2.0 M during backward extraction step (with addition of alcohol) had no effects on TV or VR. TV for all samples was 6.5 ml and VR was 0.77. The reverse micelles emulsion structure was destroyed in the present of alcohol.

4. Increasing the pH-2 from 6.5 to 7.5 increased A_E by up to 47.06%, Cp by up to 30.0%, SA by up to 27.0%, PF by up to 26.9% and RY by up to 18.47% and they all then declined with further increases in the pH-2. Similarly, increases in the KCl concentration from 0.5 to 1.0 M increased A_E by up to 192.9%, the Cp by up to 93.2% and RY by up to 50.97% and they all then decreased with further increases in the KCl concentration. SA and PF continued to increase up to 93.3% when the KCl concentration increases from 0.5 to 1.5 M and then decreased with further increases in the KCl concentration.

   (a) The decreases in A_E, Cp, SA, PF and RY were due to the denaturation of protein under a relatively high pH-2 and the ionic strength caused by high pH and KCl concentration in backward extraction step.

   (b) The effects of pH-2 and KCl concentration on A_E were highly significant at the 0.0001 level and an interaction between the pH-1 and concentration was observed.

   (c) The effects of pH-2 and KCl concentration on Cp were highly significant at the 0.0001 level and a significant interaction between the pH-2 and concentration was observed.

   (d) The effects of pH-2 and KCl concentration on SA were highly significant at the 0.0001 level and a significant interaction between the pH-1 and concentration at the 0.005 was observed.

   (e) The effects of pH-2 and KCl concentration on PF were highly significant at the 0.0001 level and a significant interaction between the pH-1 and concentration was observed.

   (f) The effects of pH-2 and KCl concentration on RY were highly significant at the 0.0001 level and a significant interaction between the pH-1 and concentration was observed.

   (g) The highest A_E, Cp and RY was achieved with pH-2 7.5 and 1.0 M KCl concentration.

(h) The highest SA and PF were achieved with pH-2 7.5 and 1.5 M KCl concentration.

5. The optimal conditions for the forward extraction step was pH-1 7.0, AOT concentration 20 mM and the optimal conditions for backward extraction step was pH-2 7.5, KCl concentration 1.0 M.

6. Addition of alcohol in the backward extraction step increased TV by 13.6%, A_E by 336.4%, TA by 342.6%, Cp by 81.1%, SA by 146.4%, PF by 146.2% and RY by 345.8%.
   (a) Alcohol reduced the interfacial resistance for the reverse micelles.
   (i) The effects of alcohol on TV, A_E, TA, Cp, SA, PF and RY were highly significant at 0.0001 level.

7. The reverse micelles method is a good choice as a crude purification step and it provided higher A_E TA, Cp, SA, PF and RY then ammonium sulphate. A_E was higher by 118.2%, TA was higher by 102.7%, SA was higher by 183.6%, PF was higher by 183.6%, RY was higher by 118.8% and Cp was lower by 23.5%.

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APPENDIX A

Forward Extraction

TV=Total volume (ml)

VR= Volume ratio (-)

A_E= Enzyme activity (Unit/ml)

Cp= Protein concentration (μg/ml)

SA= specific activity (Unit/mg)

PF= Purification fold (-)

RY= Recovery yield (%)

Table A1: AOT concentration (1 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	9.86	1.02	0.11	14.22	8.02	16.75	24.09
6.0	2	9.84	1.02	0.11	13.89	8.22	17.15	24.09
	3	9.83	1.01	0.09	14.42	6.47	13.52	19.71
Average		9.84 (0.02)	1.02 (0.01)	0.11 (0.01)	14.18 (0.27)	7.57 (0.95)	15.81 (1.99)	22.63 (2.53)
	1	9.88	1.01	0.10	16.50	5.93	12.37	20.65
6.5	2	9.83	1.00	0.14	15.46	9.20	19.21	30.04
	3	9.84	1.00	0.16	16.07	9.68	20.21	32.85
Average		9.85 (0.03)	1.00 (0.00)	0.13 (0.03)	16.01 (0.52)	8.27 (2.04)	17.27 (4.27)	27.85 (6.39)
	1	9.86	1.00	0.21	18.02	11.74	24.52	44.68
7.0	2	9.88	1.00	0.20	20.37	9.68	20.20	41.61
	3	9.87	1.00	0.19	19.76	9.45	19.73	39.42
Average		9.87 (0.01)	1.00(0.00)	0.20 (0.01)	19.38 (1.22)	10.29 (1.26)	21.48 (2.64)	41.90 (2.64)
	1	9.87	1.00	0.16	15.63	9.96	20.78	32.85
7.5	2	9.87	1.00	0.11	12.77	8.94	18.65	24.09
	3	9.90	1.01	0.11	14.64	7.79	16.27	24.09
Average		9.88 (0.02)	1.00 (0.01)	0.13 (0.02)	14.35 (1.45)	8.90 (1.08)	18.57 (2.26)	27.01 (5.06)
	1	9.91	0.99	0.12	13.97	8.91	18.60	26.28
8.0	2	9.90	1.00	0.09	14.27	8.72	18.21	19.71
	3	9.86	1.00	0.10	14.64	7.26	15.15	21.90
Average		9.89 (0.03)	1.00 (0.00)	0.11 (0.02)	14.29 (0.34)	8.30 (0.91)	17.32 (1.89)	22.63 (3.35)
	1	9.91	0.99	0.09	14.27	6.54	13.66	19.71
8.5	2	9.93	0.99	0.12	14.20	8.77	18.30	26.28
	3	9.88	1.00	0.10	14.54	7.13	14.89	21.90
Average		9.91 (0.03)	0.99 (0.00)	0.11 (0.02)	14.34 (0.18)	7.48 (1.15)	15.62 (2.40)	22.63 (3.35)

Table A2: AOT concentration (5 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	9.81	0.99	0.20	18.33	10.75	22.45	41.62
6.0	2	9.81	1.01	0.21	21.22	9.84	20.54	44.07
	3	9.75	1.01	0.20	18.12	10.88	22.71	41.62
Average		9.79 (0.03)	1.00 (0.01)	0.20 (0.01)	19.22 (1.73)	10.49 (0.57)	21.90 (1.19)	42.44 (1.41)
	1	9.69	1.00	0.21	19.12	10.92	22.79	44.07
6.5	2	9.75	0.99	0.20	19.22	10.26	21.41	41.62
	3	9.80	1.00	0.20	18.89	10.44	21.79	41.62
Average		9.75 (0.06)	1.00 (0.00)	0.20 (0.01)	19.08 (0.17)	10.54 (0.34)	22.00 (0.71)	42.44 (1.41)
	1	9.81	1.01	0.28	22.33	12.46	26.02	58.76
7.0	2	9.81	1.00	0.26	21.84	11.68	24.39	53.86
	3	9.74	1.02	0.26	22.74	11.22	23.42	53.86
Average		9.79 (0.04)	1.01 (0.01)	0.26 (0.01)	22.30 (0.45)	11.79 (0.63)	24.61 (1.31)	55.49 (2.83)
	1	9.85	0.99	0.23	21.67	10.70	22.34	48.97
7.5	2	9.84	1.00	0.23	22.12	10.48	21.89	48.97
	3	9.90	0.99	0.21	19.88	10.50	21.92	44.07
Average		9.86 (0.03)	1.00 (0.01)	0.22 (0.01)	21.22 (1.18)	10.56 (0.12)	22.05 (0.25)	47.33 (2.83)
	1	9.84	1.00	0.15	18.79	8.02	16.75	31.83
8.0	2	9.82	1.00	0.15	19.22	7.84	16.37	31.83
	3	9.86	1.00	0.21	18.83	11.09	23.14	44.07
Average		9.84 (0.02)	1.00 (0.00)	0.17 (0.03)	18.95 (0.24)	8.98 (1.82)	18.76 (3.80)	35.91 (7.07)
	1	9.87	1.00	0.15	18.67	8.07	16.86	31.83
8.5	2	9.89	1.00	0.15	19.97	7.55	15.76	31.83
	3	9.88	1.00	0.20	17.92	11.00	22.97	41.62
Average		9.88 (0.01)	1.00 (0.00)	0.17 (0.03)	18.85 (1.04)	8.87 (1.86)	18.53 (3.88)	35.09 (5.65)

Table A3: AOT concentration (10 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	9.75	1.02	0.27	24.09	11.23	23.45	57.13
6.0	2	9.61	1.01	0.20	20.01	10.14	21.17	42.84
	3	9.81	1.00	0.27	24.17	11.19	23.37	57.13
Average		9.72 (0.10)	1.01 (0.01)	0.25 (0.04)	22.76 (2.38)	10.86 (0.62)	22.66 (1.29)	52.37 (8.25)
	1	9.77	1.00	0.30	24.39	12.20	25.48	62.84
6.5	2	9.77	1.01	0.26	25.29	10.16	21.22	54.27
	3	9.72	0.99	0.31	24.84	12.53	26.15	65.70
Average		9.75 (0.03)	1.00 (0.01)	0.29 (0.03)	24.84 (0.45)	11.63 (1.28)	24.28 (2.67)	60.93 (5.95)
	1	9.82	1.01	0.30	25.09	11.86	24.77	62.84
7.0	2	9.79	0.98	0.34	24.39	13.87	28.95	71.41
	3	9.87	0.98	0.31	25.79	12.07	25.19	65.70
Average		9.83 (0.04)	0.99 (0.02)	0.32 (0.02)	25.09 (0.70)	12.60 (1.10)	26.30 (2.30)	66.65 (4.36)
	1	9.71	1.00	0.32	25.37	12.80	26.72	68.55
7.5	2	9.67	1.01	0.28	22.94	12.38	25.86	59.98
	3	9.86	1.01	0.30	24.22	12.29	25.66	62.84
Average		9.75 (0.10)	1.01 (0.00)	0.30 (0.02)	24.18 (1.22)	12.49 (0.27)	26.08 (0.57)	63.79 (4.36)
	1	9.88	1.00	0.26	21.34	12.05	25.15	54.27
8.0	2	9.81	1.00	0.22	19.97	10.84	22.63	45.70
	3	9.82	0.99	0.18	18.59	9.46	19.75	37.13
Average		9.83 (0.04)	1.00 (0.00)	0.22 (0.04)	19.97 (1.38)	10.78 (1.29)	22.51 (2.70)	45.70 (8.57)
	1	9.84	1.00	0.15	16.28	9.14	19.08	31.42
8.5	2	9.82	1.00	0.18	16.93	10.39	21.69	37.13
	3	9.81	1.00	0.15	16.22	9.18	19.15	31.42
Average		9.82 (0.02)	1.00 (0.00)	0.16 (0.02)	16.48 (0.39)	9.57 (0.71)	19.98 (1.48)	33.32 (3.30)

Table A4: AOT concentration (15 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	9.58	1.02	0.26	25.02	10.55	22.02	55.71
6.00	2	9.33	1.02	0.24	24.83	9.81	20.48	51.43
	3	9.30	1.00	0.25	24.34	10.42	21.76	53.57
Average		9.40 (0.15)	1.01 (0.01)	0.25 (0.01)	24.73 (0.35)	10.26 (0.39)	21.42 (0.82)	53.57 (2.14)
	1	9.59	1.01	0.27	23.77	11.53	24.07	57.86
6.50	2	9.53	1.01	0.29	25.54	11.52	24.06	62.14
	3	9.52	1.02	0.30	25.73	11.83	24.71	64.28
Average		9.55 (0.04)	1.01 (0.00)	0.29 (0.02)	25.01 (1.08)	11.63 (0.18)	24.28 (0.37)	61.43 (3.27)
	1	9.64	1.00	0.34	26.33	12.85	26.82	71.43
7.00	2	9.70	0.99	0.35	27.02	13.02	27.19	74.28
	3	9.67	1.01	0.32	26.58	12.22	25.51	68.57
Average		9.67 (0.03)	1.00 (0.01)	0.34 (0.01)	26.64 (0.35)	12.70 (0.42)	26.51 (0.88)	71.43 (2.86)
	1	9.57	1.00	0.32	26.52	12.24	25.56	68.56
7.50	2	9.72	1.00	0.32	26.47	12.23	25.53	68.33
	3	9.50	1.00	0.30	26.00	11.71	24.45	64.29
Average		9.60 (0.11)	1.00 (0.00)	0.32 (0.01)	26.33 (0.29)	12.06 (0.30)	26.18 (0.63)	67.06 (2.40)
	1	9.68	1.00	0.23	21.64	10.63	22.19	48.57
8.00	2	9.74	1.00	0.24	21.92	11.11	23.20	51.43
	3	9.75	1.00	0.27	22.22	12.18	25.43	57.14
Average		9.72 (0.04)	1.00 (0.00)	0.25 (0.02)	21.93 (0.29)	11.31 (0.79)	23.61 (1.66)	52.38 (4.36)
	1	9.69	0.99	0.20	20.32	9.99	20.86	42.86
8.50	2	9.66	1.00	0.20	19.32	10.51	21.93	42.86
	3	9.71	1.00	0.23	20.62	11.16	23.29	48.57
Average		9.69 (0.02)	1.00 (0.00)	0.21 (0.02)	20.09 (0.68)	10.55 (0.58)	22.03 (1.22)	44.76 (3.30)

Table A5: AOT concentration (20 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	9.60	1.01	0.26	26.42	9.69	20.22	54.02
6.0	2	9.73	1.01	0.30	27.02	11.01	22.98	62.78
	3	9.61	1.01	0.28	27.11	10.20	21.30	58.40
Average		9.65 (0.07)	1.01 (0.00)	0.28 (0.02)	26.85 (0.38)	10.30 (0.67)	21.50 (1.39)	58.40 (4.38)
	1	9.60	1.00	0.34	28.03	12.03	25.11	71.18
6.5	2	9.68	1.01	0.29	28.03	10.36	21.63	61.32
	3	9.62	1.00	0.26	26.64	9.73	20.32	54.75
Average		9.63 (0.04)	1.01 (0.00)	0.30 (0.04)	27.57 (0.80)	10.71 (1.18)	22.36 (2.47)	62.42 (8.27)
	1	9.64	0.99	0.41	29.45	14.09	29.41	87.58
7.0	2	9.58	1.01	0.44	30.03	14.68	30.66	93.10
	3	9.66	1.01	0.40	29.30	13.81	28.83	85.41
Average		9.63 (0.04)	1.00 (0.01)	0.42 (0.02)	29.59 (0.39)	14.19 (0.45)	29.63 (0.94)	88.70 (3.96)
	1	9.71	1.01	0.34	30.00	11.41	23.82	72.27
7.5	2	9.71	1.00	0.41	29.45	14.05	29.34	87.39
	3	9.65	1.01	0.40	29.13	13.57	28.33	83.44
Average		9.69 (0.03)	1.01 (0.00)	0.38 (0.04)	29.53 (0.44)	13.01 (1.41)	27.16 (2.94)	81.03 (7.84)
	1	9.69	1.00	0.35	29.09	12.03	25.11	73.87
8.0	2	9.76	1.00	0.34	28.83	11.66	24.34	70.96
	3	9.72	1.00	0.35	28.96	12.10	25.27	74.01
Average		9.72 (0.04)	1.00 (0.00)	0.35 (0.01)	28.96 (0.13)	11.93 (0.24)	24.91 (0.50)	72.95 (1.72)
	1	9.72	0.99	0.30	28.12	10.70	22.33	63.52
8.5	2	9.73	1.00	0.33	29.28	11.24	23.46	69.46
	3	9.75	1.00	0.26	28.34	9.15	19.10	54.75
Average		9.74 (0.02)	1.00 (0.00)	0.30 (0.04)	28.58 (0.62)	10.36 (1.08)	21.63 (2.26)	62.58 (7.40)

Table A6: AOT concentration (35 mM).

pH	Replicates	TV	VR	A_E	Cp	SA	PF	RY
	1	8.86	1.02	0.20	20.64	10.55	22.03	45.99
6.0	2	8.88	1.01	0.19	18.73	11.08	23.12	43.80
	3	8.79	1.00	0.14	18.92	8.22	17.17	32.85
Average		8.84 (0.05)	1.01 (0.01)	0.18 (0.03)	19.43 (1.05)	9.95 (1.52)	20.78 (3.17)	40.88 (7.04)
	1	8.97	1.00	0.25	22.35	12.07	25.19	56.94
6.5	2	8.92	1.02	0.17	20.88	8.94	18.67	39.42
	3	8.95	1.01	0.19	19.98	9.97	20.81	42.05
Average		8.94 (0.03)	1.01 (0.01)	0.20 (0.04)	21.07 (1.20)	10.33 (1.59)	21.56 (3.33)	46.14 (9.45)
	1	9.10	1.00	0.25	21.68	12.44	25.97	56.94
7.0	2	8.96	1.01	0.24	22.57	13.25	27.67	54.75
	3	9.05	1.01	0.17	19.57	9.54	19.92	39.42
Average		9.04 (0.07)	1.01 (0.01)	0.22 (0.04)	21.27 (1.54)	11.74 (1.95)	24.52 (4.07)	50.37 (9.55)
	1	9.03	1.01	0.18	16.70	11.80	24.64	41.61
7.5	2	9.06	1.01	0.16	15.88	11.11	23.18	37.23
	3	9.05	1.00	0.15	16.41	10.11	21.12	35.04
Average		9.05 (0.01)	1.01 (0.00)	0.17 (0.01)	16.33 (0.42)	11.01 (0.85)	22.98 (1.77)	37.96 (3.35)
	1	9.11	1.00	0.15	16.22	10.23	21.36	35.04
8.0	2	9.17	0.99	0.14	16.11	9.66	20.16	32.85
	3	9.13	1.00	0.13	15.58	8.66	18.07	28.47
Average		9.14 (0.03)	1.00 (0.01)	0.14 (0.01)	15.97 (0.34)	9.52 (0.80)	19.87 (1.67)	32.12 (3.35)
	1	9.22	0.99	0.12	15.66	7.95	16.59	26.28
8.5	2	9.18	1.00	0.12	14.99	8.30	17.34	26.28
	3	9.14	1.00	0.11	13.38	8.53	17.80	24.09
Average		9.18 (0.04)	1.00 (0.00)	0.11 (0.01)	14.68 (1.17)	8.26 (0.29)	17.25 (0.61)	25.55 (1.26)

APPENDIX B

Backward Extraction

A_E= Enzyme activity (Unit/ml)

Cp= Protein concentration (μg/ml)

SA= specific activity (Unit/mg)

PF= Purification fold (-)

RY= Recovery yield (%)

Table B1: KCl concentration (0.5 M).

pH	Replicates	A_E	Cp	SA	PF	RY
	1	0.160	15.03	10.65	22.24	34.08
6.5	2	0.170	15.20	11.21	23.40	36.24
	3	0.166	15.87	10.46	21.84	35.31
Average		0.165 (0.005)	15.37 (0.44)	10.77 (0.39)	22.49 (0.81)	35.21 (1.09)
	1	0.208	20.20	10.31	21.52	44.30
7.0	2	0.218	19.53	11.15	23.28	46.35
	3	0.207	19.03	10.88	22.72	44.06
Average		0.211 (0.006)	19.59 (0.59)	10.78 (0.43)	22.51 (0.90)	44.90 (1.26)
	1	0.238	19.53	12.20	25.46	50.68
7.5	2	0.250	20.20	12.39	25.87	53.26
	3	0.249	20.20	12.32	25.73	52.97
Average		0.246 (0.007)	19.98 (0.38)	12.30 (0.10)	25.69 (0.21)	52.31 (1.41)
	1	0.217	18.33	11.86	24.76	46.27
8.0	2	0.220	18.33	12.01	25.08	46.85
	3	0.211	18.10	11.68	24.39	44.99
Average		0.216 (0.004)	18.26 (0.13)	11.85 (0.16)	24.74 (0.34)	46.04 (0.95)
	1	0.190	17.17	11.07	23.12	40.45
8.5	2	0.187	17.53	10.65	22.23	39.73
	3	0.180	17.20	10.49	21.91	38.40
Average		0.186 (0.005)	17.30 (0.20)	10.74 (0.30)	22.42 (0.63)	39.53 (1.04)

Table B2: KCl concentration (1.0 M).

pH	Replicates	A_E	Cp	SA	PF	RY
	1	0.412	29.87	13.81	28.82	87.73
6.5	2	0.394	29.2	13.49	28.16	83.79
	3	0.409	30.03	13.62	28.43	87.02
Average		0.405 (0.010)	29.70 (0.44)	13.64 (0.16)	28.47 (0.33)	86.18 (2.10)
	1	0.457	31.87	14.33	29.93	97.19
7	2	0.446	30.44	14.65	30.59	94.91
	3	0.435	32.2	13.51	28.2	92.54
Average		0.446 (0.011)	31.50 (0.93)	14.17 (0.59)	29.57 (1.24)	94.88 (2.33)
	1	0.488	30.53	15.97	33.34	103.73
7.5	2	0.479	32.2	14.87	31.03	101.85
	3	0.475	30.67	15.5	32.36	101.13
Average		0.481 (0.006)	31.13 (0.93)	15.44 (0.55)	32.24 (1.15)	102.24 (1.35)
	1	0.372	30.22	12.32	25.72	79.22
8	2	0.391	31.2	12.53	26.15	83.15
	3	0.382	29.2	13.09	27.33	81.35
Average		0.382 (0.009)	30.21 (1.00)	12.65 (0.40)	26.40 (0.84)	81.24 (1.97)
	1	0.353	29.87	11.83	24.71	75.2
8.5	2	0.342	30.53	11.21	23.41	72.83
	3	0.331	28.53	11.61	24.23	70.47
Average		0.342 (0.011)	29.64 (1.02)	11.55(0.31)	24.12 (0.66)	72.83 (2.37)

Table B3: KCl concentration (1.5 M).

pH	Replicates	A_E	Cp	SA	PF	RY
	1	0.372	21.20	17.56	36.66	79.22
6.5	2	0.385	18.53	20.77	43.36	81.90
	3	0.386	21.20	18.19	37.98	82.06
Average		0.381 (0.008)	20.31 (1.54)	18.84 (1.70)	39.34 (3.55)	81.06 (1.60)
	1	0.436	21.20	20.55	42.90	92.70
7.0	2	0.424	21.20	19.99	41.74	90.17
	3	0.436	19.87	21.97	45.86	92.86
Average		0.432 (0.007)	20.76 (0.77)	20.84 (1.02)	43.50 (2.13)	91.91 (1.51)
	1	0.478	22.53	21.19	44.24	101.61
7.5	2	0.456	19.87	22.94	47.89	96.95
	3	0.470	22.53	20.87	43.56	100.04
Average		0.468 (0.011)	21.64 (1.54)	21.67 (1.11)	45.23 (2.32)	99.53 (2.37)
	1	0.333	18.20	18.32	38.24	70.92
8.0	2	0.342	16.87	20.30	42.37	72.83
	3	0.331	16.87	19.61	40.95	70.39
Average		0.335 (0.006)	17.31 (0.77)	19.41 (1.01)	40.52 (2.10)	71.38 (1.29)
	1	0.343	18.03	19.02	39.72	72.99
8.5	2	0.333	17.20	19.34	40.38	70.79
	3	0.321	16.87	19.02	39.71	68.26
Average		0.332 (0.011)	17.37 (0.60)	19.13 (0.18)	39.94 (0.39)	70.68 (2.37)

Table B4: KCl concentration (2.0 M).

pH	Replicates	A_E	Cp	SA	PF	RY
	1	0.193	16.00	12.07	25.20	41.08
6.5	2	0.187	16.87	11.07	23.11	39.73
	3	0.187	18.03	10.35	21.62	39.73
Average		0.189 (0.004)	16.97 (1.02)	11.16 (0.86)	23.31 (1.80)	40.18 (0.78)
	1	0.249	17.60	14.15	29.53	52.97
7.0	2	0.239	19.07	12.51	26.12	50.76
	3	0.237	17.60	13.44	28.05	50.32
Average		0.241 (0.007)	18.09 (0.85)	13.37 (0.82)	27.90 (1.71)	51.35 (1.42)
	1	0.259	18.53	13.99	29.21	55.18
7.5	2	0.249	18.53	13.43	28.04	52.97
	3	0.259	17.20	15.08	31.48	55.18
Average		0.256 (0.006)	18.09 (0.77)	14.17 (0.84)	29.58 (1.75)	54.44 (1.27)
	1	0.239	16.87	14.17	29.58	50.84
8.0	2	0.239	16.87	14.15	29.53	50.76
	3	0.228	18.20	12.54	26.18	48.56
Average		0.235 (0.006)	17.31 (0.77)	13.62 (0.93)	28.43 (1.95)	50.05 (1.30)
	1	0.207	17.53	11.83	24.70	44.14
8.5	2	0.197	17.20	11.46	23.92	41.94
	3	0.218	16.87	12.92	26.96	46.35
Average		0.207 (0.010)	17.20 (0.33)	12.07 (0.76)	25.20 (1.58)	44.14 (2.21)

APPENDIX C

Effect of Alcohol on Backward Extraction

TV=Total volume (ml)

A_E= Enzyme activity (Unit/ml)

TA= Total activity

Cp= Protein concentration (μg/ml)

SA= specific activity (Unit/mg)

PF= Purification fold (-)

RY= Recovery yield (%)

Table C1: Backward extraction with and without alcohol.

Parameter	Replicates	TV	A_E	TA	Cp	SA	PF	RY
	1	4.5	0.10	0.52	16.92	6.09	12.71	21.78
Without Alcohol	2	4.4	0.12	0.58	17.83	6.45	13.47	24.31
	3	4.3	0.10	0.52	18.05	5.71	11.91	21.78
Average		4.4 (0.1)	0.11 (0.01)	0.54 (0.03)	17.60 (0.60)	6.08 (0.37)	12.70 (0.78)	22.62 (1.46)
	1	5.0	0.48	2.39	30.77	15.53	32.43	101.06
With Alcohol	2	5.0	0.49	2.44	33.32	14.62	30.51	102.96
	3	5.0	0.47	2.33	31.52	14.78	30.86	98.52
Average		5.0 (0.0)	0.48 (0.01)	2.39 (0.05)	31.87 (1.31)	14.98 (0.49	31.27 (1.02)	100.85 (2.23)

APPENDIX D

Ammonium Sulphate Method

A_E= Enzyme activity (Unit/ml)

TA= Total activity

Cp= Protein concentration (μg/ml)

SA= specific activity (Unit/mg)

PF= Purification fold (-)

RY= Recovery yield (%)

Table D1: Purification of crude extract by using ammonium sulphate method.

Method	Replicates	A_E	TA	Cp	SA	PF	RY
	1	0.22	1.10	42.55	5.17	10.79	46.51
Ammonium Sulphate	2	0.21	1.06	40.33	5.23	10.92	44.61
	3	0.23	1.15	42.04	5.52	11.52	49.05
Average		0.22 (0.01)	1.10 (0.05)	41.64 (1.16)	5.31 (0.19)	11.08 (0.39)	46.72 (2.23)