PARTITION OF PEPSINOGEN FROM THE STOMACH OF RED PERCH (*SEBASTES MARINUS*) BY AQUEOUS TWO PHASE SYSTEMS

by

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for the degree of Master of Science

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The undersigned hereby certify that they have read and recommend to the Faculty of Graduate Studies for acceptance a thesis entitled "PARTITION OF PEPSINOGEN FROM THE STOMACH OF RED PERCH (*SEBASTES MARINUS*) BY AQUEOUS TWO PHASE SYSTEMS" by Lisha Zhao in partial fulfilment of the requirements for the degree of Master of Science.

Dated: November 29, 2011

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Signature of Author

DEDICATION

TO:

My supervisors:
Dr. Abdel Ghaly and Dr. Suzanne Budge

My parents:
Nana Qi and Jichao Zhao

My friends:
Lin Lu, Juan Li, Shengnan Xu,
Yannan Huang and Xiao Chen

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LIST OF ABBREVIATIONS AND SYMBOLS USED

A_E	Enzyme activity
ANOVA	Analysis of variance
ASF	Ammonium sulfate fractionation
ASP	Acid-solublization process
ATPS	Aqueous two phase system
BSA	Bovine serum albumin
C	Salt (PEG) concentration
C_P	Protein content
CV	Coefficient of Variation
DF	Degree of freedom
E-64	L-3-carboxy-trans-2,3-epoxy-propionyl-L-leucin-4-guanidinobutylamide
GFC	Gel filtration chromatography
IEC	Ion exchange chromatography
K*i*	Dissociation constant of enzyme-inhibitor complex
K_P	Partition coefficient
M	PEG molecular weight
MC	Interaction of PEG molecular weight and concentration
MS	Mean of squares
Native PAGE	Native polyacrylamide gel electrophoresis
OC	Optimal concentration of the best salt
PEG	Polyethylene glycol
PF	Purification fold
PG	Pepsinogen
PMSF	Phenylmethylsulfonyl fluoride
RY	Recovery yield
SA	Specific activity
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SS	Sum of squares
T	Salt type
TC	Interaction of salt type and concentration
TCA	Trichloroacetic acid
TV	Total volume
V_R	Volume ratio

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ABSTRACT

The purification of pepsinogen from the stomach of red perch using aqueous two phase systems (ATPS) formed by polyethylene glycol (PEG) and salt at 4°C was optimized. Salt type, salt concentration, PEG molecular weight and PEG concentration had significant effects on total volume (TV), volume ratio (V_R), enzyme activity (A_E), protein content (C_P), specific activity (SA), purification fold (PF) and recovery yield (RY). $(NH_4)_2SO_4$ at 15% w/w concentration was selected as the optimum salt type and concentration. PEG 1500 at 18% w/w concentration was selected as the optimum PEG molecular weight and concentration. 15% $(NH_4)_2SO_4$−18% PEG 1500, the optimal ATPS, was compared with ammonium sulfate fractionation (ASF). ATPS gave better partition of pepsinogen (SA of 5.40 U/mg, PF of 5.20 and RY of 86.6%) than ASF (SA of 2.55 U/mg, PF of 2.46, RY of 70.4%).

CHAPTER 1. INTRODUCTION

The increasing consumption of fish has led to a thriving fish processing industry worldwide. With greater production of fish and fish products, a large fraction (30–80%) of fish (flesh, heads, bones, fins, skin, tails and viscera) is generated as waste. Fish wastes are usually disposed of in landfills or poured directly into the sea, which results in high disposal cost and causes environmental problems.

Conventional disposal of fish wastes underscores the need for a more reasonable utilization approach of fish wastes as well as effective recovery of valuable ingredients from these wastes. Fish wastes can be utilized as animal feed ingredients as well as organic fertilizers (Gildberg and Raa, 1977; El-Beltagy et al., 2004). The recovery of valuable biomolecules such as collagen (Nagai and Suzuki, 2000; Ogawa et al., 2004), ω-3 fatty acids (Yoshida et al., 1999), trypsin (Genicot et al., 1996; Klomklao et al., 2006), chymotrypsin (Castillo-Yáñezet al., 2006) and elastase (Gildberg and Overbo, 1990) have also been reported. Among the valuable products that can be recovered from fish, pepsin is one of the abundant and useful biomolecules that can be effectively recovered from fish viscera.

Pepsin is an important acidic protease widely applied in the hydrolysis of proteins in the food and manufacturing industries. It can be used in collagen extraction (Jongjareonrak et al., 2005; Zhang et al., 2007; Nalinanon et al., 2007), in gelatin extraction (Nalinanon et al., 2008), in cheese making (Aehle, 2007) and in regulating digestibility (Thorne Research, 2010). Pepsins recovered from fish viscera not only significantly reduce the capital costs of enzyme production, but also partially reduce the cost of disposal of fish wastes and minimize environmental pressures associated with it.

Pepsin as well as its zymogen, pepsinogen (PG), was widely purified from several fish species including arctic fish capelin (*Mallotus villosus*) (Gildberg and Raa, 1983), rainbow trout (*Salmo gairdneri*) (Twining et al., 1983), Atlantic cod (*Gadus morhua*) (Gildberg, 2004), bolti fish (*Tilapia nilotica*) (El-Beltagy et al., 2004), Antarctic rock cod (*Trematomus bernacchii*) (Brier et al., 2007), sea bream (*Sparuslatus Houttuyn*) (Zhou et al., 2007), African coelacanth (*Latimeria chalumnae*) (Tanji et al., 2007), Mandarin fish (*Siniperca chuatsi*) (Zhou et al., 2008), smooth hound (*Mustelus mustelus*) (Bougatef et

al., 2008), orange-spotted grouper (*Epinephelus coioides*) (Feng et al., 2008), albacore tuna (*Thunnus alalunga*) (Nalinanon et al., 2009) and European eel (*Anguilla anguilla*) (Wu et al., 2009). During the purification of proteases, several conventional techniques such as ammonium sulfate fractionation (ASF), gel filtration chromatography (GFC) and ion exchange chromatography (IEC) were frequently performed. Conventional purification methods can give good enzyme purity but are very complex, time-consuming and expensive. Therefore, an innovative, efficient and economical method for the purification of pepsin and PG that gives both high yield and high purity is in demand. Recently, ATPS has been established as an effective pathway to purify proteins but its efficacy and feasibility for pepsin and PG purification is less investigated. The aim of this study was to evaluate the effectiveness of PG purification using the ATPS method with the goal of optimizing it to achieve highest yield of PG recovered from the stomachs of red perch (*Sebastes marinus*).

CHAPTER 2. OBJECTIVES

The aim of this study was to purify PG from the stomach of red perch (*Sebastes marinus*) using ATPS. The specific objectives were:

1. To optimize the ATPS purification of PG from the stomach of red perch (*Sebastes marinus*) using polyethylene glycol (PEG)-salt combinations
   - investigate the effects of salt type and concentration on the total volume (V_T), volume ratio (V_R), specific activity (SA), purification fold (PF), partition coefficient (K_p) and recover yield (RY).
   - investigate the effects of PEG molecular weight and concentration on the V_T, V_R, SA, PF, K_P and RY.
   - determine the optimal PEG-salt combination for PG purification on the basis of SA, PF and RY.

2. To compare the efficiency of ATPS and ASF methods in purifying PG on the basis of SA, PF and RY.

CHAPTER 3. LITERATURE REVIEW

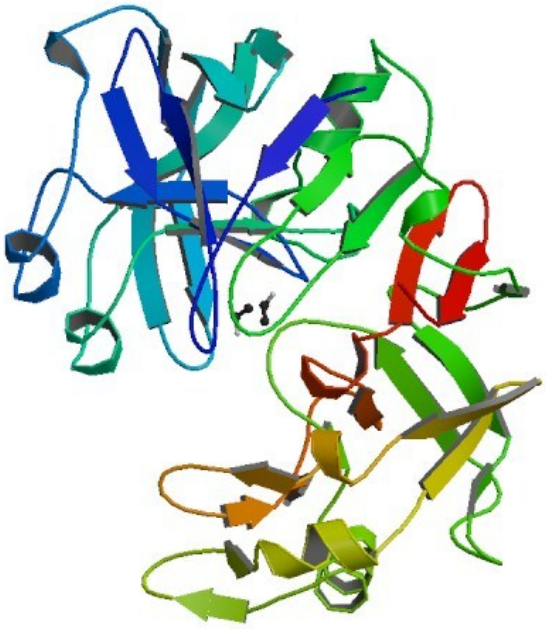
3.1 Definition and History of Pepsinogen (PG) and Pepsin

Pepsin, with approximate molecular mass of 36 kDa, is an enzyme in the aspartic protease family which helps in the digestion of proteins in animals (Haard and Simpson, 2000). As an active enzyme, pepsin can be found primarily in the gastric juice of the stomach lumen (Effront et al., 2007). With this protease, peptide bonds can be readily cleaved and ingested proteins can be degraded and peptonized under acidic conditions (Haard and Simpson, 2000).

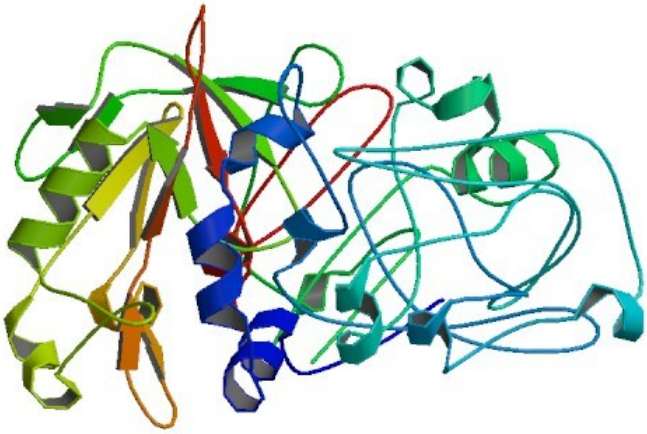
Pepsin is synthesized and secreted by cells in the gastric membrane (mucosa of stomach) in an inactive state called PG. PG is the proenzyme of pepsin with an approximate molecular weight of 40 kDa. Compared with its active state, PG contains an additional 44 amino acids as an inhibitory section at the N-terminal location and is stable in neutral and alkaline environment (Raufman, 2004). The structure of PG and the structure of pepsin show great similarity (Figure 3.1). When exposed to the hydrochloric acid present in gastric juice, PG is activated and the 44 amino acids are proteolytically removed in an autocatalytic way to produce pepsin (Raufman, 2004). The newly created pepsin can also help the cleavage of PG to generate more pepsin (Moffatt, 1997).

Under neutral conditions, pepsin and PG are both quite stable and maintain their natural molecular structures. Under acidic conditions (pH range of 1.5 ~ 2.5) PG is transformed to pepsin. Under alkaline conditions, pepsin's structure is easily destroyed and its activity is lost.

Pepsin's main role in protein proteolysis is to cleave aromatic amino acids, such as phenylalanine and tyrosine, from the N-terminus of proteins (Raufman, 2004). The effect of stomach juice in the process of digestion was first noticed by Réamur in 1752 (Effront et al., 2007). At about the same time, Spallanzani discovered the ability of gastric juices to digest meat, which laid an important foundation for the study of pepsin (Effront et al., 2007). In 1836, pepsin was first discovered by Theodor Schwann, who named it with the word "pepsin" which means "to digest". In 1930, crystallized pepsin was isolated by John H. Northrop and identified as protein for the first time (Aehle, 2007). Since then, pepsins from different animals, especially mammals, have been investigated



(1) Pepsin



(2) PG

Figure 3.1: Three-dimensional structure of porcine pepsin and PG (Protein Databank, 2010; Hartsuck et al., 1992).

extensively. Characteristics and properties of pepsin are still being discovered and new methods for analysis of pepsin are being developed.

3.2 Classification of Pepsin and PG

The classification of pepsin and PG depends primarily on the characteristics and properties of the enzymes. The important characteristics include: (a) activity, which can be influenced by pH, temperature and inhibitors, (b) stability and (c) kinetics (Benjakulet al., 2009; El-Beltagy et al., 2004; Luca et al., 2009). As a member of the aspartic family, pepsin has several special properties including: (a) aspartate residues for catalysis of substrates, (b) protein degradation at acidic pH and (c) a typical tertiary structure (Foltmann, 1981; Cooper et al., 1990; Davies, 1990; Dunn and Corbett, 1992). In mammals, these properties have been investigated extensively, which contribute to a definite classification (Kageyama, 2002). Mammalian PG is divided into two groups: the major group, including PG-A, PG-C (or progastricsin) and PG-Y (or prochymosin), and the minor group including PG-B and PG-F (Foltmann, 1981; Kageyama et al., 1990; Kageyama, 2002; Tanji et al., 2009; Kageyama et al., 2007). The corresponding pepsin for each type of mammalian PG is also expressed and labeled with letter A to F.

In contrast, the classifications of fish pepsin and PG vary among researchers. Some researchers followed the category of mammal PG and pepsins and designated the fish PG as PG-A, PG-B and PG-C and its active form as Pepsin A, Pepsin B, Pepsin C and Pepsin D (Klomklao et al., 2007; Gildberg, 1990; Feng et al., 2008; Gilberg and Raa, 1983). Other literature shows that there are up to four types of pepsins in fishes (Pepsin I, Pepsin II, Pepsin III and Pepsin IV), derived from the conversion of different PG (PG-I, PG-II, PG- III and PG-IV), and every type has its own characteristics and enzymatic properties (Shahidi and Kamil, 2001; Tanji et al., 2007; Bougatef et al., 2008; Zhou et al., 2007; Wu et al., 2009). Compared to those in mammals, PG and pepsins in fish have some distinct characteristics including: (a) less acidity (Norris and Mathies, 1953), (b) higher specific activity (Norris and Mathies, 1953), (c) low optimum temperature (Simpson and Haard, 1987) and (d) higher heat sensitivity (Martinez and Olsen, 1989).

3.3 Sources of Pepsin

Pepsin can be found in both invertebrate and vertebrate animals. In mammals (such as cattle, sheep and pig), the main location is the stomach, including both the membrane and the gastric juice (Effront et al., 2007). PG is mainly synthesized in the stomach mucosa. Usually, there are distinct types of pepsins and PG in a mammal's stomach. For instance, there are four PG and four corresponding pepsins detected in the body of Japanese monkey while there are six different ones in the rabbit; every isoform of pepsin has distinct protein structures as well as enzymatic properties (Kageyama, 2006). Apart from the stomach, pepsin can also be found in limited amounts in the blood, muscle and the urine (Effront et al., 2007).

In fish, most of the pepsins and PG are present in the stomach. Pepsin can also be found in ovaries (brook trout pepsin) and skin (pufferfish pepsin) of some fish species (Bobe and Goetz, 2001; Kurokawa et al., 2005). Fish without stomachs (such as carp) have a totally different mechanism of digestion and contain no pepsin in the body. Pepsin and PG have been characterized in various fish species including: arctic fish capelin (Gildberg and Raa, 1983), rainbow trout (Twining et al., 1983), Atlantic cod (Gildberg, 2004), bolti fish (El-Beltagy et al., 2004), Antarctic rock cod (Brier et al., 2007), sea bream (Zhou et al., 2007), African coelacanth (Tanji et al., 2007), Mandarin fish (Zhou et al., 2008), smooth hound (Bougatef et al., 2008), orange-spotted grouper (Feng et al., 2008), albacore tuna (Nalinanon et al., 2009) and European eel (Wu et al., 2009).

Different fishes have different amounts of PG and pepsins and some have more than one type of pepsin. Bougatef et al. (2008) isolated only one type of PG in the stomach of smooth hound (7.33mg/100g stomach). Gildberg and Raa (1983) successfully isolated two PG in the arctic fish capelin with amounts of 13.9 and 0.5 mg per 100 g gastric membranes. Tanji et al. (2007) found three types of PG (PG-I, PG-II and PG-III), which corresponded to three different types of pepsins, in African coelacanth stomach. Their amounts were 12.1, 8.57 and 16.7 mg per 100 g stomach mucosa, respectively. Wu et al. (2009) found three kinds of PG (PG-I, PG-II and PG- III) in the stomach of European eel with a total amount (of all three) of only 3.63 mg per 100 g stomach. Sea bream stomachs contained four types of PG with a total amount of 35.5 mg/100 g stomach (Zhou et al., 2007). The mucosa of mandarin fish contained 4 PG with a total

amount of 36.6 mg/100 g (Zhou et al. 2008).Among all the fish, pepsins and PG from cod and tuna were the most investigated.

Cod have been studied and sequenced by several researchers (Brewer et al., 1984; Gildberg et al., 1990; Karlsen et al., 1998). As a cold water fish, cod has pepsins and PG which are more active at low temperature than those in warm water fishes, which can be particularly useful in food processing (Gidlberg, 2004). Bjellandet al. (1988) stated that there are two different types of pepsins Pepsin I and Pepsin II contained in cod stomach. Pepsin I functions under relatively weak acidic condition (pH=4.0) while Pepsin II is similar to mammals' pepsin and is more active in strong acidic environment (pH=2.0). These two forms are quite similar to mammalian pepsins in structure, but they are more active at low temperatures and weak acid conditions and more easily inactivated by moderate heating (Martinez and Olsen, 1989). Brier et al. (2007) reported that these two types of pepsins and PG have greater sensitivity to inhibitors such as pepstatin.

Albacore tuna have only one type of PG and pepsin in their body (Nalinanon et al., 2009). This pepsin had similar properties to those found in tropical fishes (Nalinanon et al., 2009). Tongol tuna contains two isoforms of pepsin while only one type of pepsin was found in skipjack tuna stomach (Nalinanon et al., 2008). Pepsin in tuna has a different molecular weight and an active temperature than those of bovine pepsin (Nalinanon et al., 2009). There are three PG (PG-I, PG-II and PG-III) detected in pacific blue fin tuna (Tanji, 2009), which contain a greater number of basic residues than mammals' PG (Tanji et al., 1988). These three pepsins in blue fin tuna have been found most active at pH 2.5 and two of them (Pepsin I and II) can be inhibited greatly by pepstatin A (Tanji et al., 1988). Pepsins in blue fin tuna have unique properties in enzymatic activity which are quite different from cod or porcine pepsin (Tanji et al., 1988; Tanji, 2009). Pepsin from yellow fin tuna hasan optimum temperature of 45°C and an optimum pH of 2.5. Its crystal structure, specificity, alkaline stability and other properties are different than swine pepsins (Norris and Mathies, 1953; Northrop et al., 1948).

3.4 Characteristics and Properties of Pepsin

The activity of pepsin which catalytically hydrolyzes proteins can be influenced by three parameters: pH, temperature and inhibitor. Each parameter has a distinctive

influence on the activity of fish pepsin.

3.4.1 Effect of pH on the Pepsin Activity

The pH has a significant effect on the activity of fish pepsin. Both the optimum pH (the pH value giving the highest enzymatic activity) and pH stability (the pH range in which good enzyme stability is shown) are very important.

Pepsin has a characteristic pH value at which it displays highest enzymatic activity. When the pH deviates from the optimum value, the activity of pepsin drops. Figure 3.2 shows that Pepsins I and II from bolti fish have the same optimum value of pH (2.5) and deviation from that pH value results in reduced activity (El-Beltagy et al., 2004). Mammals' pepsins are usually most active at pH of 1.5 – 2.0 while most fish pepsins show high activity in less acidic conditions (Gildberg, 1988; Gildberg, 2004). A summary of optimum pH values for different fishes is displayed in Table 3.1. Pepsin from smooth hound shows optimum activity at pH 2.0 while pepsins from sea bream are most active at pH 3.7 (Bougatef et al., 2008; Zhou et al., 2007). In general, if the fish has more than one type of pepsin, their optimum pH will be similar. However, this fact does not apply to some the fishes such as Arctic capelin.

The effect of pH on the pepsin stability is shown in Figure 3.3 (Klomklao et al., 2007). The activity of both pepsins dropped dramatically when pH exceeded 6. Similar results were prepared for most fish pepsins (Xu et al., 1996; Castillo-Yanez et al., 2004; Gildberg, 1988; Kubota and Ohnuma, 1970). The depression of fish pepsin stability was attributed to the denaturation of proteins. Since pepsin is a type of acidic protease, its protein structures are transformed readily under neutral or alkaline condition (Castillo-Yanez et al., 2004). A summary of pH ranges at which pepsin is stable is shown in Table 3.2.

3.4.2 Effect of Temperature on the Pepsin Activity

Temperature has a great influence on the activity of fish pepsin. The optimum temperature (the temperature giving the highest enzymatic activity) and thermal stability (the temperature range in which good enzyme stability is shown) are very important.

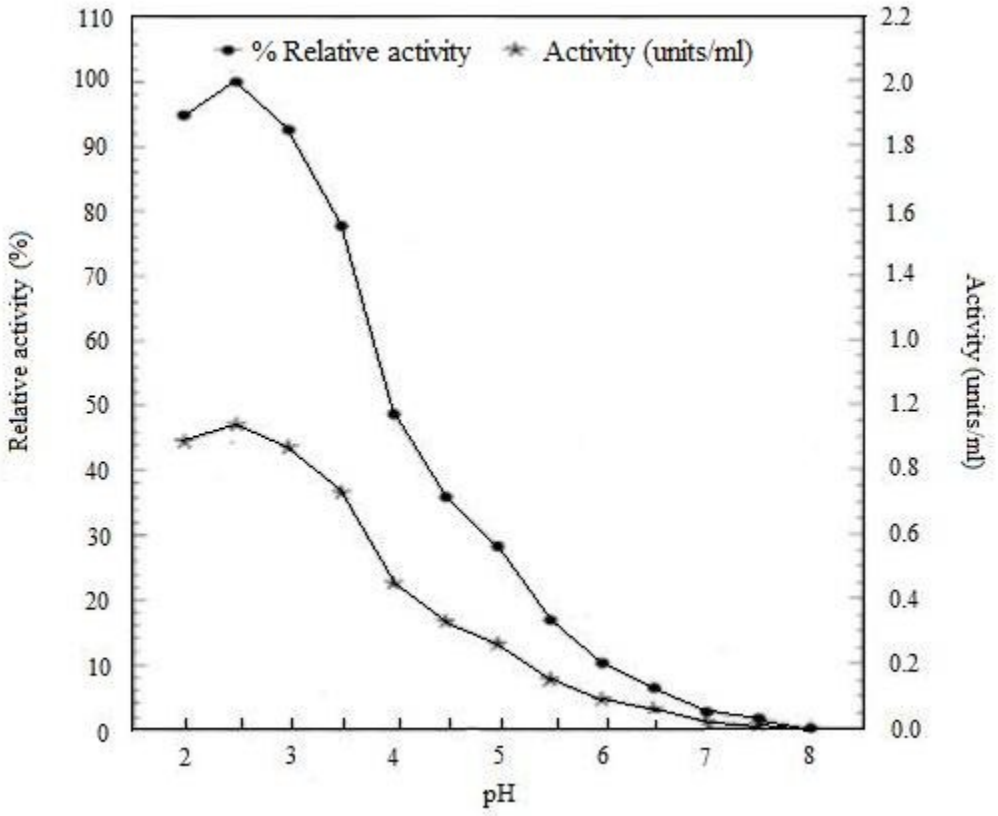


Figure 3.2: The effect of pH on the activity of Pepsins I and II from the freshwater bolti fish (*Tilapia nilotica*). (El-Beltagy et al., 2004).

Table 3.1: The optimum pH for activity of pepsin from different fish species.

Enzyme	Identified Species	Optimum pH	Reference
Pepsin	Smooth hound	2.0	Bougatef et al. (2008)
Pepsin I	Arctic capelin	3.7	Gildberg and Raa (1981)
Pepsin II	Arctic capelin	2.5	Gildberg and Raa (1981)
Pepsin I	European eel	3.5	Wu et al. (2009)
Pepsin II	European eel	2.5	Wu et al. (2009)
Pepsin III	European eel	2.5	Wu et al. (2009)
Pepsin	Greenland cod	3.5	Squires et al. (1986)
Pepsin	Greenland cod	3.0-3.5	Haard et al. (1982)
Pepsin	Arctic cod	3.0-3.5	Haard et al. (1982)
Pepsin I	Atlantic cod	3.5	Gildberg et al. (2004)
Pepsin II	Atlantic cod	3.0	Martinez and Olsen (1989)
Pepsin	palometa	3.0-3.5	Pavlisko et al. (1997)
Pepsin I	Sea bream	3.0	Zhou et al. (2007)
Pepsin II	Sea bream	3.5	Zhou et al. (2007)
Pepsin III	Sea bream	3.5	Zhou et al. (2007)
Pepsin IV	Sea bream	3.5	Zhou et al. (2007)
Pepsin	Albacore tuna	2.0	Nalinanon et al. (2009)
Pepsin I	Mandarin fish	3.5	Zhou et al. (2008)
Pepsin II	Mandarin fish	3.5	Zhou et al. (2008)
Pepsin III	Mandarin fish	3.5	Zhou et al. (2008)
Pepsin IV	Mandarin fish	3.5	Zhou et al. (2008)
Pepsin I	African coelacanth	2.0	Tanji et al. (2007)
Pepsin II	African coelacanth	2.0	Tanji et al. (2007)
Pepsin III	African coelacanth	2.5	Tanji et al. (2007)

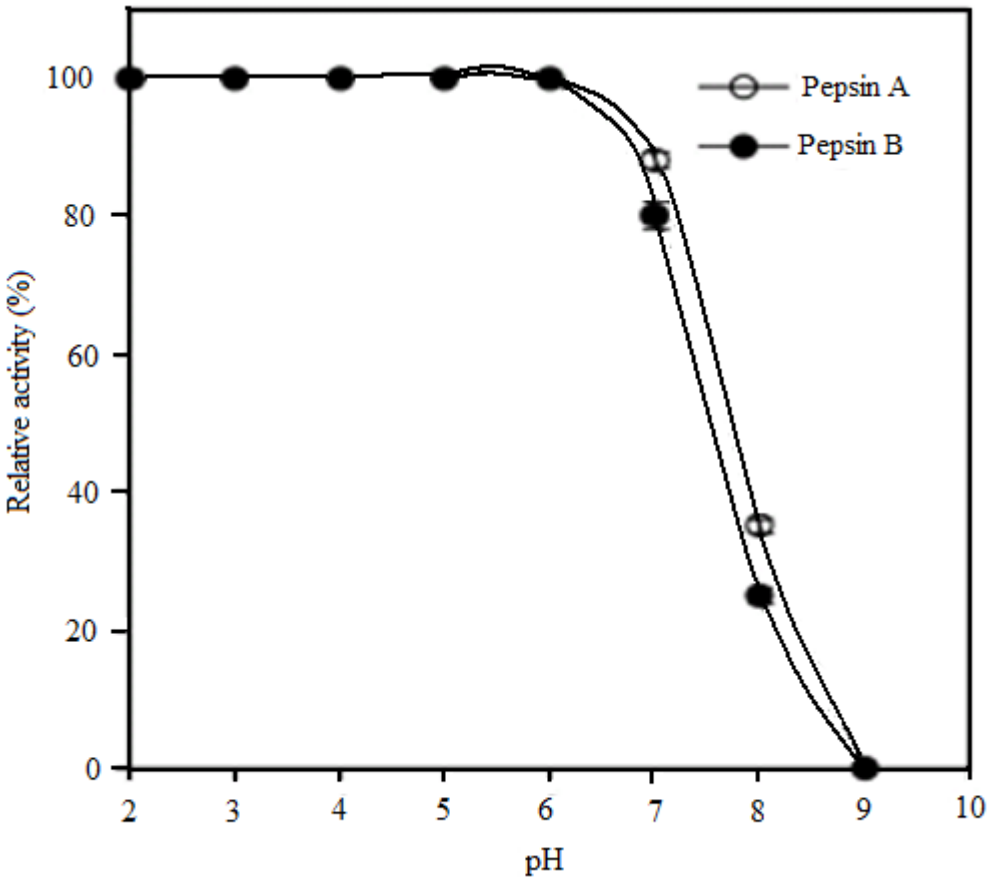


Figure 3.3: The pH stability of pepsins from the northern Pacific Ocean pectoral rattail (*Coryphaenoides pectoralis*). (Klomklao et al., 2007).

Table 3.2: The pH range of stable fish pepsin.

Enzyme	Identified Species	pH range	Activity range (%)	Reference
Pepsin	Albacore tuna	2~5	100~85	Nalinanon et al. (2009)
Pepsin	Orange roughy	2~6	≈ 100	Xu et al. (1996)
Pepsin	Sardine	2~6	/	Noda and Murakami (1981)
Pepsin	Monterey sardine	3~6	120~95	Yanez et al.(2004)
Pepsin I	capelin	2~5	/	Gildberg and Raa (1983).
Pepsin II	capelin	2~5	/	Gildberg and Raa (1983).
Pepsin I	Antarctic rock cod	1~4	105~75	Brier et al. (2007)
Pepsin II	Antarctic rock cod	1~2.5	115~100	Brier et al. (2007)
Pepsin A	pectoral rattail	2~6	100~98	Klomklao et al. (2007)
Pepsin B	pectoral rattail	2~6	100~98	Klomklao et al. (2007)
Pepsin I	European eel	2~4	100~75	Wu et al. (2009)
Pepsin II	European eel	1.5~4	100~80	Wu et al. (2009)
Pepsin III	European eel	2~3	100~80	Wu et al. (2009)

Pepsin has an optimum temperature at which it displays highest enzymatic activity. When the temperature deviates from the optimum value, the activity of pepsin drops. Figure 3.4 shows that pepsin from monastery sardine has the greatest activity at about 45°C, which drops significantly when reaching 60°C. The optimum temperature of fish pepsin depends greatly on fish species and fish habitat (such as cold or warm water species) (Gilderg, 1988; Pavlisko et al., 1997; Shahidi and Kamil, 2001). Fishes from cold water habitats were found to have lower optimum temperature than those from warm aquatic environments (Bjelland et al., 1988; Noda and Murakami, 1981; Squires et al., 1986; Chiang et al., 1987). For example, the warm species sardine has a relatively high temperature optima (40°C and 55°C) for two pepsins, Pepsin I and Pepsin II, while pepsins from arctic capelin (cold water species) shows greatest activity at 38 and 43°C, respectively (Noda and Murakami, 1981; Gildberg and Raa, 1983). A summary of optimum temperatures for fish pepsin is given in Table 3.3. The optimum temperature of fish pepsin ranges from 30°C to 55°C. Every type of pepsin corresponded to unique thermal properties and enzymes from cold water species had a relatively lower optimum temperature. Cold water fish enzymes have a low Arrhenius activation energy, explaining their low optimal temperature and high heat liability compared with warm water counterparts (Simpson and Haard, 1987). Genes in different fish species also account for the diversity of the enzymic characteristics (Haard, 1992).

The effect of temperature on pepsin stability is shown in Figure 3.5. Pepsin from warm water albacore tuna retained its stability within the temperature range of 20~50°C (80% activity left at 50°C after which the stability decreased significantly). The pepsins from other warm water species such as dogfish, pectoral rattail and smooth hound have good stability below the temperature upper limit 50°C, 40°C and 50°C, respectively (Guerard and Le Gal, 1987; Klomklao et al, 2007; Bougatef et al., 2008). Pepsin from cold water species is quite susceptible to higher temperature (Tanji et al., 2007; Twining et al., 1983; Gildberg, 2004; Brier et al., 2007) and the sharp decrease of thermal stability was attributed to the destruction of structure and denaturation of pepsin (Haard, 1988; Nalinanon et al., 2009).

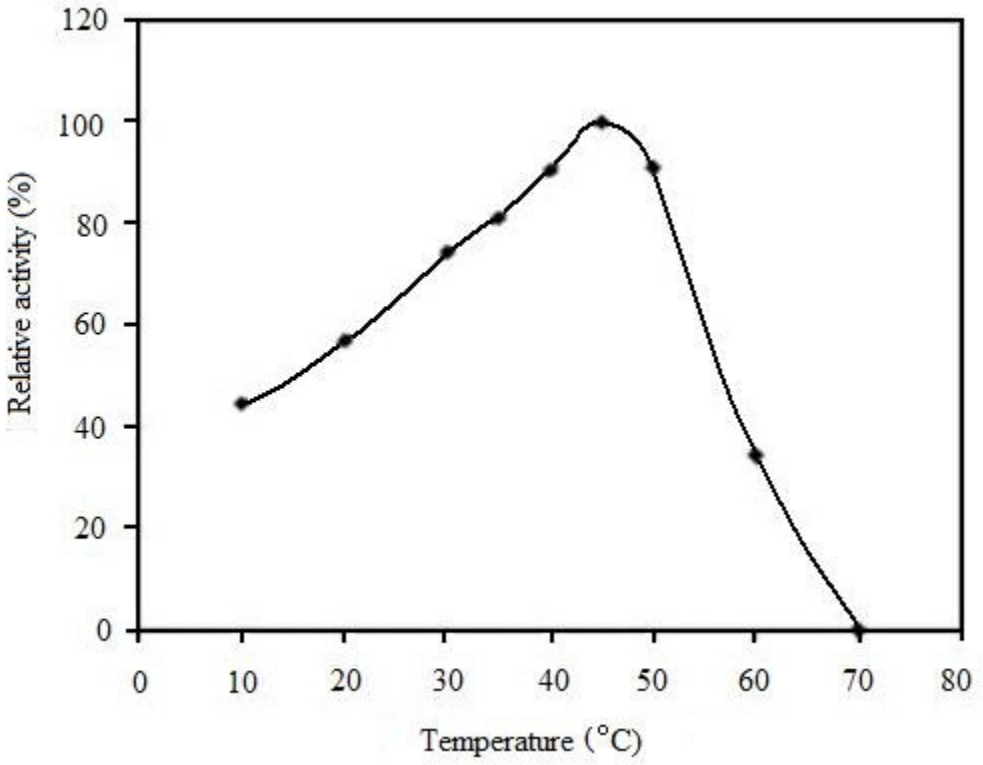


Figure 3.4: The effect of temperature on pepsin activity from Monterey sardine (*Sardinops sagax caerulea*). (Castillo-Yaneza et al., 2009).

Table 3.3: The optimum temperature for pepsin activity from different fishes.

Enzyme	Identified Species	Habitat	Optimum Temperature (°C)	Reference
Pepsin I	Sardine	Warm water	55	Noda and Murakami (1981)
Pepsin II	Sardine	Warm water	40	Noda and Murakami (1981)
Pepsin I	Arctic capelin	Cold water	38	Gildberg and Raa (1981)
Pepsin II	Arctic capelin	Cold water	43	Gildberg and Raa (1981)
Pepsin I	European eel	Warm water	40	Wu et al. (2009)
Pepsin II	European eel	Warm water	40	Wu et al. (2009)
Pepsin III	European eel	Warm water	35	Wu et al. (2009)
Pepsin	Greenland cod	Cold water	30	Squires et al. (1986)
Pepsin	Arctic cod	Cold water	32	Haard et al. (1982)
Pepsin	Polar cod	Cold water	37	Arunchalam and Haard (1985)
Pepsin I	Atlantic cod	Cold water	40	Gildberg et al. (2004)
Pepsin II	Atlantic cod	Cold water	40	Martinez and Olsen (1989)
Pepsin	Palometa	Warm water	37	Pavlisko et al. (1997)
Pepsin I	Sea bream	Warm water[a]	45	Zhou et al. (2007)
Pepsin II	Sea bream	Warm water	50	Zhou et al. (2007)
Pepsin III	Sea bream	Warm water	50	Zhou et al. (2007)
Pepsin IV	Sea bream	Warm water	50	Zhou et al. (2007)
Pepsin	Albacore tuna	Warm water	50	Nalinanon et al. (2009)
Pepsin I	Mandarin fish	Warm water	40	Zhou et al. (2008)
Pepsin II	Mandarin fish	Warm water	45	Zhou et al. (2008)
Pepsin III	Mandarin fish	Warm water	40	Zhou et al. (2008)
Pepsin IV	Mandarin fish	Warm water	45	Zhou et al. (2008)
Pepsin I	Pectoral rattail	Warm water	45	Klomklao et al. (2007)
Pepsin II	Pectoral rattail	Warm water	45	Klomklao et al. (2007)

a. Sea bream can be found in all types of water temperatures, depending on the species. This type of sea bream refers to *Sparuslatus Houttuyn*, a warm-water species.

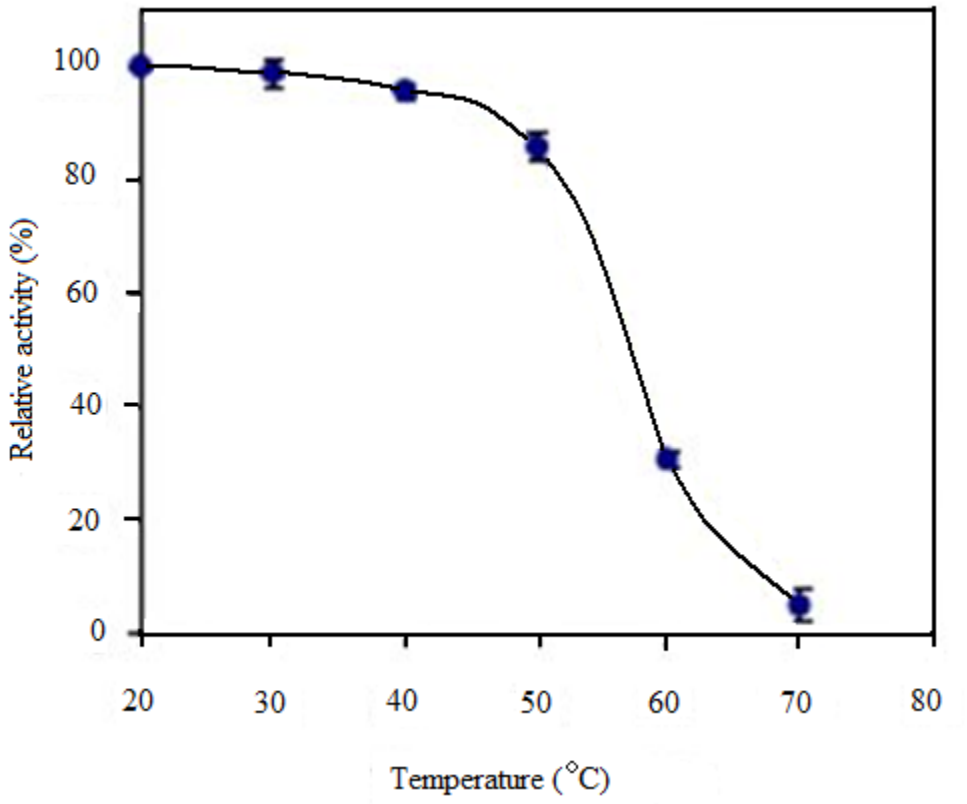


Figure 3.5: The thermal stability of pepsin from albacore tuna. (Nalinanon et al., 2009).

3.4.3 Effect of Inhibitors on the Pepsin Activity

Fish pepsin can combine with pepstatin A, a typical aspartic proteinase inhibitor, which can prevent the binding between enzyme and substrate, resulting in a complete inhibition of its activity (Davis, 1990; Athauda et al., 2004; Copeland, 2005; Zhou et al., 2007; Wu et al., 2009). Pepstatin A is a peptide isolated from cultures of several species of actinomyces such as Streptomyces spp. (Cammack et al., 2006). As a very specific inhibitor, pepstatin A has one of the lowest known inhibition constants, the dissociation constant of the enzyme-inhibitor complex (K*i*) for pepsin (45 pM) (Zollner, 1993; Copeland, 2005). The inhibitory effect on sea bream fish pepsin is displayed in Figure 3.6. The pepstatin was in a complex with Pepsin-I, Pepsin-III, and Pepsin-IV at a ratio of 1:1 but with Pepsin-II in a ratio of 2:1.

This molar ratio of pepstatin: pepsin plays an essential role in the inhibition. A ratio of about 1:1 gives an entire inhibition of Pepsins III and IV from mandarin fish while a ratio of 10:1 was necessary to inhibit Pepsins I and II. Pepsin from smooth hound was found to be completely inhibited with a molar ratio of 16:1 (Bougatef et al., 2008). Molar ratios of pepstatin: pepsin for bluefin tuna (*Thunnus thynuus orientalis*), two pepsins from bullfrog and two pepsins from Antarctic rock cod were found to be 17:1, 17:1 and 1:1, respectively (Tanji et al., 1988; Yakabe et al., 1991; Brier et al., 2007).

Not every protease inhibitor has the same effect on pepsin. It has been shown that typical inhibitors such as phenylmethylsulfonyl fluoride (PMSF) (serine proteinases inhibitor), L-3-carboxy-trans-2, 3- epoxy-propionyl-L-leucin-4-guanidino-butylamide (E-64) (cysteine proteinases inhibitor) and EDTA do not have any effect of inhibition on pepsins (Bougatef et al., 2008; Zhou et al. 2007; Wu et al., 2008; Yanez et al., 2004; Klomklao et al., 2007). In addition to protease inhibitors, some potentially inhibitory chemicals were investigated for their influence on the activity of fish pepsin. The chemical SDS (sodium dodecyl sulfate) at 0.05% – 0.10% (w/v) had a strong inhibitory effect on the activity of pepsins from albacore, skipjack and tongol tuna. Cysteine (5 – 50 mM) also showed inhibitory effects while ATP, molybdate, NaCl, $MgCl_2$, and $CaCl_2$ did not have any impact (Nalinanon et al., 2008). Aliphatic alcohols (methanol, ethanol, amylalcohol) have been found to competitively inhibit the activity of pepsin at low concentrations. For instance, the inhibition of pepsin by isoamyl alcohol occurs at

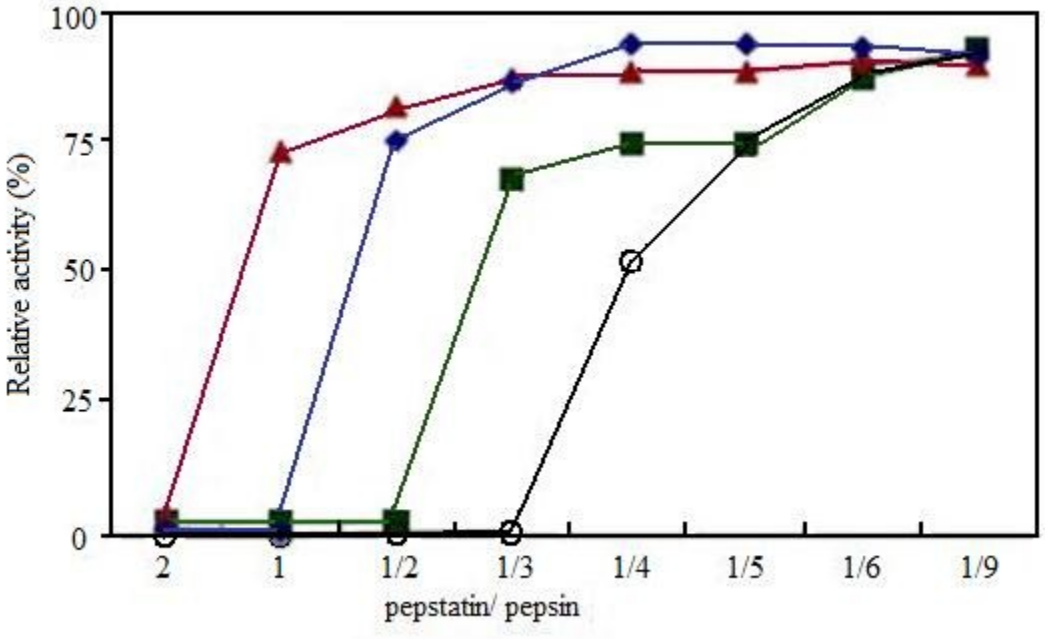


Figure 3.6: The inhibitory effect of pepstatin on pepsin from sea bream (*Sparuslatus Houttuyn*). Pepsin-I (◊); Pepsin-II (▲); Pepsin-III (■) and Pepsin-IV (○). (Zhou et al., 2007).

concentration less than 0.09 M (Tang, 1965). With the size of the hydrocarbon group of the alcohols increasing, the inhibitory activity increases (K*i* is lowered) (Tang, 1965). However, dialysis can completely remove this inhibition effect.

3.5 Industrial Applications of Pepsin

3.5.1 Collagen Extraction

Fish pepsin has been applied in collagen extraction and was regarded as a promising enzyme (Benjakul et al., 2009). Usually, collagen is extracted by the acid-solublization process (ASP), in which pepsin assistance brings higher collagen yield (Jongjareonrak et al., 2005; Nalinanon et al., 2007; Benjakul et al., 2009). Compared with the collagen yield of 5.31% from bigeye snapper skin using ASP, a yield of 18.74% was obtained by addition of pepsin (Nalinanon et al., 2007). By using abundant fish stomach as a source of fish pepsin to produce collagen, the cost of pepsin and collagen production can be reduced significantly.

3.5.2 Rennet Substitutes in Cheese–making

Pepsin can be applied as a rennet ingredient for the production of good quality cheese. There are two types of commercial coagulants available in cheese processing: animal rennet and microbial coagulants (Aehle, 2007). Pepsin mixed with chymosin in a standardized ratio forms the animal rennet. Until now, pepsins from calf, bovine and porcine stomach membranes have had limited use in commercial purpose. Cod pepsin and tuna pepsin have been proved to be feasible in cheese production (Han, 1993; Tavares et al., 1997; Brewer et al., 1984). However, cheese production based on fish pepsin has not been commercialized (Gildberg, 1992; Aehle, 2007).

3.5.3 Fish Silage and Fish Sauce

Fish pepsin can help in the production of fish silage and fish sauce. Both fish silage and fish sauce are highly nutritious protein hydrolysates made from whole fish or fish viscera by fermentation (Raa and Gildberg, 1982; Wignall and Tatterson, 1976; Saisithi, 1994; Gildberg, 2004; Murado et al., 2009). Pepsins in cod viscera have been proven to function well under acidic conditions in the aqueous phase of silage processing

(Gildberg and Almas,1986). Because pepsin is naturally present in fish stomach, minced fish material can be degraded and fermented with its help.

3.5.4 Therapeutic Enzymes

Pepsin is utilized in the regulation of digestion, as a dental antiseptic and in the treatment of ailments including dyspepsia, gastralgia, obstinate vomiting, infantile diarrhea, apepsia and some cancers (Gorgas, 2009). Combined with HCl, many pepsin tablets and capsules are developed to support the digestibility of the gastrointestinal tract as well as to enhance patients' appetite (Murado et al., 2009). Apart from this function, pepsin from porcine stomach is used for the treatment of gastric ulcers with bismuth complexes added (Almas, 1990). Pepsin was also added for better digestibility of proteins in animal feed.

3.5.5 Other Applications

Pepsin from cold-water species such as Atlantic cod and orange roughy (*Hoplostecthus atlanticus*) have also been used for caviar production from the roe of the same species in New Zealand (Xu et al., 1996). Some fish raw materials were gently processed with the help of pepsins. Cod pepsin was tested for the deskinning of herring (Joakimsson, 1984). Pepsin was also used to descale hake and haddock in weak acidic conditions, acquiring good and rapid removal of scales by a rapid passage through a water jet system (Svenning et al., 1993).

3.6 Recovery of PG and Pepsin

PG and pepsins have been extensively investigated. Several methods for mammals' pepsins recovery can be found and most of these approaches have been successfully extended to fish pepsin recovery. Generally, methods for fish pepsin recovery can be divided into two distinct procedures: (a) conventional methods which are used in the laboratory and (b) innovative methods which have potential for industrial application.

3.6.1 The Conventional Method of Recovery

The conventional method is most commonly used for PG and pepsin recovery from different fish stomach samples. This method has several steps for preparation of crude enzymes: homogenization, centrifugation and purification of crude enzymes using ammonium sulfate fractionation (ASF), gel filtration chromatography (GFC) and/or Ion exchange chromatography (IEC). With this method, highly homogenous PG are first obtained and then converted to pure pepsin. In this way, the characteristics of PG and pepsin from a fish species can both be elucidated, as shown in Figure 3.7.

Zhou et al. (2007) homogenized stomach samples using a homogenizer with phosphate buffer, centrifuged the homogenate to collect the crude enzymes and used 20–60% ASF and several chromatographic techniques to purify four types of PG and pepsins from sea bream. Tanji et al. (1988) used a Warring blender to homogenize fish stomach sample in sodium phosphate buffer, removed the insoluble residues, isolated the crude enzyme by centrifugation and purified three types of PG and corresponding pepsins from north pacific bluefin tuna. In addition to these two fishes, similar approaches were applied in zymogen and enzyme purification from other fish species including palometa (*Purona signufu*) (Pavlisko et al., 1997), smooth hound (Bougatef et al., 2008), European eel (Wu et al., 2009) and mandarin fish (Zhou et al., 2008).

A modified version of this procedure was developed in which an acidic buffer was used instead of the neutral one. In this case, only pure pepsin can be obtained and characterized in three steps: preparation of the fish stomach, preparation of crude enzyme extract and purification of pepsin (Figure 3.8). This method was applied in the purification of pepsin from the fish species Arctic capelin (Gildberg and Raa, 1983), cod (Bjelland et al., 1988), orange roughy (Xu et al., 1996) and Monterey sardine (Castillo-Yaneza et al., 2004).

Another version of the conventional method has been developed in which a fine powder is prepared and solubilized in a designated buffer (Figure 3.9). The neutral buffer (pH 6.0-7.5) gives PG while the acidic one (2.0-2.5) gives pepsin. One way to prepare this powder is to lyophilize stomachs in liquid nitrogen. Nalinanon et al. (2010) and Feng et al. (2008) homogenized albacore tuna stomach and orange-spotted grouper stomach by grinding them in liquid nitrogen and then freeze-drying to yield a fine powder before the

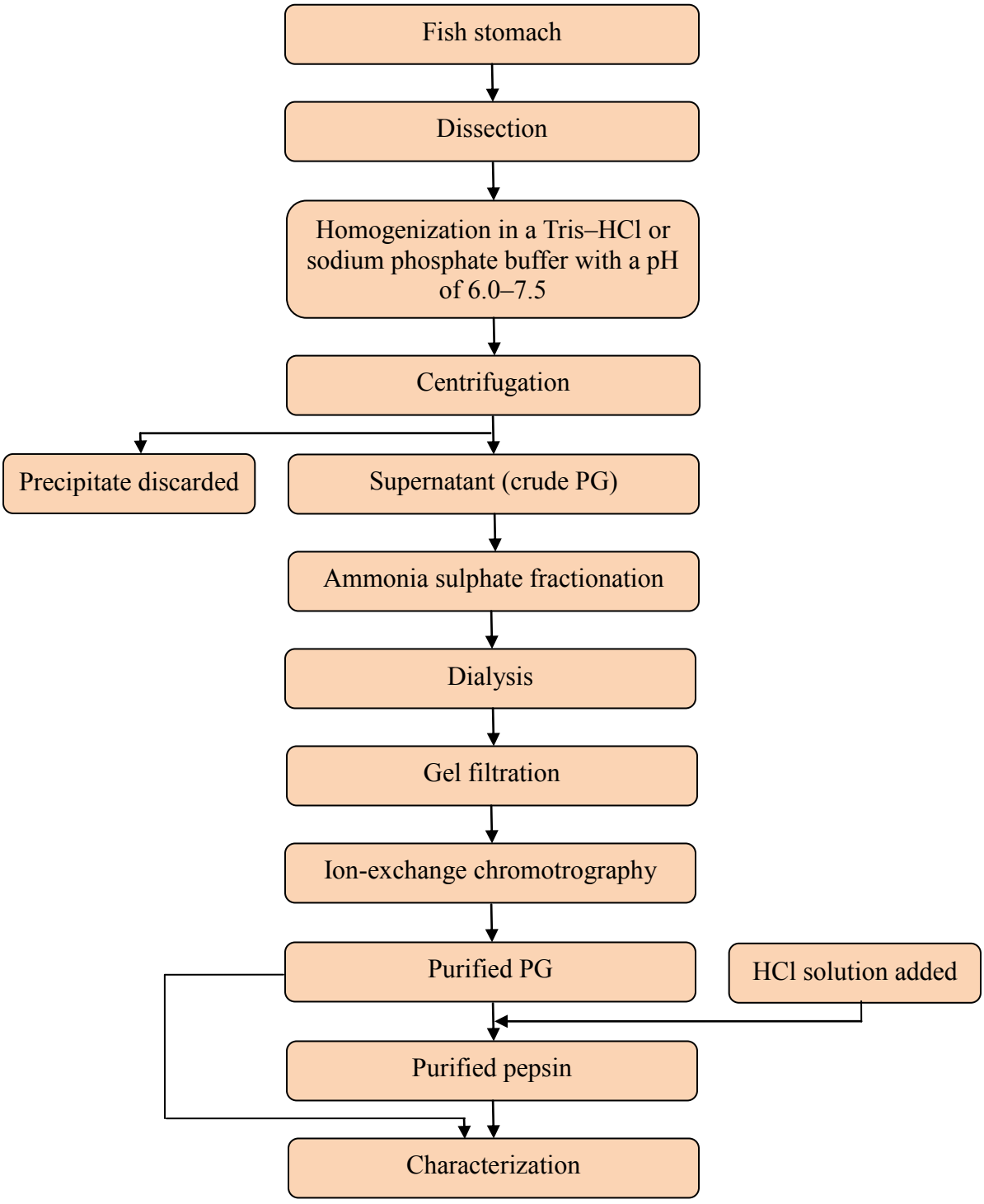


Figure 3.7: The procedure of the conventional method for PG and pepsin recovery with a neutral buffer. (Zhou et al., 2007).

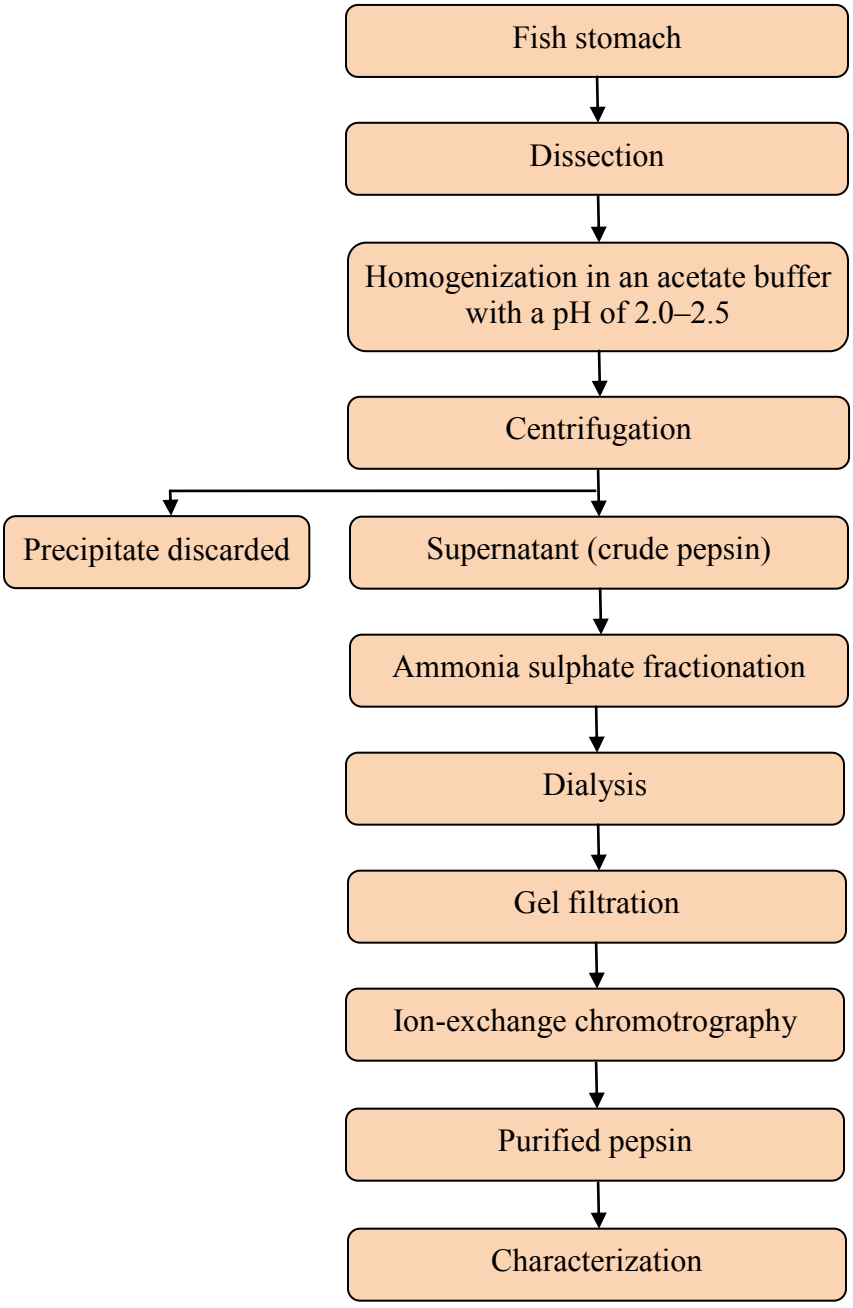


Figure 3.8: The modified procedure of the conventional method for pepsin recovery with an acidic buffer. (El-Beltagy et al., 2004).

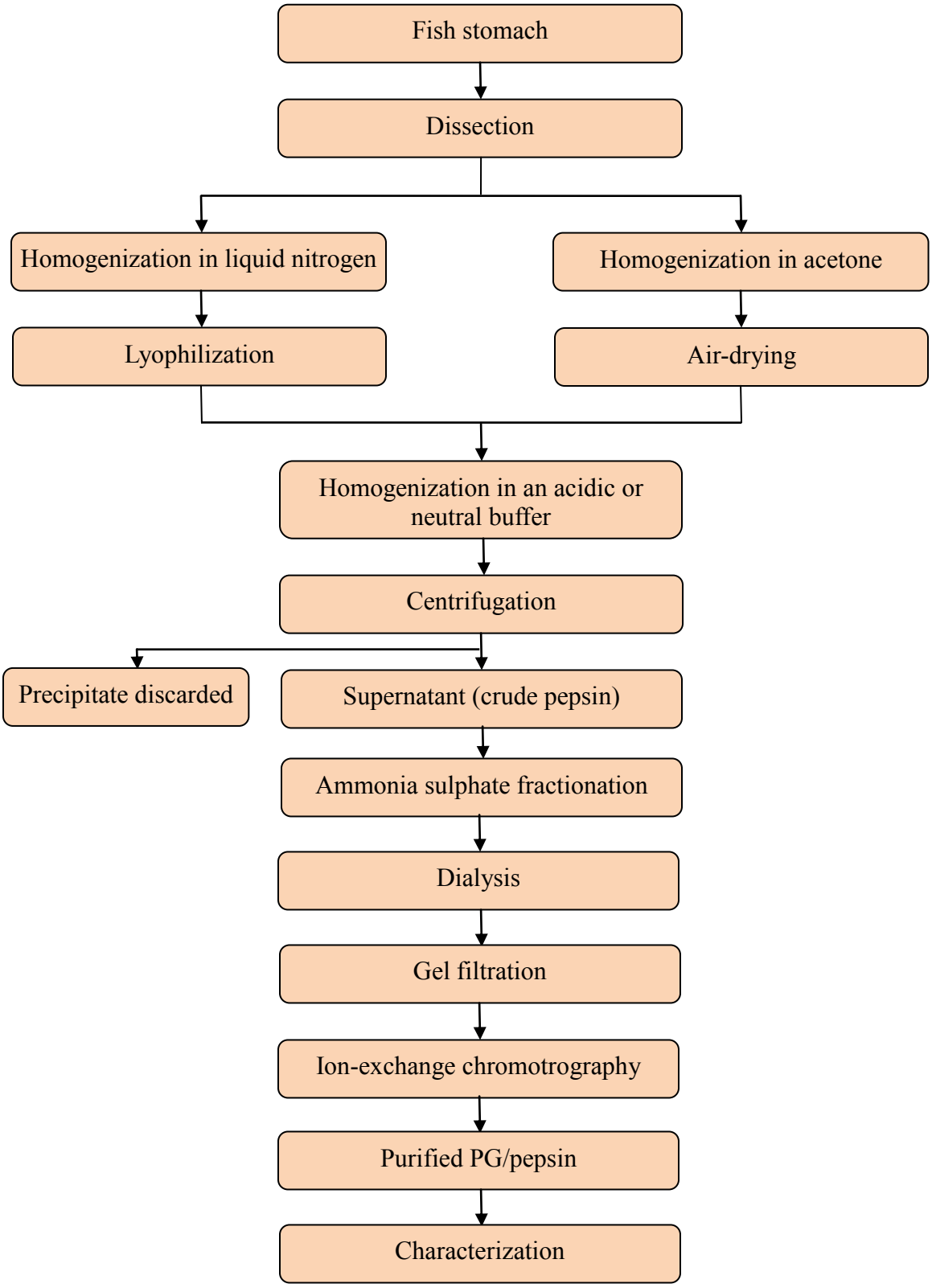


Figure 3.9: The modified procedure of the conventional method for PG and pepsin recovery with a fine powder. (Nalinanon et al., 2010).

buffering and centrifugation steps. Another way, which is more common, involves homogenizing in acetone at -20°C and air-drying at ambient temperature for preparation of crude enzyme powder. This approach was applied to recover pepsin from bolti fish (El-Beltagy et al., 2004), eel (Chiu and Pan, 2002) and pectoral rattail (Klomklao et al., 2007). This method is advantageous because some cells, salts and blood are soluble in acetone while pepsin are not. Therefore, impurities can be removed and the percentage of the pepsin in the supernatant obtained in the next step increased.

3.6.2 The ATPS Method

Although the conventional method gives high homogeneity of enzymes, its length of time and high expense (especially the purification step) make it unfeasible for application in the industry. Therefore, in the enzyme processing industry, an alternative method which gives greater economic feasibility, high yield and excellent purity is needed. The ATPS method is the most promising one for PG and pepsin recovery.

In the ATPS method, the crude enzyme is first prepared in the same manner as the conventional method. However, the purification of enzymes in ATPS is totally different. The formation of ATPS is based on two immiscible aqueous solutions, with a polymer and a salt dispersed in two phases individually (Spelzini et al., 2005; Tubío et al., 2007; Nalinanon et al., 2010). During the partition process, enzymes (pepsins) are transferred in a large proportion towards the polymer-rich phase in the whole system, which indicates a strong protein–polymer interaction due to the highly hydrophobic groups of the protein surface exposed to the solvent (Imelio et al. 2008), while the impurities (including soluble cells, blood, polysaccharides, pigments, more hydrophilic proteins) stay in the salt phase (Nalinanon et al., 2009). In this way, most of the enzymes can be isolated from other materials. The procedure of ATPS method is shown in Figure 3.10.

The ATPS method has several advantages for PG and pepsin recovery: (a) the ATPS is quick to prepare by low speed centrifugation, (b) it yields an excellent and suitable environment for maintaining the native structure and stabilizing the enzymes and (c) due to their existence in different aqueous phases, the polymers and salt can be readily recycled (Tanuja et al., 1997; Spelzini et al., 2005; Imelio et al., 2008; Nalinanon et al., 2009). These merits as well as the ease of scale-up, continuous operation, low capital cost

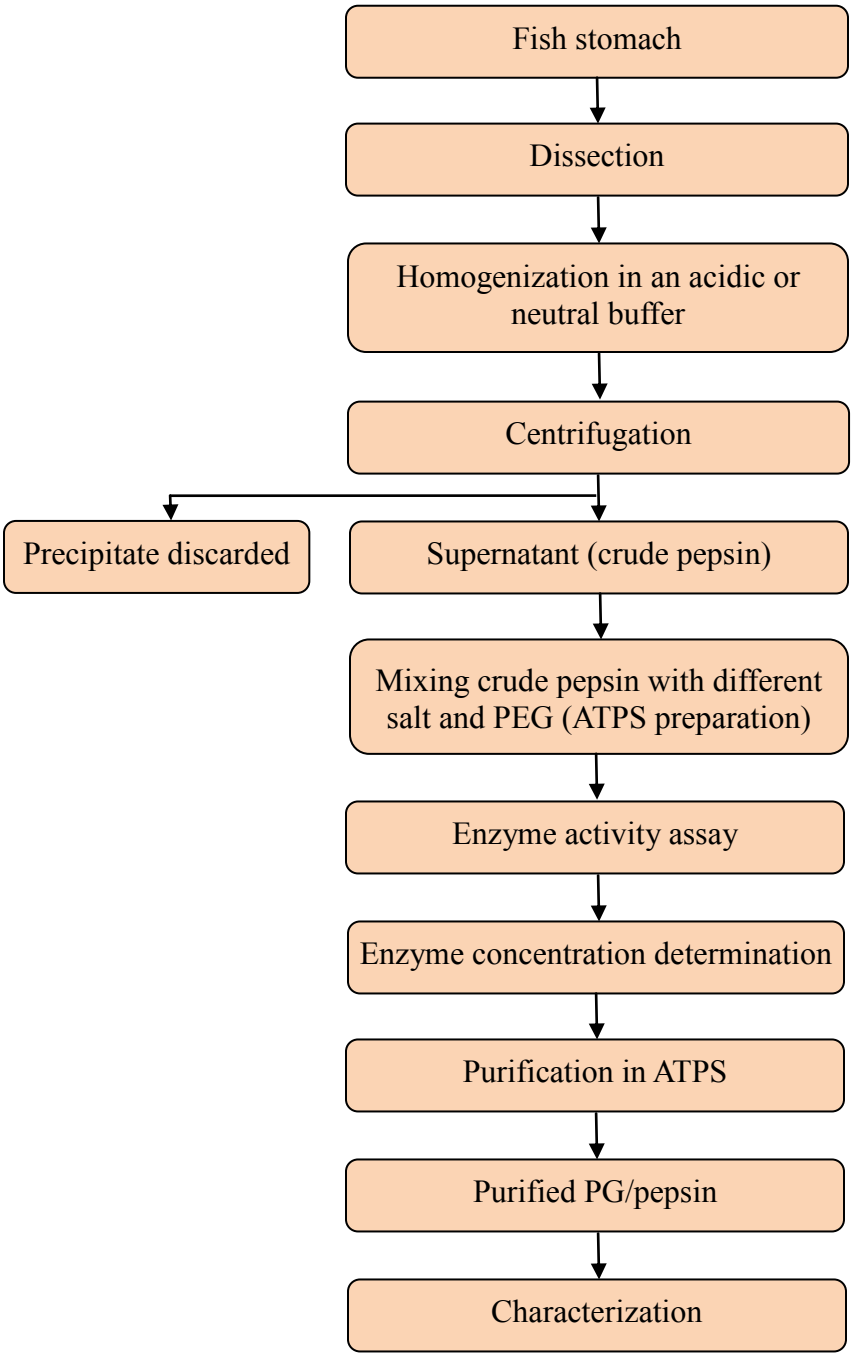


Figure 3.10: The procedure of ATPS method for PG and pepsin recovery. (Nalinanon et al., 2009).

and good feasibility give ATPS great potential for industrial application. It is recognized as a promising and versatile technique for downstream processing of proteins (Albertsson et al., 1987).

Several researchers used the ATPS method for the recovery of proteases such as trypsin (Tubío et al., 2007; Klomklao et al., 2005), α-chymotrypsin (Oliveira et al., 2002) and chymosin (Spelzini et al., 2005). Klomklao et al. (2005) purified trypsin from yellowfin tuna by the ATPS method and found 20% $MgSO_4$ and 15% PEG1000 as the best conditions for trypsin recovery (recovered activity of 69%). Rawdkuen et al. (2010) purified proteases from *Calotropis procera* latex and found that the highest protease recovery (74.6%) was produced in the PEG-rich phase of the system, comprising of 14% $MgSO_4$ and 18% PEG 1000.

Although the ATPS method has been recognized as an excellent way to partition enzymes, only two reports were found for pepsin recovery. Nalinanon et al. (2009) prepared ATPS using several polymer-salt combinations and found the optimum to be 20% $MgSO_4$ – 25% PEG 1000 and 15% $MgSO_4$ – 15% PEG 2000 for recovering PG and pepsin from albacore tuna, with recovered activity of 85.7% and 89.1%, respectively. Imelio et al. (2008) conducted bovine pepsin partition and found that the PEG 600/K_3PO_4 gave highest purity. However, it is important to note that ATPS is not a final solution to the recovery and purification of pepsin. The ATPS method is not selective enough to provide the extreme purity achieved with chromatography techniques used in conventional methods. To meet the stringent final product purity specifications, some other downstream steps are still required (Raghavarao et al., 1995).

3.6.3 Comparing the Effectiveness of ASF and ATPS Methods

The effectiveness of ASF and ATPS methods are mainly evaluated by two parameters: purification fold (PF) and recovery yield (RY). The recovery effects of the two types of methods are displayed in Tables 3.4 and 3.5. Table 3.4 shows the PF and RY of the different recovery processes, composed of ASF and several chromatography steps. The PF of recovered enzymes ranged from 7.1 to 87.2. However, a low range of RY (0.5%–38.36%) was obtained in this process. Different experimental conditions (including purification time of purification steps, ASF saturation, gel column type and

Table 3.4: Protease recovery from various fish species by conventional methods.

Enzyme	Fish Species	Method	PF[a]	RY[b] (%)	Reference
PG I	Sea bream	ASF-GFC-IEC	9.0	4.0	Zhou et al. (2007)
PG II	Sea bream	ASF-GFC-IEC	9.9	4.2	Zhou et al. (2007)
PG III	Sea bream	ASF-GFC-IEC	9.9	4.4	Zhou et al. (2007)
PG IV	Sea bream	ASF-GFC-IEC	9.6	4.8	Zhou et al. (2007)
PG I	Mandarin fish	ASF-GFC-IEC	25.6	2.8	Zhou et al. (2008)
PG II	Mandarin fish	ASF-GFC-IEC	11.5	13.3	Zhou et al. (2008)
PG III	Mandarin fish	ASF-GFC-IEC	15.5	6.5	Zhou et al. (2008)
PG IV	Mandarin fish	ASF-GFC-IEC	16.2	14.0	Zhou et al. (2008)
PG	Smooth hound	ASF-GFC-IEC	9.48	38.36	Bougatef et al.(2008)
PG I	European eel	ASF-IEC-GFC-GFC	28.3	2.4	Wu et al. (2009)
PG II	European eel	ASF-IEC-GFC-GFC	36.3	1.2	Wu et al. (2009)
PG III	European eel	ASF-IEC-GFC-GFC	64.2	1.3	Wu et al. (2009)
Pepsin I	Sardine	ASF-IEC-GFC	65.0	3.4	Noda and Murakamia (1981)
Pepsin II	Sardine	ASF-IEC-GFC	38.0	1.9	Noda and Murakamia (1981)
Pepsin I	Capelin	ASF-IEC-IEC-GFC	42.6	13.9	Gildberg and Raa (1983)
Pepsin II	Capelin	ASF-IEC-GFC	76.9	0.5	Gildberg and Raa (1983)
PG	Albacore tuna	ASF-GFC-GFC-IEC	87.2	16.5	Nalinanon et al. (2010)
Pepsin I	Pectoral rattail	ASF-GFC-IEC-GFC	7.1	5.7	Klomklao et al. (2007)
Pepsin II	Pectoral rattail	ASF-GFC-IEC-GFC	13.0	2.2	Klomklao et al. (2007)

a.PF = specific activity of protease purified/specific activity of crude protease.

b.RY = 100 × Total protease activity after purification/crude protease activity.

Table 3.5: Protease recovery from different sources by ATPS method.

Enzyme	Source	Optimal Condition	PF[a]	RY[b] (%)	Reference
PG	Albacore tuna	20% $MgSO_4$–25% PEG 1000	7.2	85.7	Nalinanon et al. (2009)
Pepsin	Albacore tuna	15% $MgSO_4$–15% PEG 2000	2.4	89.1	Nalinanon et al. (2009)
Trypsin	Yellowfin tuna	20% $MgSO_4$–15% PEG 1000	6.6	69.2	Klomklao et al. (2005)
Trypsin	Skipjack tuna	20% $MgSO_4$–15% PEG 1000	3.5	73.6	Klomklao et al. (2005)
Trypsin	Tongol tuna	20% $MgSO_4$–15% PEG1000	3.6	89.5	Klomklao et al. (2005)
Pepsin	Cattle	28% K_3PO_4–40% PEG 600	2.4	98.5	Imelio et al. (2008)
Protease	Latex of *Calotropisprocera*	14% $MgSO_4$–18% PEG1000	4.08	74.6	Rawdkuen et al. (2011)

a. PF = purification fold (Purification fold = specific activity of protease purified/specific activity of crude protease).
b. RY = recovery yield (Yield= 100 × total protease activity after ATPS/crude protease activity).

anion exchange chromatography column type) and people's skills may account for the differences in RY and PF of proteases from different fish species. For example, the PF of PG II in European eel (36.3) was achieved by a four step purification scheme (ASF-IEC-GF-GF) and is higher than that obtained (11.5) through a three step purification in Mandarin fish. However, its RY (1.2%) is much lower than the corresponding one (13.3%) in Mandarin fish. This example also indicated that PF increased while RY decreased as the purification times increased. Therefore, when a higher enzyme purity is required, less enzyme is retained during the recovery process.

The RY recovered by the ATPS method is shown in Table 3.5. Only a few data were available for RY and PF of ATPS in the literature. PF ranged from 2.4 to 7.2 while RY ranged from 69.2% to 98.5%. These data indicates that a moderate purity and high RY of enzymes can be obtained by the ATPS method. A good example involves the protease data of albacore tuna (Nalinanon et al, 2009). Its optimal recovery condition (20% $MgSO_4$ – 25% PEG 1000 combination) finally yielded PG with 85.7% of the enzymatic activity and the 7.2 times the purity of the crude enzyme.

CHAPTER 4. EXPERIMENTAL MATERIALS

4.1 Fish Sample

Red perch (*Sebastes marinus*) was purchased from the Fisherman's Market (607 Bedford Highway, Halifax, Nova Scotia, Canada). Samples (100 lb) were collected and packed in polyethylene bag and transported in ice to the Biotechnology Laboratory, Department of Process Engineering and Applied Science, Dalhousie University, in Halifax. Fish were separated into individual organs and the stomachs were collected. The undigested food in the stomach was removed and the stomach mucosa was rinsed with cold distilled water, then immediately frozen and stored at -20°C to minimize autolysis of enzymes until used in the experiments.

4.2 Chemicals

Polyethylene glycols (PEG1000, PEG1500, PEG3000 and PEG4000) were obtained from Sigma-Aldrich Canada Ltd. (Oakville, Ontario, Canada). Hemoglobin, bovine serum albumin, trichloroacetic acid (TCA) and Coomassie Brilliant Blue G-250 were purchased from Fisher Scientific Company (Ottawa, Ontario, Canada). Analytical grade salts of $(NH_4)_2SO_4$, $MgSO_4$, $Na_3C_6H_5O_7$, K_2HPO_4, K_3PO_4 and Na_2SO_4 were procured from Fisher Scientific Company (Ottawa, Ontario, Canada).

4.3 Buffers

The buffer solutions were prepared as described by Ruzin (1999) to obtain the required pH values shown in Tables 4.1 and 4.2. To obtain 50 mM sodium phosphate buffer with pH 7.0 as a crude enzyme solvent in the PG extraction, 200 ml of 0.1 M NaH_2PO_4 and Na_2HPO_4 solutions were prepared separately. Two specific volumes of both solutions were mixed together to give the desired pH as shown in Table 4.1. After preparation, sodium phosphate buffer was placed in the refrigerator at 4°C until used. Cold buffer was used to maintain the required pH and prevent protein denaturation for PG extraction. 100 mM phosphate-citrate buffer with pH 2.5 as an enzymatic reaction solvent in the determination of enzyme activity was prepared and stored in a similar way

as sodium phosphate buffer. The amounts of Na_2HPO_4 and $C_6H_8O_7$ solutions mixed together are shown in Table 4.2.

4.4 Equipment

A homogenizer (PowerGen Model 1800, Fisher Scientific, Ottawa, Ontario, Canada) was used to break fish stomach mucosa and release the enzymes. A refrigerated ultracentrifuge (Beckamn Model L-2, Beckman Coulter Canada Inc., Mississauga, Ontario Canada) was used to separate crude enzyme from stomach mucosa precipitates. A refrigerated high speed table top centrifuge (IEC Centra-MP4R, International Equipment Company, Needham, Massachusetts, USA) was used in ATPS preparation. A water bath (Precision Microprocessor Controlled 280 Series, Fisher Scientific Company, Ottawa, Ontario, Canada) was used to maintain constant reaction temperature while assaying enzyme activity. A pH meter (Accumet Model 15, Fisher Scientific, Ottawa, Ontario, Canada) was used to measure the pH of samples. A laboratory balance (Mettler Toledo PB4002-S FACT, Fisher Scientific, Ottawa, Ontario, Canada) was used for weighing all the samples. A vortex mixer (Fisher G-560 Vortex Genie 2, Fisher Scientific, Ottawa, Ontario, Canada) was used for mixing samples and reagents. A UV/Visible spectrophotometer (Ultrospec 1100 pro, Amersham Pharmacia Biotech, Inc., Schenectady, NY, USA) was used for absorbance measurement of samples in the analysis of enzyme activity and protein content.

Table 4.1: The buffer table for preparation of sodium phosphate buffer (Ruzin, 1999).

0.2 M NaH_2PO_4 solution (ml)	**0.2 M Na_2HPO_4 solution (ml)**	**pH**
92.0	8.0	5.8
87.7	12.3	6.0
81.5	18.5	6.2
68.5	31.5	6.5
62.5	37.5	6.6
56.5	43.5	6.7
51.0	49.0	6.8
45.0	55.0	6.9
39.0	61.0	7.0
33.0	67.0	7.1
28.0	72.0	7.2
23.0	77.0	7.3
19.0	81.0	7.4
16.0	84.0	7.5
8.5	91.5	7.8
5.3	94.7	8.0

Table 4.2: The buffer table for preparation of phosphate–citrate buffer (Ruzin, 1999).

0.2 M Na_2HPO_4 solution (ml)	**0.1 M $C_6H_8O_7$ solution 0.2 (ml)**	**pH**
5.4	44.6	2.6
7.8	42.2	2.8
10.2	39.8	3.0
12.3	37.7	3.2
14.1	35.9	3.4
16.1	33.9	3.6
17.7	32.3	3.8
19.3	30.7	4.0
20.6	29.4	4.2
22.2	27.8	4.4
23.3	26.7	4.6
24.8	25.2	4.8
25.7	24.3	5.0
26.7	23.3	5.2
27.8	22.2	5.4
29.0	21.0	5.6
30.3	19.7	5.8
32.1	17.9	6.0
33.1	16.9	6.2
34.6	15.4	6.4
36.4	13.6	6.6
40.9	9.1	6.8
43.6	6.5	7.0

CHAPTER 5. EXPERIMENTAL DESIGN

The experimental work was performed at the laboratory scale to purify proteases from the stomach of red perch by the ATPS and ASF methods. PG was chosen for purification instead of pepsin because of its higher stability in ATPS. The work was divided into two parts: (a) optimization of ATPS for PG purification and (b) comparing the ATPS method with the ASF method.

To obtain the optimum purification of the ATPS method, the effects of salt type and concentration and PEG molecular weight and concentration were evaluated. Four salts ($MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, K_2HPO_4), each with eleven concentrations were finally determined. Similarly, PEG with four molecular weights (1000, 1500, 3000, 4000), each with five concentrations (16%, 18%, 20%, 22%, 24%) worked and were chosen. The study was designed in two steps as shown in Figure 5.1 and Tables 5.1 and 5.2. In the first step, 18% PEG1000 was used and the effects of salt type and concentration were evaluated. In the second step, the optimum salt type and concentration were used and the effects of PEG molecular weight and concentration were evaluated. After the optimal purification conditions of the ATPS was established, the ATPS and ASF methods were compared as shown in Figure 5.2.

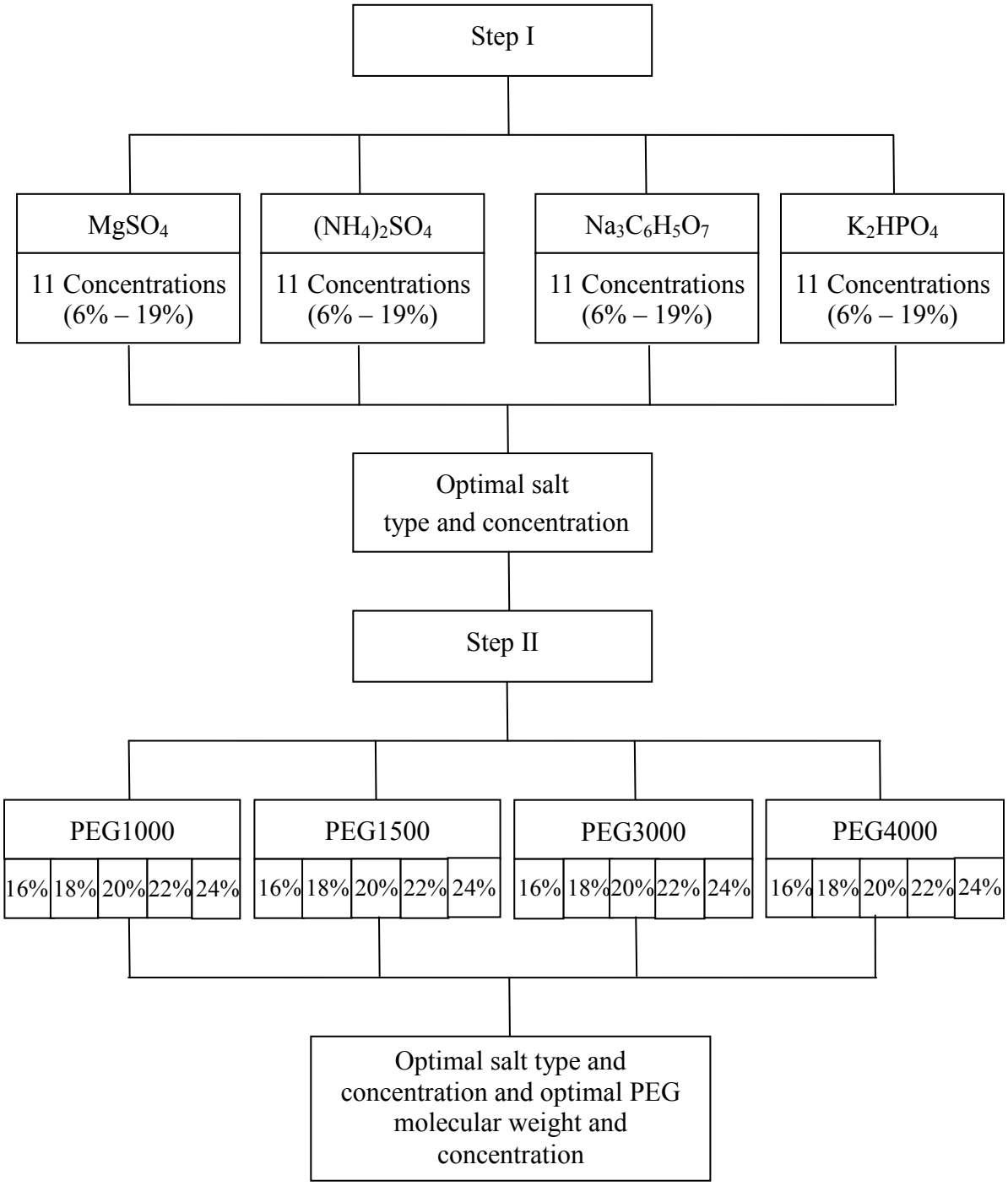


Figure 5.1: The flowchart for optimization of ATPS for PG purification.

Table 5.1: Phase compositions for the effects of salt type and concentration in various systems.

System	PEG (%, w/w)	Salt (%, w/w)	PG (%, w/w)	H_2O (%, w/w)
PEG1000/ $MgSO_4$	18	6	10	66
PEG1000/ $MgSO_4$	18	7	10	65
PEG1000/ $MgSO_4$	18	8	10	64
PEG1000/ $MgSO_4$	18	9	10	63
PEG1000/ $MgSO_4$	18	10	10	62
PEG1000/ $MgSO_4$	18	11	10	61
PEG1000/ $MgSO_4$	18	12	10	63
PEG1000/ $MgSO_4$	18	13	10	59
PEG1000/ $MgSO_4$	18	15	10	57
PEG1000/ $MgSO_4$	18	17	10	55
PEG1000/ $MgSO_4$	18	19	10	53
PEG1000/$(NH_4)_2SO_4$	18	6	10	66
PEG1000/$(NH_4)_2SO_4$	18	7	10	65
PEG1000/$(NH_4)_2SO_4$	18	8	10	64
PEG1000/$(NH_4)_2SO_4$	18	9	10	63
PEG1000/$(NH_4)_2SO_4$	18	10	10	62
PEG1000/$(NH_4)_2SO_4$	18	11	10	61
PEG1000/$(NH_4)_2SO_4$	18	12	10	60
PEG1000/$(NH_4)_2SO_4$	18	13	10	59
PEG1000/$(NH_4)_2SO_4$	18	15	10	57
PEG1000/$(NH_4)_2SO_4$	18	17	10	55
PEG1000/$(NH_4)_2SO_4$	18	19	10	53
PEG1000/$Na_3C_6H_5O_7$	18	6	10	66
PEG1000/$Na_3C_6H_5O_7$	18	7	10	65
PEG1000/$Na_3C_6H_5O_7$	18	8	10	64
PEG1000/$Na_3C_6H_5O_7$	18	9	10	63
PEG1000/$Na_3C_6H_5O_7$	18	10	10	62
PEG1000/$Na_3C_6H_5O_7$	18	11	10	61
PEG1000/$Na_3C_6H_5O_7$	18	12	10	60
PEG1000/$Na_3C_6H_5O_7$	18	13	10	59
PEG1000/$Na_3C_6H_5O_7$	18	15	10	57
PEG1000/$Na_3C_6H_5O_7$	18	17	10	55
PEG1000/$Na_3C_6H_5O_7$	18	19	10	53

Table 5.1: Continued.

System	PEG (%, w/w)	Salt (%, w/w)	PG (%, w/w)	H_2O (%, w/w)
PEG1000/K_2HPO_4	18	6	10	66
PEG1000/K_2HPO_4	18	7	10	65
PEG1000/K_2HPO_4	18	8	10	64
PEG1000/K_2HPO_4	18	9	10	63
PEG1000/K_2HPO_4	18	10	10	62
PEG1000/K_2HPO_4	18	11	10	61
PEG1000/K_2HPO_4	18	12	10	60
PEG1000/K_2HPO_4	18	13	10	59
PEG1000/K_2HPO_4	18	15	10	57
PEG1000/K_2HPO_4	18	17	10	55
PEG1000/K_2HPO_4	18	19	10	53

Table 5.2: Phase compositions for the effects of PEG molecular weight and concentration in various systems.

System	PEG (%, w/w)	Salt (%, w/w)	PG (%, w/w)	H_2O (%, w/w)
PEG1000/Optimal Salt	16	OC[a]	10	74−OC
PEG1000/Optimal Salt	18	OC	10	72−OC
PEG1000/Optimal Salt	20	OC	10	70−OC
PEG1000/Optimal Salt	22	OC	10	68−OC
PEG1000/Optimal Salt	24	OC	10	66−OC
PEG1500/Optimal Salt	16	OC	10	74−OC
PEG1500/Optimal Salt	18	OC	10	72−OC
PEG1500/Optimal Salt	20	OC	10	70−OC
PEG1500/Optimal Salt	22	OC	10	68−OC
PEG1500/Optimal Salt	24	OC	10	66−OC
PEG3000/Optimal Salt	16	OC	10	74−OC
PEG3000/Optimal Salt	18	OC	10	72−OC
PEG3000/Optimal Salt	20	OC	10	70−OC
PEG3000/Optimal Salt	22	OC	10	68−OC
PEG3000/Optimal Salt	24	OC	10	66−OC
PEG4000/Optimal Salt	16	OC	10	74−OC
PEG4000/Optimal Salt	18	OC	10	72−OC
PEG4000/Optimal Salt	20	OC	10	70−OC
PEG4000/Optimal Salt	22	OC	10	68−OC
PEG4000/Optimal Salt	24	OC	10	66−OC

a. OC = optimal concentration of the best salt.

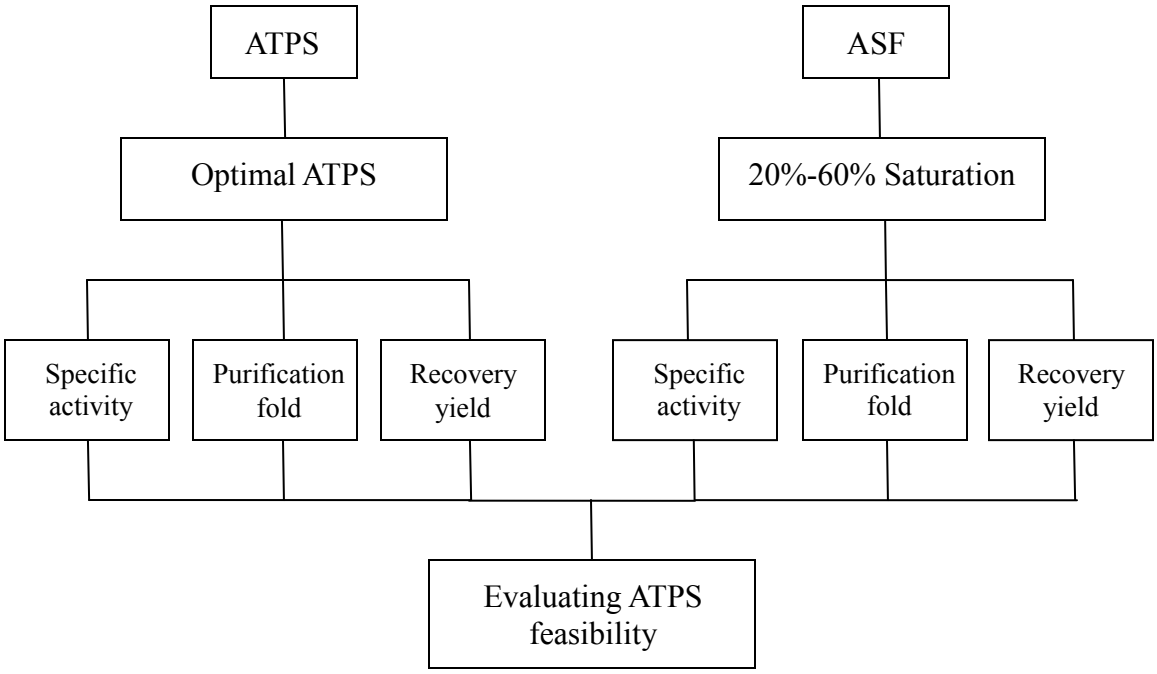


Figure 5.2: The flowchart for the comparison between ATPS and ASF purifications.

CHAPTER 6. EXPERIMENTAL PROCEDURES

The experimental procedure for ATPS recovery and ASF are summarized in Figures 6.1–6.3.

6.1 Extraction of Crude PG

Frozen stomachs (35 g) were thawed using running water (4°C) until the core temperature reached −2 to 0°C. The samples were cut into pieces with a thickness of 1.0-1.5 cm and homogenized in four volumes of 50 mM sodium phosphate buffer at pH 7.0. The homogenate was centrifuged in a refrigerated centrifuge (IEC Centra-MP4R refrigerated high speed table top centrifuge, International Equipment Company, Needham, Massachusetts, USA) at 15,000 g and 4°C for 20 min to remove the tissue debris. The supernatant was collected and referred to as crude extract (crude PG). Crude extract was divided and stored in 4 ml vials and stored at -20°C.

6.2 ATPS Purification

All the steps were performed at 4°C. ATPS were prepared in 15 ml centrifuge tubes by mixing PEG, salts and crude extract according to the methods described by Klomklao et al. (2005) and Nalinanon et al. (2010). The effects of salts (type and concentration) and PEG (molecular weight and concentration) were investigated according to the experimental design shown in Tables 5.1 and 5.2. ATPS were initially prepared at room temperature but the PG extract had poor stability at that temperature, showing a rapid decrease in activity. Thus, 4°C was used in these experiments to reduce the autolysis or self-digestion of enzyme that resulted in the activity drop.

6.2.1 Effect of Salt Type and Concentration on PG Purification

To investigate the effect of salt on PG purification, one solid salt selected from $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$, $MgSO_4$ and K_2HPO_4 and 50% stock PEG 1000 solution were weighted and mixed to achieve the designated concentrations in an aqueous system at room temperature. Although attempts were made to use buffers, it was difficult to maintain a pH of 7.0 for all salts in the bottom phase. Therefore, distilled water was used

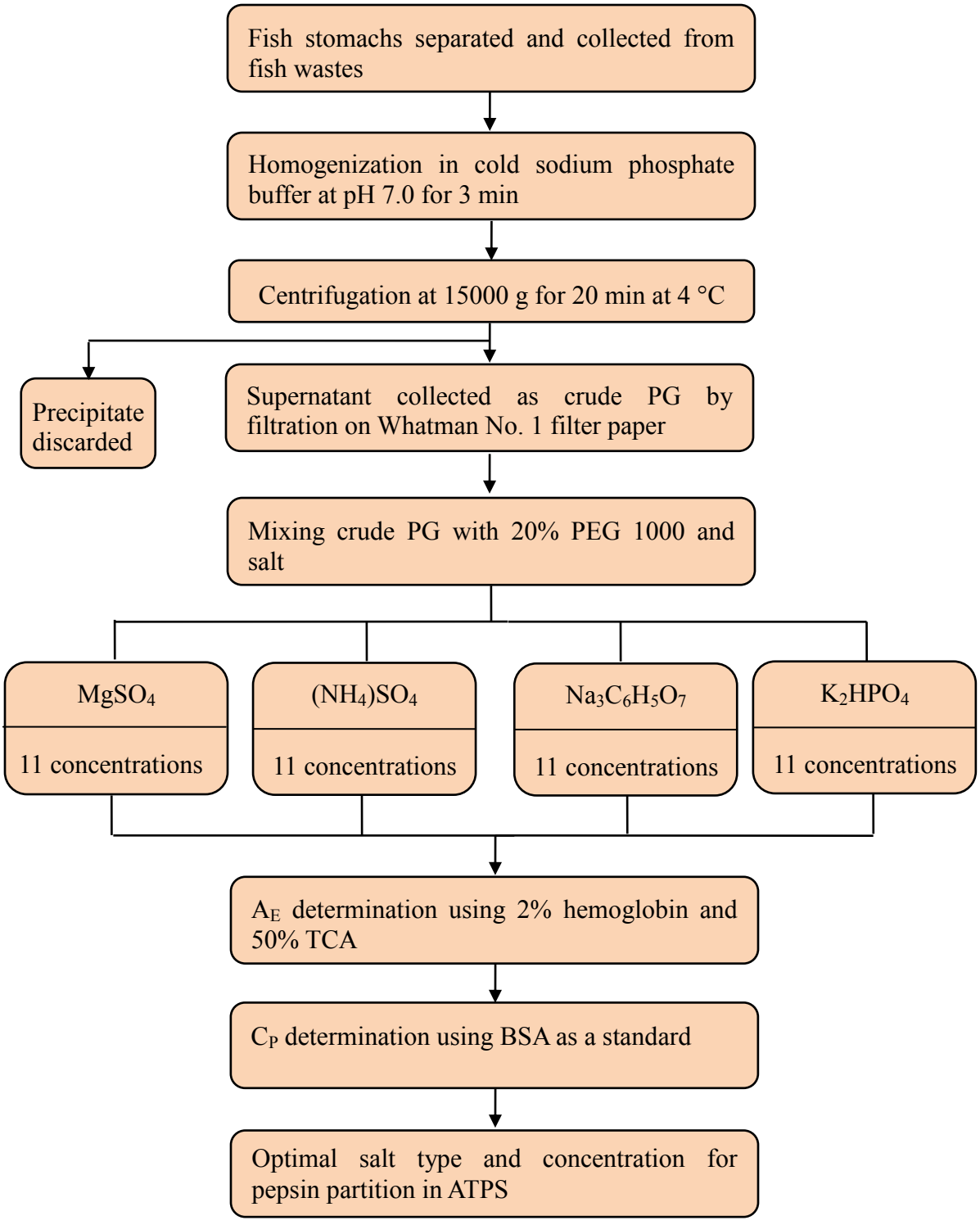


Figure 6.1: Salt optimization for pepsin partition by the ATPS method.

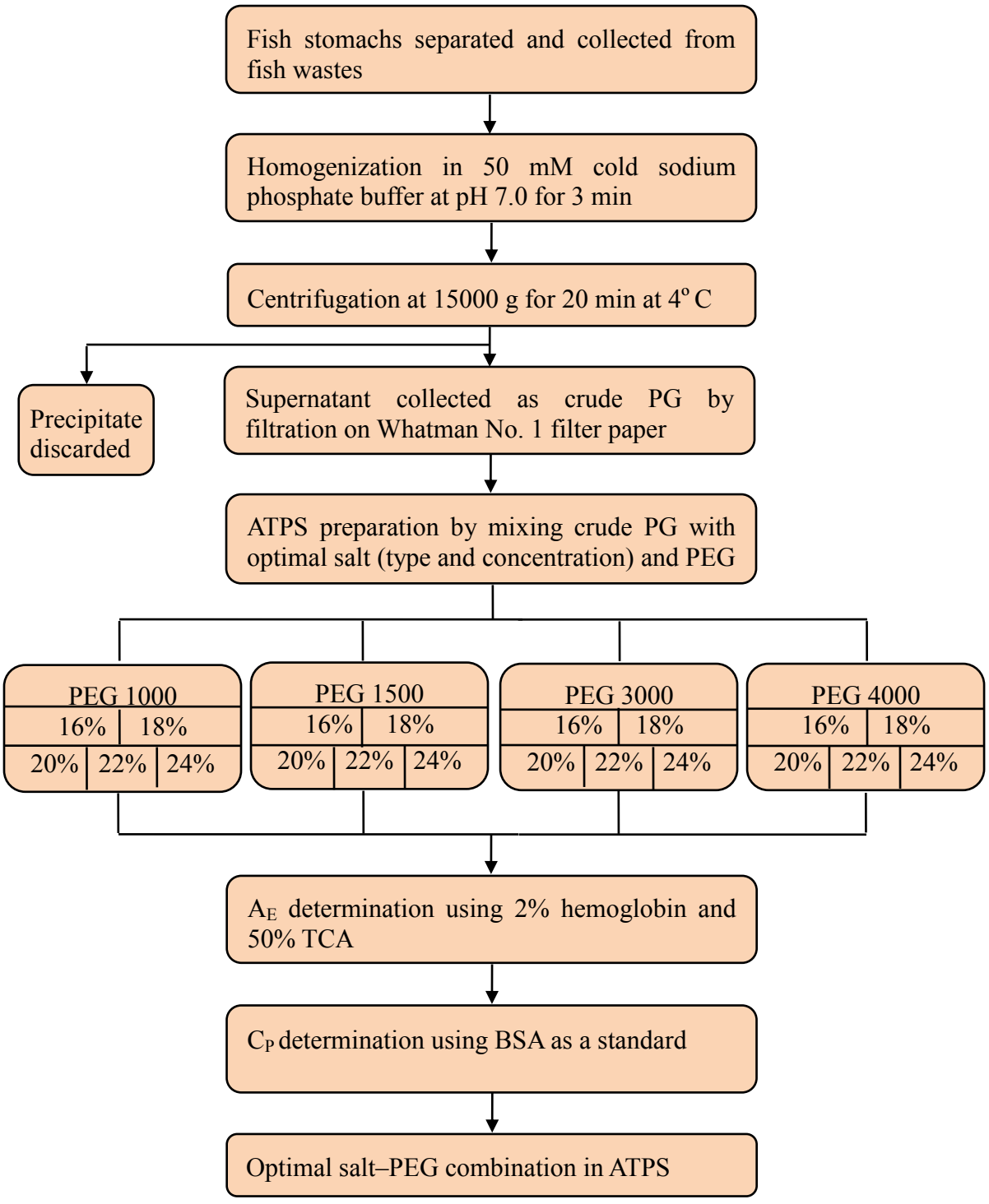


Figure 6.2: PEG optimization for pepsin partition by the ATPS method.

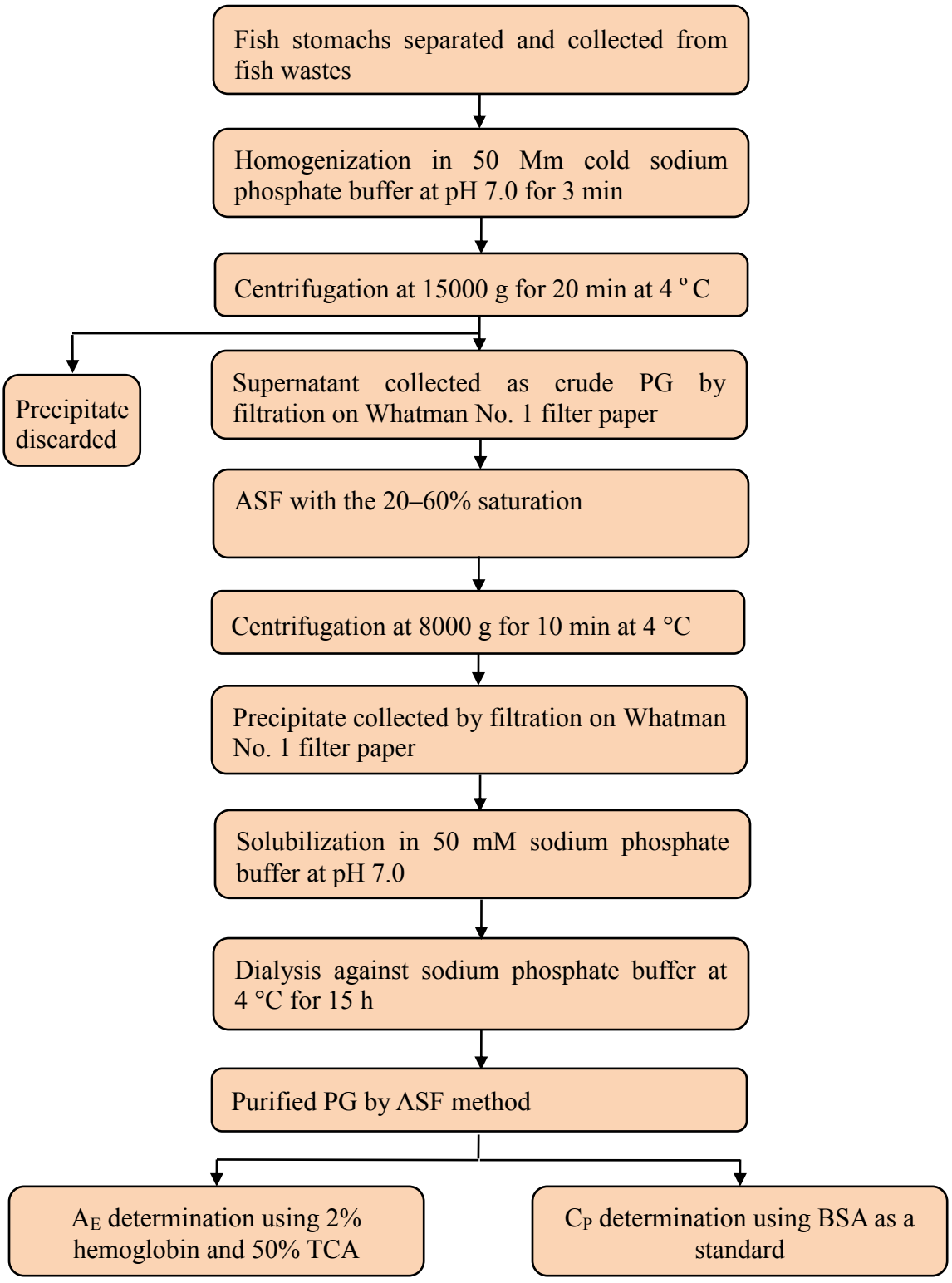


Figure 6.3: Flowchart of pepsin partition by the ASF method.

to adjust the system to obtain the final weight of 9 g (pH range in the top phase: 5.83-6.22 for $MgSO_4$, 6.08-6.34 for $(NH_4)_2SO_4$, 8.23-8.86 for $Na_3C_6H_5O_6$; 9.21-9.53 for K_2HPO_4). The mixtures were mixed completely using a vortex mixer (Fisher G-560 Vortex Genie 2, G-560, Fisher Scientific, Ottawa, Ontario) until the salt was completely solubilized and then stored at 4°C. Attempts were also made to use Na_2SO_4 and K_3PO_4 in ATPS. However, at 4°C, Na_2SO_4 failed to form an ATPS at concentrations of 9-19% due to low solubility and high viscosity. K_3PO_4 did form an ATPS within this salt concentration range and temperature but gave very low RY (<10%), which may be attributed to the destruction and denaturation of PG at too basic of a pH created by K_3PO_4. Therefore, neither Na_2SO_4 nor K_3PO_4 were suitable for PG purification with ATPS.

Approximately 1 g crude PG extract (thawed overnight) was added into the cold salt and PEG mixture and mixed by inversion several times. The cold mixture was transported in ice for centrifugation. After centrifugation for 5 min at 2000 g and 4°C, the phases separated. For each tube, the top phase (polymer phase) and bottom phase (salt phase) were carefully separated using a pre-chilled pipette and the interface ($\leq$0.05ml) was discarded. Volumes of the separated phases were measured using a 10ml graduate cylinder. Based on literature, 60-95% PG was partitioned in the top phase (Imelio et al., 2008; Nalinanon et al., 2009). Aliquots of the top phase were taken for determination of enzyme activity and aliquots in both phases were take for determination of protein content (see below). Based on purity and yield, the salt type and concentration which gave the highest RY was selected as the most effective purification salt for further study.

6.2.2 Effect of PEG Molecular Weight and Concentration on PG Purification

To study the effect of the polymer on PG purification, one 50% stock solution of PEG selected from either one of PEG 1000, PEG 2000, PEG 3000 and PEG 4000 were mixed with the optimal solid salt (selected in the previous step) to achieve the designated concentrations in the aqueous system at room temperature. All the steps for ATPS formation were performed in the same way as described in Section 6.2.1. Based on SA and PF, the PEG molecular weight and concentration and best PEG–salt composition were selected.

6.3 ASF Purification

Approximately 4 g PG crude extract were used for ASF purification. Ammonium sulfate powder was added very carefully and slowly and the precipitates in the saturation range of 20 – 60% were collected. The solution was brought to 20% saturation first and centrifuged in a refrigerated centrifuge at 10,000 g for 15 min. The supernatant was collected and volume was measured. Then the supernatant was brought to 60% saturation and centrifuged in the same way. After centrifugation, the precipitate was collected. The purified protease was dialyzed against 50mM sodium phosphate buffer with pH 7.0 overnight during which time the buffer was changed three times. The enzymes were stored at 4°C for assay and comparison with ATPS.

6.4 Protease Assay

The protease assay for the ATPS and ASF methods are different as the two phases in ATPS account for more parameters in the assay process. For the ATPS method, the protease assay procedures determined total volume (TV), volume ratio (V_R), enzyme activity (A_E), protein content (C_P), specific activity (SA), partition coefficient (K_P), purification fold (PF) as well as recovery yield (RY). For the conventional method, only A_E, C_P, SA, PF and RY were assessed.

6.4.1 Determination of Enzyme Activity (A_E)

Aliquots in PEG-rich top phase of the ATPS were taken for determination of A_E. Potential pepsin activity of PG was determined as described by Anson and Mirsky (1932) with a minor modification. Crude or purified PG (0.5 ml) was added into 2.5 ml of 2% (w/v) hemoglobin in the phosphate-citrate buffer. The reaction was conducted at a pH of 2.5 and a temperature of 37°C for 10 min. To terminate the enzymatic reaction, 5 ml of 5% (w/v) trichloroacetic acid (TCA) were added and nonhydrolyzed substrates were filtrated and removed. The clear filtrate was collected and the absorbance was measured at 280 nm. One unit was defined as an increase of 1.0 in absorbance at 280 nm per minute at pH 2.5 and 37°C. A blank was conducted in a similar way and protease was added into the reaction mixture after the addition of 5% TCA (w/v). The activity was assayed in triplicate. Aliquots from ASF purification were assayed in a similar way.

6.4.2 Determination of Protein Content (C_P)

Aliquots of both phases were taken for determination of C_P. C_P was measured by the method described by Bradford (1976) using BSA as a standard. A standard curve for BSA was constructed for the determination of C_P. BSA solutions (0.1 ml) with concentrations of 0, 100, 200, 400, 600 and 800 µg/ml were prepared in test tubes by mixing stock BSA solution (1 mg/ml) with enzyme buffer. Sample containing protein (0.1 ml) was pipetted into the same test tube. Each tube containing BSA solutions and protein samples were to 5 ml Bradford reagent and mixed using a vortex mixer. The color reaction was conducted at room temperature for 5 min. The absorbance was measured at 595 nm. A standard curve of absorbance of BSA solution against C_P of BSA was plotted. A blank was conducted and triplicates were performed. PEG mixed with the protein samples consistently caused a small reduction in absorbance. According to Barbosa et al. (2009), this effect can be reduced if the PEG concentration is diluted below 10% (w/w). Therefore, this dilution was made for all samples and the dilution fold was taken into account in the calculation of the original C_P.

6.4.3 Determination of Specific Activity (SA)

The SA of recovered proteases in the top PEG phase was determined in units/mg protein as follows (Nalinanon et al., 2009):

$$SA = \frac{A_E}{C_P} \ (units/mg\ protein) \qquad (1)$$

Where:

A_E is the enzyme activity in the top phase (U)

C_P is the protein content in the top phase (mg)

6.4.4 Determination of Purification Fold (PF)

The PF (also called purification factor) of PG in the top phase was defined as (Nalinanon et al., 2009):

$$PF = \frac{SA_p}{SA_c} \qquad (2)$$

Where:

SA_p is the SA purified in ATPS top phase (U/mg)

SA_c is the SA of crude PG extract (U/mg)

6.4.5 Determination of Partition Coefficient (K_P)

The K_P of recovered proteases for the ATPS was defined as (Nalinanon et al., 2009):

$$K_P = \frac{C_T}{C_B} \tag{3}$$

Where:

C_T is the C_P in top phase (mg)

C_B is the C_P in bottom phase (mg)

6.4.6 Determination of Volume Ratio (V_R)

The V_R of recovered proteases for the ATPS was defined as (Nalinanon et al., 2009):

$$V_R = \frac{V_T}{V_B} \tag{4}$$

Where:

V_T is the volume of top phase (ml)

V_B is the volume of bottom phase (ml)

6.4.7 Determination of Total Volume (TV)

The TV) of recovered proteases in the ATPS was defined as (Nalinanon et al., 2009):

$$TV = V_T + V_B \tag{5}$$

Where:

V_T is the volume of top phase (ml)

V_B is the volume of bottom phase (ml)

6.4.8 Determination of Recovery Yield (RY)

The protease RY was calculated using the ratio of protease activities and defined as (Nalinanon et al., 2009):

$$RY\ (\%) = \frac{A_t}{A_i} \times 100 \quad (6)$$

Where:

A_t is A_E in top phase (U)

A_i is the A_E of crude PG extract (U)

6.5 Statistical Analysis

The mean values and standard deviations were calculated from triplicate assays. All the data was subjected to analysis of variance (ANOVA) using Minitab 16 (Minitab Inc., State College, PA, USA). Evaluations of differences and Duncan Multiple Comparisons were based on a 5% of significance level. Coefficient of variation (CV), the ratio of the standard deviation to the mean, was calculated to evaluate degree of variation in the data.

6.6 Comparison between the ATPS and ASF methods

For the ATPS method, the crude and recovered proteases were investigated and different A_E and C_E, as well as phase volumes, were determined. All required data on SA, PF, K_P, V_R and RY were calculated and collected. For the ASF method, crude and recovered proteases were evaluated in a similar way. Only A_E and C_E were measured and data on SA, PF and RY were calculated. Parameters including SA, PF and RY of ATPS (optimal polymer-salt combination) and ASF were then compared to determine the feasibility of the ATPS method.

CHAPTER 7. RESULTS

Crude PG was extracted from the stomachs of red perch and was purified using the ATPS and ASF method. In the ATPS purification, the parameters included salt type, salt concentration, PEG molecular weight and PEG concentration.

7.1 Extraction Profiles

The results of the extraction of crude PG from the stomach (35 g) of red perch are summarized in Table 7.1. During the extraction process, the volumes of crude extract first decreased from 175 to 121 ml after centrifugation due to the removal of the mucosa residues and then increased to 152 ml after dialysis because of the absorption of some water. The A_E and C_P decreased from 2154 to 1655 U and from 3871 to 1595 mg, respectively. The SA and PF increased from 0.56 to 1.04 U/mg and from 1.00 to 1.90, respectively. The RY decreased from 100.0 to 86.6% during extraction process.

7.2 Purification by ATPS

7.2.1 Effect of Salt Type and Concentration

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on ATPS purification using 18% PEG 1000 are presented in Tables 7.2 – 7.5. $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 started to form two phases at salt concentrations of 9, 12, 12 and 10%, respectively. They were able to form ATPS at 4°C from their starting salt concentration up to 19%. Although higher salt concentrations could form ATPS, a low recovery yield was achieved in preliminary experiments. Therefore, 19% was selected as an upper level for purification by ATPS.

7.2.1.1 TV

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on TV are shown in Figure 7.1. All TV decreased with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.6 and 7.7), salt type and salt concentration had significant effects on TV. There was also a significant interaction between salt type and salt concentration. Grouping data showed that TV of $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and one salt

Table 7.1: Extraction profiles of PG from the red perch.

Extraction step	Total extract volume	Total activity (U)*	Total protein (mg)	Specific activity (U/mg protein)	Purification fold	Recovery yield (%)
After homogenation	175	2154	3871	0.56	1.00	100.0
After centrifugation	121	1969	2022	0.97	1.75	91.41
After dialysis	152	1655	1595	1.04	1.90	86.55

Sample size = 35 g

Table 7.2: Effects of magnesium sulfate ($MgSO_4$) on partition of 1 g PG using 18% PEG 1000.

$MgSO_4$ concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
6	No phase separation											
7	No phase separation											
8	No phase separation											
9	6.77±0.01	1.95±0.01	8.72±0.02	3.47±0.02	6.56±0.12	0.93±0.05	1.32±0.04	2.26±0.02	7.05±0.35	0.70±0.06	6.80±0.34	60.2±0.93
10	5.87±0.01	2.75±0.01	8.62±0.02	2.14±0.01	6.80±0.16	0.96±0.03	1.41±0.03	2.37±0.03	7.08±0.25	0.68±0.03	6.82±0.24	62.4±1.47
11	5.05±0.01	3.49±0.01	8.54±0.01	1.45±0.01	6.75±0.20	0.95±0.04	1.41±0.02	2.36±0.03	7.11±0.25	0.67±0.03	6.85±0.24	61.8±1.85
12	4.32±0.01	4.16±0.01	8.48±0.02	1.04±0.01	6.42±0.21	0.91±0.02	1.35±0.04	2.26±0.02	7.09±0.23	0.67±0.05	6.83±0.22	59.0±1.93
13	3.95±0.01	4.48±0.01	8.43±0.02	0.88±0.01	5.99±0.27	0.85±0.05	1.25±0.03	2.10±0.06	7.05±0.28	0.68±0.04	6.79±0.27	55.1±2.50
15	3.83±0.02	4.52±0.01	8.35±0.03	0.84±0.01	5.32±0.19	0.77±0.04	1.08±0.04	1.85±0.01	6.91±0.11	0.71±0.02	6.66±0.11	48.9±1.76
17	3.73±0.01	4.56±0.01	8.29±0.01	0.82±0.00	4.62±0.10	0.69±0.04	0.86±0.03	1.55±0.03	6.70±0.29	0.80±0.07	6.45±0.28	42.4±0.93
19	3.69±0.01	4.57±0.01	8.26±0.01	0.81±0.00	3.38±0.26	0.52±0.04	0.57±0.05	1.09±0.09	6.50±0.26	0.91±0.04	6.26±0.46	31.0±2.41

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.3: Effects of ammonium sulfate ($(NH_4)_2SO_4$) on partition of 1g PG using 18% PEG 1000.

$(NH_4)_2SO_4$ concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
6	No phase separation											
7	No phase separation											
8	No phase separation											
9	No phase separation											
10	No phase separation											
11	No phase separation											
12	6.97±0.01	1.96±0.01	8.93±0.02	5.56±0.02	7.04±0.20	1.72±0.06	0.32±0.02	2.04±0.08	4.09±0.26	5.38±0.23	3.94±0.25	64.7±1.85
13	5.52±0.01	3.22±0.01	8.74±0.01	1.71±0.01	7.25±0.12	1.74±0.06	0.36±0.02	2.10±0.08	4.17±0.19	4.83±0.16	3.97±0.18	66.6±1.11
15	4.51±0.01	4.10±0.01	8.61±0.02	1.10±0.01	7.81±0.16	1.79±0.03	0.38±0.03	2.17±0.06	4.36±0.14	4.71±0.30	4.20±0.13	71.7±1.48
17	4.07±0.01	4.45±0.01	8.52±0.02	0.92±0.01	6.69±0.19	1.69±0.03	0.30±0.02	1.99±0.04	3.96±0.12	5.63±0.26	3.81±0.12	61.4±1.76
19	3.75±0.02	4.72±0.02	8.47±0.04	0.79±0.00	5.57±0.20	1.60±0.05	0.23±0.02	1.84±0.08	3.48±0.21	6.96±0.43	3.35±0.20	51.2±1.85

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.4: Effects of sodium citrate tribasic ($Na_3C_6H_5O_7$) on partition of 1g PG using 18% PEG 1000.

$Na_3C_6H_5O_7$ concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
6	No phase separation											
7	No phase separation											
8	No phase separation											
9	No phase separation											
10	No phase separation											
11	No phase separation											
12	7.30±0.01	1.48±0.02	8.78±0.03	4.93±0.04	3.63±0.13	2.46±0.04	0.57±0.02	3.02±0.06	1.48±0.08	4.32±0.08	1.42±0.08	33.3±1.20
13	5.55±0.01	3.03±0.01	8.58±0.01	1.83±0.01	3.72±0.15	2.49±0.07	0.61±0.04	3.11±0.06	1.49±0.11	4.08±0.16	1.43±0.11	34.2±1.39
15	4.85±0.01	3.63±0.01	8.48±0.02	1.34±0.01	3.85±0.17	2.54±0.05	0.64±0.04	3.18±0.09	1.52±0.10	3.97±0.23	1.46±0.10	35.3±1.57
17	4.32±0.01	4.08±0.01	8.40±0.02	1.06±0.00	3.47±0.20	2.47±0.06	0.53±0.04	3.00±0.10	1.40±0.12	4.66±0.24	1.35±0.12	31.8±1.85
19	4.15±0.02	4.18±0.01	8.33±0.03	0.99±0.00	2.88±0.23	2.38±0.04	0.41±0.03	2.79±0.05	1.21±0.12	5.80±0.33	1.17±0.12	26.5±2.11

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.5: Effects of potassium phosphate dibasic (K_2HPO_4) on partition of 1g PG using 18% PEG 1000.

K_2HPO_4 concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
6	No phase separation											
7	No phase separation											
8	No phase separation											
9	No phase separation											
10	6.99±0.01	1.87±0.01	8.86±0.02	3.74±0.02	4.21±0.16	2.75±0.02	0.24±0.01	3.00±0.02	1.53±0.08	11.5±0.40	1.35±0.08	38.7±1.47
11	5.86±0.01	2.78±0.01	8.64±0.01	2.11±0.01	4.50±0.12	2.88±0.05	0.29±0.02	3.17±0.06	1.58±0.07	9.93±0.52	1.52±0.07	41.3±1.10
12	5.29±0.01	3.25±0.01	8.54±0.02	1.63±0.01	4.40±0.15	2.84±0.04	0.29±0.02	3.13±0.05	1.55±0.05	9.97±0.39	1.49±0.05	40.4±1.38
13	5.03±0.01	3.45±0.01	8.48±0.02	1.46±0.01	4.13±0.09	2.77±0.05	0.26±0.01	3.04±0.06	1.50±0.06	10.7±0.18	1.45±0.06	37.9±0.83
15	4.60±0.01	3.75±0.01	8.35±0.02	1.23±0.01	3.53±0.25	2.57±0.05	0.21±0.03	2.78±0.07	1.43±0.07	12.9±0.78	1.38±0.07	32.4±2.30
17	4.23±0.02	4.03±0.01	8.27±0.03	1.05±0.00	3.34±0.17	2.43±0.05	0.17±0.02	2.60±0.07	1.38±0.04	15.2±0.75	1.33±0.04	30.7±1.56
19	4.04±0.01	4.15±0.01	8.19±0.02	0.97±0.00	3.01±0.08	2.26±0.04	0.13±0.02	2.39±0.06	1.26±0.03	15.2±0.49	1.28±0.03	27.7±0.73

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

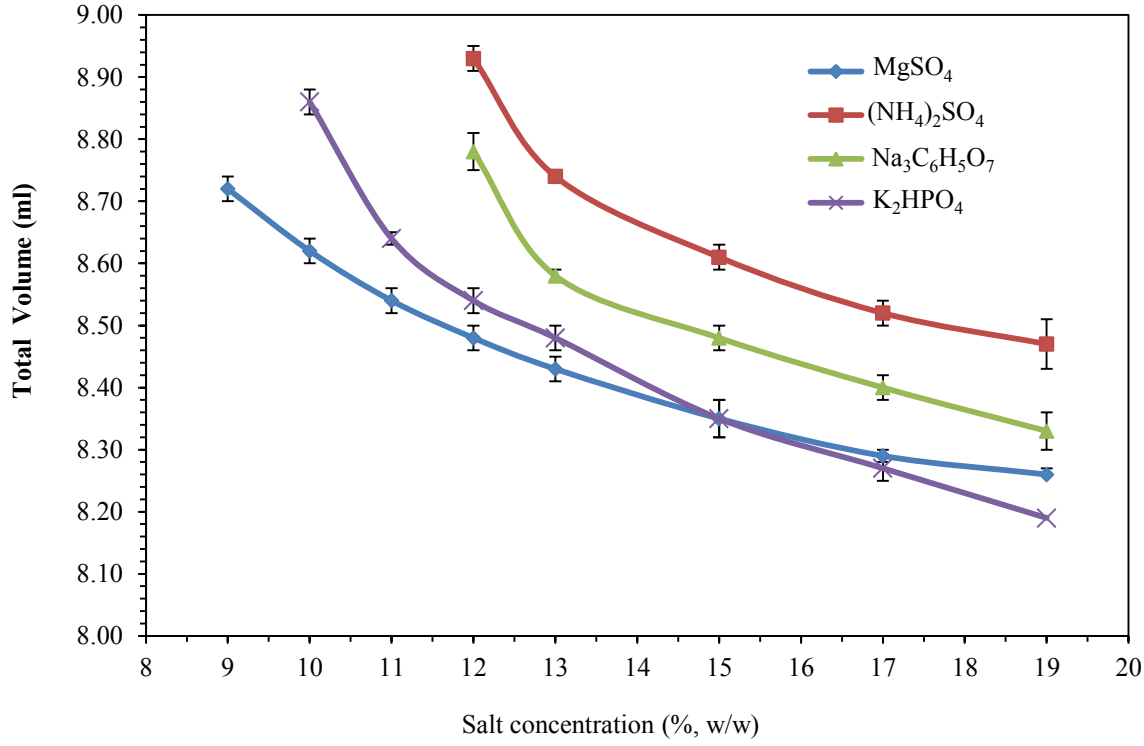


Figure 7.1: TV as a function of salt concentration (mean ± S.D, n = 3).

Table 7.6: ANOVA of TV (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	2.03830			
T	3	0.89119	0.297064	522.69	0.0001
C	4	1.04756	0.261889	460.80	0.0001
TC	12	0.07682	0.006401	11.26	0.0001
Error	40	0.02273	0.000568		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV: = 2.19%

Table 7.7: The effects of salt type and concentration on TV.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$(NH_4)_2SO_4$	15	8.65	A
$Na_3C_6H_5O_7$	15	8.51	B
$MgSO_4$	15	8.36	C
K_2HPO_4	15	8.37	C
Salt concentration (%)			
12	12	8.68	A
13	12	8.56	B
15	12	8.45	BC
17	12	8.38	CD
19	12	8.31	D

from $MgSO_4$ and K_2HPO_4 were significantly different from one another at the 0.05 level while TV of $MgSO_4$ and K_2HPO_4 were not significantly different. TV of $MgSO_4$ decreased from 8.72 to 8.26 ml (5.28%) when its concentration increased from 9 to 19% (111.11%). The TV of $(NH_4)_2SO_4$ decreased from 8.93 to 8.47 ml (5.15%) when its concentration increased from 12 to 19% (58.33%). The TV of $Na_3C_6H_5O_7$ decreased by from 8.78 to 8.33 ml (5.13%) when its concentration increased from 12 to 19 % (58.33%). The TV of K_2HPO_4 decreased from 8.86 to 8.19 ml (7.56%) when its concentration increased from 10 to 19% (90.0%). $(NH_4)_2SO_4$ at 12% concentration gave the highest TV value (8.93 ml) while K_2HPO_4 at 19% concentration gave the lowest TV value (8.19 ml).

7.2.1.2 V_R

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on V_R are shown in Figure 7.2. All V_R sharply decreased first with increased salt concentration (up to 13%) and then slowly decreased with further increases in salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.8 and 7.9), salt type and salt concentration had significant effects on V_R. There was also significant interaction between salt type and salt concentration. Grouping data showed that V_R of $Na_3C_6H_5O_7$ was significantly different from V_R of K_2HPO_4 and $MgSO_4$ at the 0.05 level.

The V_R of $MgSO_4$ decreased from 3.47 to 1.04 (70.03%) when its concentration increased from 9 to 12% (33.33%) and then decreased from 1.04 to 0.81 (6.63%) when its concentration further increased from 12 to 19% (77.78%). The V_R of $(NH_4)_2SO_4$ decreased from 5.56 to 1.71 (69.24%) when its concentration increased from 12 to 13% (8.33%) and then decreased from 1.71 to 0.79 (16.55%) when its concentration further increased from 13 to 19% (50.00%). The V_R of $Na_3C_6H_5O_7$ decreased from 4.93 to 1.83 (62.88%) when its concentration increased from 12 to 13% (8.33%) and then decreased from 1.83 to 0.99 (17.04%) when its concentration further increased from 13 to 19% (50.00%). The V_R of K_2HPO_4 decreased from 3.74 to 1.63 (56.42%) when its concentration increased from 10 to 12% (20.00%) and then decreased from 1.63 to 0.97 (17.65%) when its concentration further increased from 12 to 19% (70.00%). This resulted in total reductions in V_R of 76.66, 85.79, 79.92 and 74.07% for the MgSO4, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO4, respectively. $Na_3C_6H_5O_7$ gave the highest V_R

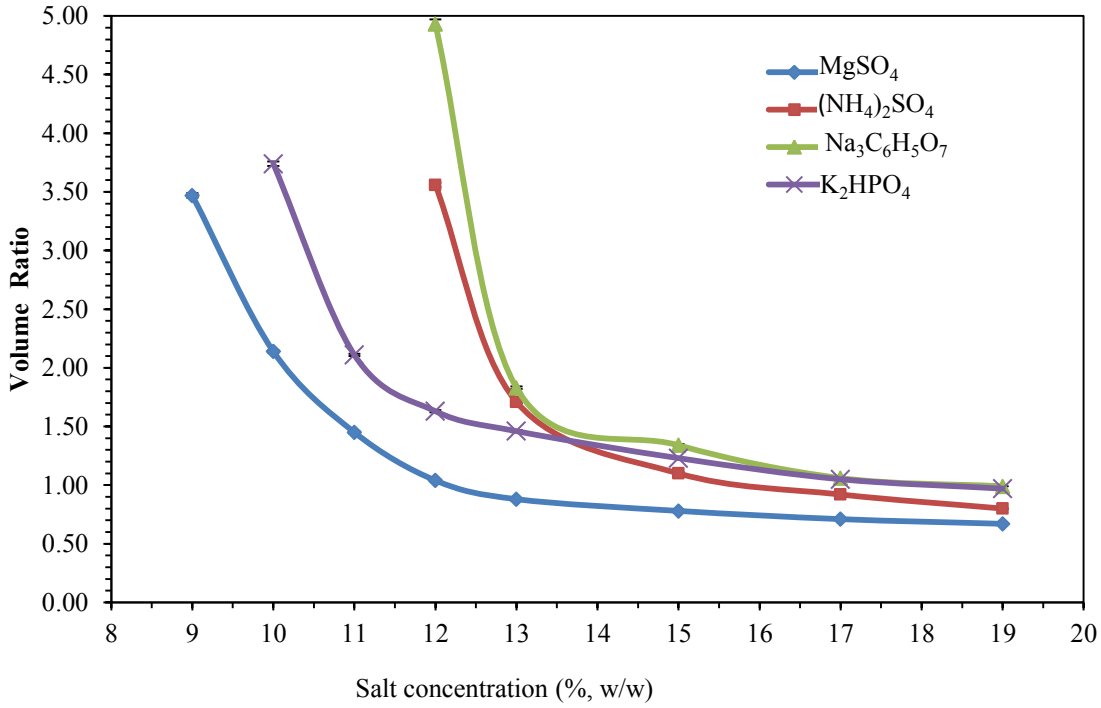


Figure 7.2: V_R as a function of salt concentration (mean ± S.D, n = 3).

Table 7.8: ANOVA of V_R (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	61.6530			
T	3	12.0097	4.00325	23493.23	0.0001
C	4	30.3149	7.57871	44476.03	0.0001
TC	12	19.3216	1.61014	9449.15	0.0001
Error	40	0.0068	0.00017		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.999$,
CV = 71.35%

Table 7.9: The effects of salt type and concentration on V_R.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$Na_3C_6H_5O_7$	15	2.03	A
$(NH_4)_2SO_4$	15	1.62	AB
K_2HPO_4	15	1.27	BC
$MgSO_4$	15	0.82	C
Salt concentration (%)			
12	12	2.79	A
13	12	1.47	B
15	12	1.11	B
17	12	0.93	B
19	12	0.86	B

value (4.93) while $MgSO_4$ gave the lowest V_R value (0.81).

7.2.1.3 A_E

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on A_E are shown in Figure 7.3. All A_E increased slightly and then decreased steadily with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.10 and 7.11), salt type and concentration had significant effects on A_E. There was also a significant interaction between salt type and concentration. Grouping results indicated that A_E of $(NH_4)_2SO_4$, $MgSO_4$ and one salt from $Na_3C_6H_5O_7$ and K_2HPO_4 were significantly different at the 0.05 level while A_E of $Na_3C_6H_5O_7$ and K_2HPO_4 were not significantly different from each other. The A_E at a concentration of 19% was significantly different from A_E at concentrations of 12, 13 and 15%.

The A_E of $MgSO_4$ increased from 6.56 to 6.80 U (3.66%) when the salt concentration increased from 9 to 10% (11.11%) and then decreased from 6.80 to 3.38 U (50.29%) when salt concentration further increased from 10 to 19% (100.00%). The A_E of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 7.04 to 7.81 U (10.94%) and from 3.63 to 3.85 U (6.06%), when the salt concentration increased from 12 to 15% (25.00%) and then decreased from 7.81 to 5.57 U (28.68%) and from 3.85 to 2.88 U (25.19%) when the salt concentration further increased from 15 to 19% (33.33%), respectively. The A_E of K_2HPO_4 increased from 4.21 to 4.50 U (6.89%) when the salt concentration increased from 10 to 11% (10.00%) and then decreased from 4.50 to 2.97 U (34.00%) when the salt concentration further increased from 11 to 19% (80.00%). Highest A_E was achieved with $(NH_4)_2SO_4$ at a concentration of 15%.

7.2.1.4 SA

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on SA are shown in Figure 7.4. All SA initially increased slightly and then decreased slowly with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.12 and 7.13), salt type had a significant effect and salt concentration also had a significant effect on SA except for $MgSO_4$. Similar to A_E, grouping results indicated that SA of $(NH_4)_2SO_4$, $MgSO_4$ and one salt from $Na_3C_6H_5O_7$ and K_2HPO_4 were significantly different at the 0.05

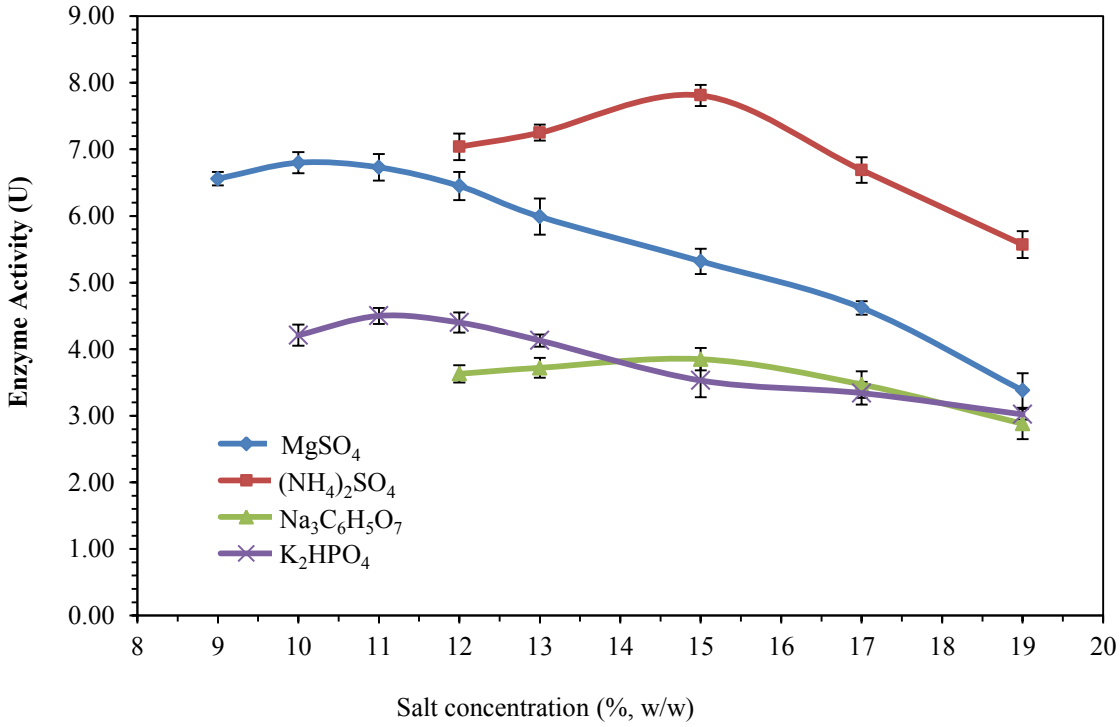


Figure 7.3: A_E in the top phase as a function of salt concentration (mean ± S.D, n = 3).

Table 7.10: ANOVA of A_E (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	142.678			
T	3	109.921	36.6403	1080.83	0.0001
C	4	23.041	5.7603	169.92	0.0001
TC	12	8.361	0.6967	20.55	0.0001
Error	40	1.356	0.0339		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 32.36%

Table 7.11: The effects of salt type and concentration on A_E.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$(NH_4)_2SO_4$	15	6.87	A
$MgSO_4$	15	5.15	B
K_2HPO_4	15	3.69	C
$Na_3C_6H_5O_7$	15	3.51	C
Salt concentration (%)			
12	12	5.38	A
13	12	5.27	A
15	12	5.13	A
17	12	4.53	AB
19	12	3.71	B

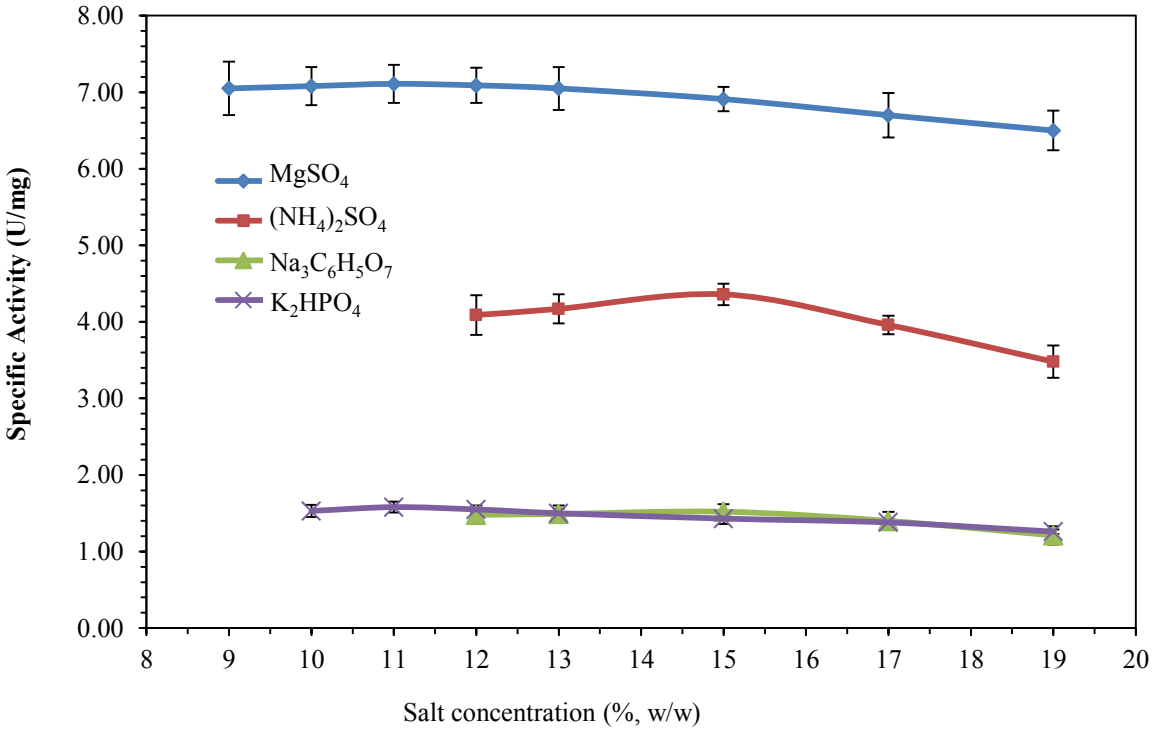


Figure 7.4: SA in the top phase as a function of salt concentration (mean ± S.D, n = 3).

Table 7.12: ANOVA of SA (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	305.021			
T	3	301.505	100.502	3610.62	0.0001
C	4	1.820	0.455	16.35	0.0001
TC	12	0.582	0.049	1.47	0.0930
Error	40	1.113			

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.996$,
CV = 66.37%

Table 7.13: The effects of salt type and concentration on SA.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$MgSO_4$	15	6.85	A
$(NH_4)_2SO_4$	15	4.01	B
K_2HPO_4	15	1.42	C
$Na_3C_6H_5O_7$	15	1.42	C
Salt concentration (%)			
12	12	3.55	A
13	12	3.55	A
15	12	3.55	A
17	12	3.36	A
19	12	3.11	A

level while SA of $Na_3C_6H_5O_7$ and K_2HPO_4 were not significantly different from each other.

The SA of $MgSO_4$ increased from 7.05 to 7.11 U/mg (0.85%) when salt concentration was increased from 9 to 11% (22.22%) and then decreased from 7.11 to 6.50 U/mg (8.58%) when salt concentration further increased from 11 to 19% (88.89%). The SA of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 4.09 to 4.36 U/mg (6.60%) and from 1.48 to 1.52 U/mg (2.70%) when salt concentration increased from 12 to 15% (25.00%) and then decreased from 4.36 to 3.48 U/mg (20.18%) and from 1.52 to 1.21 U/mg (20.39%) when salt concentrations further increased from 15% to 19% (33.33%), respectively. The SA of K_2HPO_4 increased from 1.53 to 1.58 U/mg (3.27%) when salt concentration increased from 10 to 11% (10.00%) and then decreased from 1.58 to 1.26 U/mg (20.25%) when salt concentration further increased from 11 to 19% (80.00%). Highest SA was achieved with $MgSO_4$ at the concentration of 12%.

7.2.1.5 C_P

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on C_P are shown in Figure 7.5. All C_P in both phases, as well as total C_P values, initially increased slightly and then decreased gradually with increased salt concentration. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.14 – 7.19), salt type and salt concentration had significant effects on C_P in top, bottom and total phases. There was significant interaction between salt type and salt concentration in all phases. Grouping results indicated that in the top and total phases C_P of the four salts were significantly different. In the bottom phase C_P of $Na_3C_6H_5O_7$, $MgSO_4$ and one salt from $(NH_4)_2SO_4$ and K_2HPO_4 were significantly different at the 0.05 level while C_P of $(NH_4)_2SO_4$ and K_2HPO_4 were not significantly different.

In the top phase, the C_P of $MgSO_4$ increased from 0.93 to 0.96 mg (3.23%) when salt concentration increased from 9 to 10% (11.11%) and then decreased from 0.96 to 0.52 mg (45.83%) when salt concentration further increased from 10 to 19% (100.00%). The C_P of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 1.72 to 1.79 mg (4.07%) and from 2.46 to 2.54 mg (3.25%) when salt concentrations increased from 12 to 15% (25.00%) and then decreased from 1.79 to 1.60 mg (10.61%) and from 2.54 to 2.38 mg (6.30%)

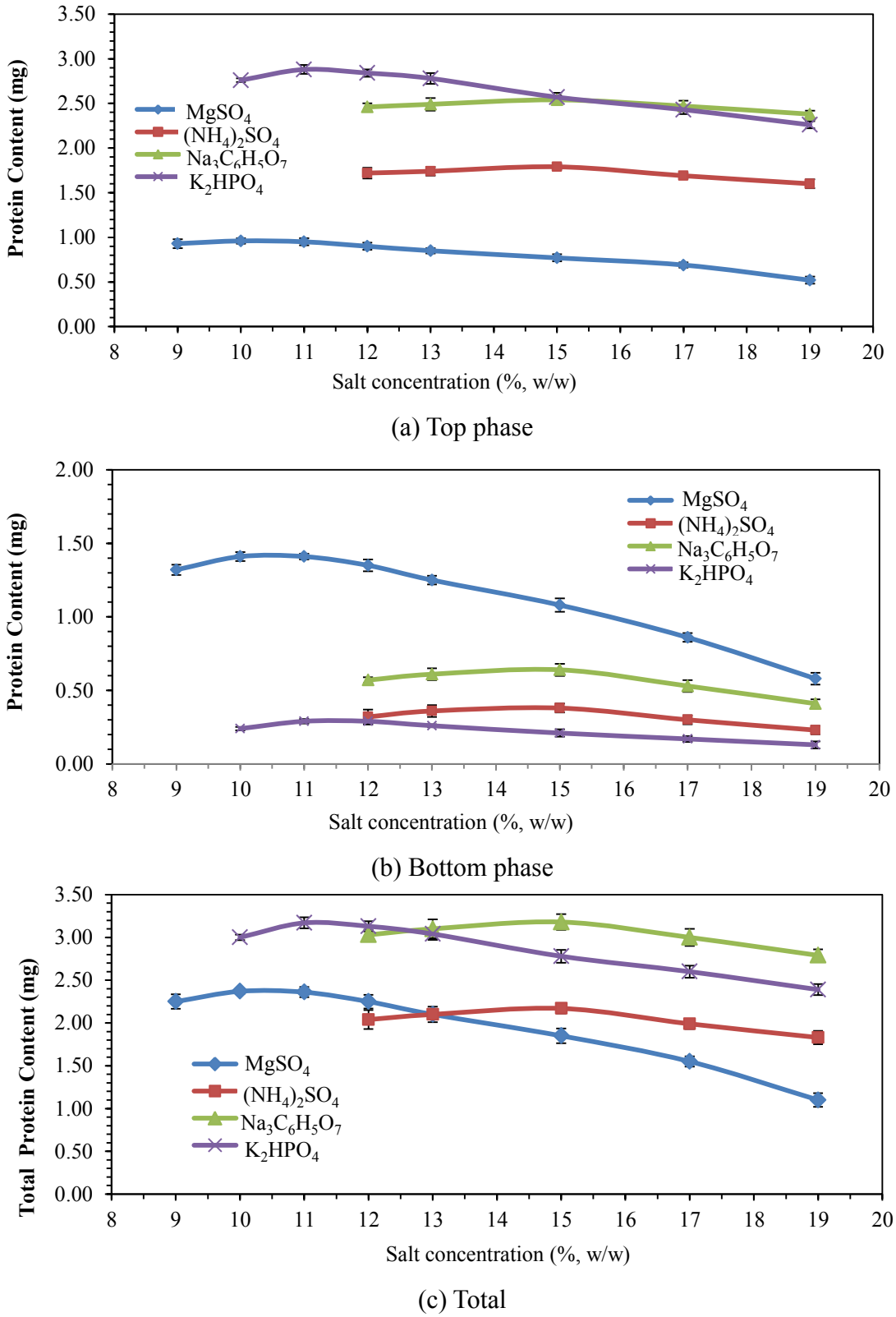


Figure 7.5: C_P in the top, bottom and total phases as a function of salt concentration (mean ± S.D, n = 3).

Table 7.14: ANOVA of C_P in the top phase (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	33.3353			
T	3	32.1837	10.7279	4989.72	0.0001
C	4	0.6982	0.1745	81.18	0.0001
TC	12	0.3674	0.0306	14.24	0.0001
Error	40	0.0860	0.0022		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.997$,
CV = 40.10%

Table 7.15: The effects of salt type and concentration on C_P in the top phase.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
K_2HPO_4	15	2.58	A
$Na_3C_6H_5O_7$	15	2.47	B
$(NH_4)_2SO_4$	15	1.71	C
$MgSO_4$	15	0.75	D
Salt concentration (%)			
12	12	1.98	A
13	12	1.97	A
15	12	1.92	A
17	12	1.82	A
19	12	1.69	A

Table 7.16: ANOVA of C_P in the bottom phase (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	7.24382			
T	3	5.85808	1.95269	1765.15	0.0001
C	4	0.74499	0.18625	168.36	0.0001
TC	12	0.59649	0.04971	44.93	0.0001
Error	40	0.04425	0.00111		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration
$R^2 = 0.99$,
CV = 66.55%

Table 7.17: The effects of salt type and concentration on C_P in the bottom phase.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$MgSO_4$	15	1.02	A
$Na_3C_6H_5O_7$	15	0.55	B
$(NH_4)_2SO_4$	15	0.32	C
K_2HPO_4	15	0.21	C
Salt concentration (%)			
12	12	0.63	A
13	12	0.62	AB
15	12	0.58	AB
17	12	0.47	AB
19	12	0.34	B

Table 7.18: ANOVA of total C_P (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	20.2894			
T	3	16.0598	5.35326	1699.06	0.0001
C	4	2.8878	0.72195	229.14	0.0001
TC	12	1.2158	0.10132	32.16	0.0001
Error	40	0.1260	0.00315		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 99.38$,
CV = 24.42%

Table 7.19: The effects of salt type and concentration on total C_P.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$Na_3C_6H_5O_7$	15	3.0199	A
K_2HPO_4	15	2.7881	B
$(NH_4)_2SO_4$	15	2.0273	C
$MgSO_4$	15	1.7701	D
Salt concentration (%)			
12	12	2.6137	A
13	12	2.5851	A
15	12	2.4950	AB
17	12	2.2849	AB
19	12	2.0279	B

when salt concentration further increased from 15 to 19% (33.33%). The C_P of K_2HPO_4 increased from 2.76 to 2.88 mg (4.35%) when salt concentration increased from 10 to 11% (10.00%) and then decreased from 2.88 to 2.26 mg (21.53%) when salt concentration further increased from 11 to 19% (another 80.00%). The Highest C_P in the top phase was given by K_2HPO_4 at 12% concentration.

In the bottom phase, the C_P of $MgSO_4$ increased from 1.32 to 1.42 mg (7.58%) when the salt concentration increased from 9 to 10% (11.11%) and then decreased from 1.42 to 0.58mg (59.15%) when salt concentration further increased from 10 to 19% (100.00%). The C_P of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 0.32 to 0.38 mg (18.75%) and from 0.57 to 0.64 mg (12.28%) when salt concentration increased from 12 to 15% (25.00%) and then decreased from 0.38 to 0.23mg (39.47%) and from 0.64 to 0.41 mg (35.94%) when salt concentration further increased from 15 to 19% (33.33%). The C_P of K_2HPO_4 increased from 0.24 to 0.29 mg (20.83%) when salt concentration increased from 10 to 11% (10.00%) and then decreased from 0.29 to 0.13mg (55.17%) when salt concentration further increased from 11 to 19% (80.00%). Highest C_P in the bottom phase was given by $MgSO_4$ at 12% concentration.

The total C_P of $MgSO_4$ increased from 2.25 to 2.37 mg (5.33%) when its concentration increased from 9 to 10% (11.11%) and then decreased from 2.37 to 1.10 mg (53.59%) when salt concentration further increased from 10 to 19% (100.00%). The total C_P of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 2.04 to 2.17 mg (6.37%) and from 3.03 to 3.18 mg (4.95%) when salt concentration increased from 12 to 15% (25.00%) and then decreased from 2.17 to 1.83 mg (15.67%) and from 3.18 to 2.79 mg (12.26%) when salt concentration further increased from 15 to 19% (33.33%). The total C_P of K_2HPO_4 increased from 3.00 to 3.17 mg (5.67%) when salt concentration increased from 10 to 11% (10.00%) and then decreased from 3.17 to 2.39 mg (78.00%) when the concentration further increased from 11 to 19% (80.00%). Highest C_P in the total phase was given by $Na_3C_6H_5O_7$ at 15% concentration.

7.2.1.6 K_P

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on K_P are shown in Figure 7.6. K_P of $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 initially decreased and then

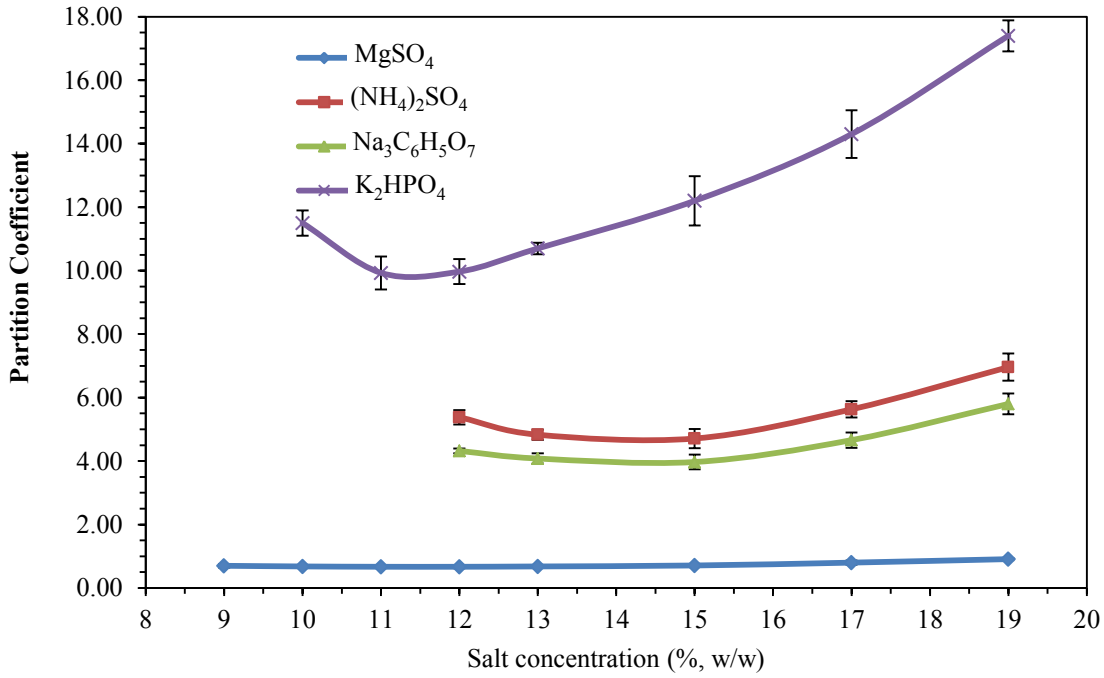


Figure 7.6: K_P as a function of salt concentration (mean ± S.D, n = 3).

Table 7.20: ANOVA of K_P (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	1284.02			
T	3	1158.41	386.137	3601.35	0.0001
C	4	61.99	15.498	144.54	0.0001
TC	12	59.33	4.944	46.12	0.0001
Error	40	4.29	0.107		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.997$,
CV = 78.71%

Table 7.21: The effects of salt type and concentration on K_P.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
K_2HPO_4	15	12.9	A
$(NH_4)_2SO_4$	15	5.50	B
$Na_3C_6H_5O_7$	15	4.57	B
$MgSO_4$	15	0.75	C
Salt concentration (%)			
12	12	5.08	A
13	12	5.07	A
19	12	7.73	A
17	12	6.35	A
15	12	5.40	A

increased gradually with increased salt concentration. K_P of $MgSO_4$ slightly decreased and then increased slightly with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.20 and 7.21), salt type and salt concentration had significant effects on K_P. There was also a significant interaction between salt type and salt concentration. Grouping results indicated that K_P of $MgSO_4$, K_2HPO_4 and one salt from $Na_3C_6H_5O_7$ and $(NH_4)_2SO_4$ were significantly different while K_P of $Na_3C_6H_5O_7$ and $(NH_4)_2SO_4$ were not.

The K_P of $MgSO_4$ decreased from 0.70 to 0.67 (4.29%) when salt concentration increased from 9 to 11% (22.22%) and then increased from 0.67 to 0.91 (35.82%) when salt concentration further increased from 11 to 19% (88.89%). The K_P of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ decreased from 5.38 to 4.71 (12.45%) and from 4.32 to 3.97 (8.10%) when the salt concentrations increased from 12 to 15% (25.00%) and then increased from 4.71 to 6.96 (47.77%) and from 3.97 to 5.80 (46.10%) when the salt concentrations further increased from 15 to 19% (33.33%). The K_P of K_2HPO_4 decreased from 11.5 to 9.97 (13.30%) when the salt concentration increased from 10 to 12% (20.00%) and then increased from 9.97 to 15.2 (52.46%) when the salt concentration further increased from 12 to 19% (70.00%). Highest K_P was achieved with K_2HPO_4 at 19% concentration.

7.2.1.7 PF

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on PF are shown in Figure 7.7. All PF increased initially and then decreased with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.22 and 7.23), salt type had a significant effect on PF and salt concentration also had a significant effect except for $MgSO_4$. Grouping data showed that PF of $MgSO_4$, $(NH_4)_2SO_4$ and one salt from $Na_3C_6H_5O_7$ and K_2HPO_4 were significantly different while PF of $Na_3C_6H_5O_7$ and K_2HPO_4 were not significantly different from each other.

The PF of $MgSO_4$ increased from 6.80 to 6.85 (0.74%) when salt concentration increased from 9 to 11% (22.22%) and then decreased from 6.85 to 6.26 (8.61%) when salt concentration further increased from 11 to 19% (another 88.89%). The PF of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 3.94 to 4.20 (6.60%) and from 1.42 to 1.46 (2.82%) when salt concentrations increased from 12 to 15% (25.00%) and was decreased

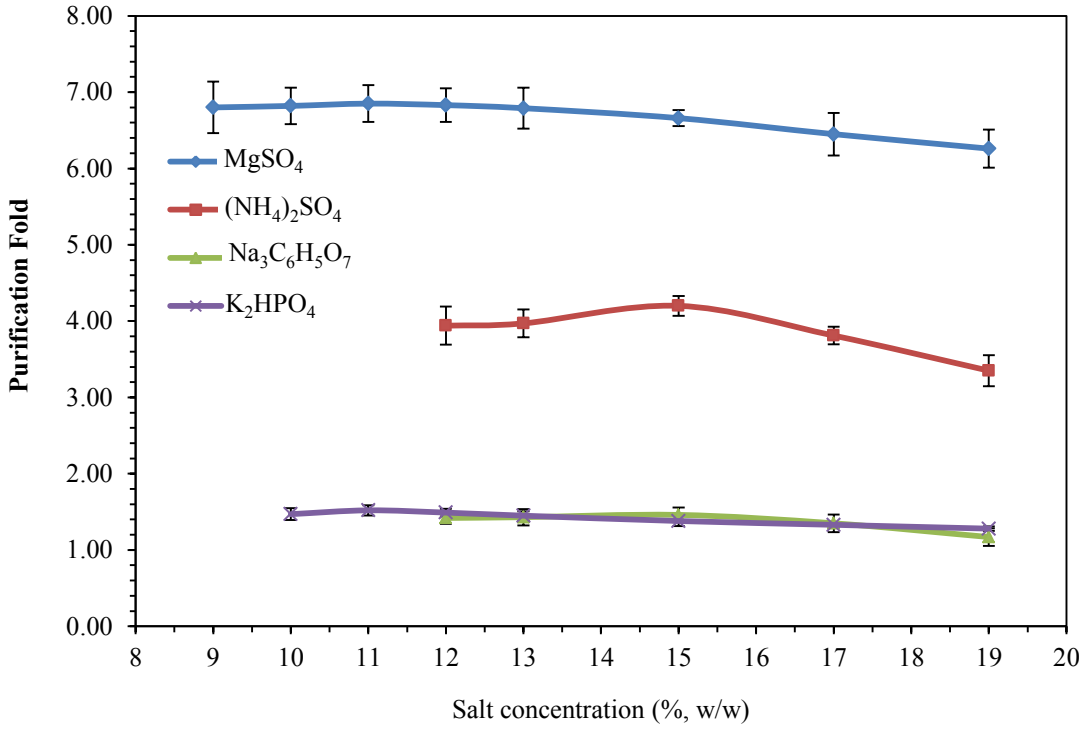


Figure 7.7: PF in the top phase as a function of salt concentration (mean ± S.D, n = 3).

Table 7.22: ANOVA of PF (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	281.986	92.9410		
T	3	278.823	0.3833	3617.56	0.0001
C	4	1.533	0.0501	14.92	0.0001
TC	12	0.602	0.0257	1.95	0.0570
Error	40	1.028			

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.996$,
CV = 66.22%

Table 7.23: The effects of salt type and concentration on PF.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$MgSO_4$	15	6.60	A
$(NH_4)_2SO_4$	15	3.85	B
K_2HPO_4	15	1.39	C
$Na_3C_6H_5O_7$	15	1.37	C
Salt concentration (%)			
12	12	3.42	A
13	12	3.41	A
15	12	3.43	A
17	12	3.24	A
19	12	3.02	A

from 4.20 to 3.35 (20.24%) and from 1.46 to 1.17 (19.86%) when salt concentrations further increased from 15 to 19% (33.33%), respectively. The PF of K_2HPO_4 increased from 1.35 to 1.52 (12.59%) when the salt concentration increased from 10 to 11% (10.00%) and then decreased from 1.52 to 1.28 (15.79%) when salt concentration further increased from 11 to 19% (80.00%). The highest PF was achieved with $MgSO_4$ at 12% concentration.

7.2.1.8 RY

The effects of $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 on RY are shown in Figure 7.8. All RY increased initially and then decreased with increased salt concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.24 and 7.25), salt type and salt concentration had significant effects on RY. There was also a significant interaction between salt type and salt concentration. Grouping data showed that PF of $MgSO_4$, $(NH_4)_2SO_4$ and one salt from $Na_3C_6H_5O_7$ and K_2HPO_4 were significantly different from one another while PF of $Na_3C_6H_5O_7$ and K_2HPO_4 were not significantly different from each other.

The RY of $MgSO_4$ increased from 60.2 to 62.4% (3.65%) when salt concentration increased from 9 to 10% (11.11%) and then decreased from 62.4 to 31.0% (50.32%) when salt concentration further increased from 10 to 19% (100.0%). The RY of $(NH_4)_2SO_4$ and $Na_3C_6H_5O_7$ increased from 64.7 to 71.7% (10.82%) and from 33.3 to 35.3% (6.01%) respectively when salt concentration increased from 12 to 15% (25.00%) and then decreased from 71.7 to 51.2% (28.59%) and from 35.3 to 26.5% (24.93%) when salt concentration further increased from 15 to 19% (33.33%). The RY of K_2HPO_4 increased from 38.7 to 41.3% (6.72%) when salt concentration increased from 10 to 11% (10.00%) and then decreased from 41.3 to 27.7% (32.93%) when salt concentration further increased by another 80.00% (from 11 to 19%). Highest RY was achieved with $(NH_4)_2SO_4$ at 15% concentration.

7.2.2 Effect of PEG Molecular Weight and Concentration

The effects of PEG 1000, PEG 1500, PEG 3000 and PEG 4000 (each at PEG concentrations of 16, 18, 20, 22 and 24%) on ATPS purification are presented in Tables

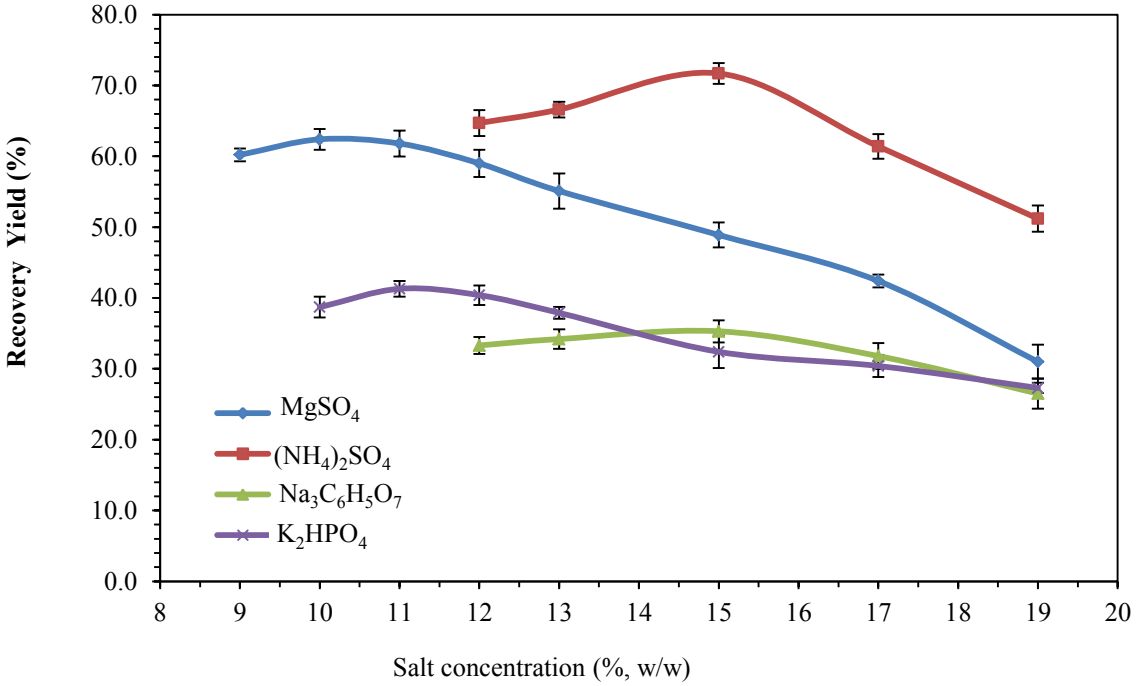


Figure 7.8: RY of PG in the top phase as a function of salt concentration (mean ± S.D, n = 3).

Table 7.24: ANOVA of RY (effects of salt type and concentration).

Source	DF	SS	MS	F	P
Total	59	13247.5			
T	3	10109.5	3369.82	438.73	0.0001
C	4	2207.8	551.94	71.86	0.0001
TC	12	623.1	51.92	6.76	0.0001
Error	40	307.2	7.68		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
T: Salt type,
C: Salt concentration,
TC: Interaction of salt type and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 33.74%

Table 7.25: The effects of salt type and concentration on RY.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
Salt type			
$(NH_4)_2SO_4$	15	64.5	A
$MgSO_4$	15	47.3	B
K_2HPO_4	15	33.7	C
$Na_3C_6H_5O_7$	15	32.2	C
Salt concentration (%)			
12	12	51.0	A
13	12	48.5	A
15	12	47.1	A
17	12	41.5	AB
19	12	34.0	B

Table 7.26: Effects of PEG 1000 on partition of 1g PG using 15% $(NH_4)_2SO_4$.

PEG 1000 concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
16	4.44±0.01	4.06±0.01	8.50±0.01	1.09±0.01	7.32±0.18	1.78±0.03	0.42±0.02	2.21±0.05	4.11±0.17	4.58±0.17	3.96±0.16	66.3±1.67
18	4.41±0.01	3.86±0.01	8.27±0.02	1.14±0.01	7.81±0.16	1.81±0.03	0.38±0.03	2.19±0.07	4.36±0.14	4.71±0.30	4.20±0.13	71.7±1.48
20	4.71±0.01	3.90±0.01	8.61±0.02	1.21±0.01	6.99±0.20	1.74±0.03	0.37±0.02	2.11±0.01	4.02±0.18	4.65±0.17	3.65±0.17	64.2±1.85
22	4.73±0.01	3.64±0.02	8.37±0.02	1.30±0.01	5.46±0.17	1.55±0.03	0.35±0.03	1.90±0.05	3.53±0.16	4.48±0.28	3.40±0.15	50.2±1.57
24	4.63±0.01	3.40±0.01	8.03±0.01	1.36±0.01	4.31±0.16	1.25±0.02	0.31±0.02	1.56±0.04	3.25±0.11	4.06±0.20	3.13±0.11	39.6±1.48

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.27: Effects of PEG 1500 on partition of 1g PG using 15% $(NH_4)_2SO_4$.

PEG 1500 concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
16	4.20±0.01	4.47±0.01	8.67±0.02	0.94±0.00	8.62±0.14	1.70±0.04	0.63±0.02	2.33±0.02	5.07±0.20	2.70±0.15	4.89±0.19	79.1±1.30
18	4.28±0.01	4.12±0.01	8.40±0.01	1.04±0.00	9.39±0.10	1.73±0.03	0.60±0.03	2.33±0.05	5.43±0.15	2.88±0.12	5.23±0.14	86.2±0.93
20	4.67±0.01	4.17±0.01	8.84±0.01	1.12±0.01	8.20±0.12	1.64±0.04	0.59±0.02	2.23±0.02	5.01±0.10	2.76±0.16	4.83±0.10	75.3±1.11
22	4.72±0.01	3.93±0.01	8.65±0.01	1.20±0.01	6.71±0.08	1.44±0.03	0.55±0.02	1.99±0.02	4.66±0.14	2.60±0.12	4.49±0.13	61.6±0.74
24	4.61±0.01	3.74±0.01	8.35±0.02	1.24±0.01	5.19±0.15	1.15±0.02	0.49±0.03	1.64±0.05	4.50±0.13	2.37±0.13	4.35±0.13	47.7±1.39

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.28: Effects of PEG 3000 on partition of 1g PG using 15% $(NH_4)_2SO_4$.

PEG 3000 concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
16	3.89±0.01	4.87±0.01	8.76±0.01	0.80±0.00	5.52±0.10	1.55±0.02	0.82±0.03	2.37±0.03	3.56±0.11	1.89±0.10	3.16±0.11	50.7±0.93
18	3.98±0.01	4.45±0.01	8.43±0.02	0.89±0.00	6.31±0.06	1.57±0.05	0.78±0.02	2.35±0.06	4.02±0.17	2.01±0.06	3.88±0.16	58.0±0.56
20	4.52±0.01	4.38±0.01	8.90±0.02	1.03±0.00	4.97±0.15	1.43±0.04	0.74±0.03	2.17±0.04	3.46±0.19	1.94±0.08	3.34±0.18	45.7±1.48
22	4.55±0.02	4.15±0.01	8.70±0.03	1.10±0.00	3.90±0.18	1.18±0.03	0.69±0.02	1.87±0.01	3.30±0.13	1.70±0.09	3.18±0.13	35.8±1.67
24	4.58±0.01	3.81±0.01	8.39±0.01	1.20±0.01	2.83±0.17	0.93±0.02	0.61±0.02	1.54±0.04	3.03±0.12	1.53±0.04	2.92±0.12	26.0±1.57

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

Table 7.29: Effects of PEG 4000 on partition of 1g PG using 15% $(NH_4)_2SO_4$.

PEG 4000 concentration (%, w/w)	Volume (ml)			V_R	A_E (U)	C_p			SA (U/mg)	K_P	PF	RY (%)
	Top phase (ml)	Bottom phase (ml)	Total (ml)			Top phase (mg)	Bottom phase (mg)	Total (mg)				
16	3.54±0.01	4.78±0.01	8.32±0.02	0.74±0.00	4.35±0.16	1.49±0.04	1.05±0.03	2.54±0.06	2.92±0.16	1.42±0.04	2.81±0.15	40.0±1.48
18	3.94±0.01	3.99±0.01	7.93±0.02	0.89±0.00	4.85±0.10	1.51±0.02	1.01±0.03	2.52±0.03	3.21±0.09	1.50±0.06	3.10±0.09	44.5±0.93
20	4.10±0.01	4.28±0.01	8.38±0.02	0.96±0.00	3.72±0.19	1.34±0.03	0.95±0.03	2.30±0.05	2.77±0.19	1.41±0.04	2.67±0.18	34.2±1.85
22	4.16±0.01	4.03±0.01	8.19±0.01	1.03±0.01	2.72±0.12	1.07±0.03	0.85±0.02	1.92±0.03	2.54±0.11	1.26±0.06	2.45±0.11	25.0±1.11
24	4.27±0.01	3.63±0.01	7.89±0.02	1.18±0.00	1.95±0.09	0.86±0.02	0.75±0.02	1.61±0.04	2.28±0.14	1.15±0.03	2.20±0.13	17.9±0.83

Top phase: PEG phase

Bottom phase: salt phase

V_R: volume ratio (the volume of top phase / the volume of bottom phase)

A_E: enzyme activity in the top phase

C_P: protein content in top and bottom phases

SA: specific activity (A_E / C_P) in the top phase

K_P: partition coefficient (C_P of top phase / C_P of bottom phase) for the overall ATPS system

PF: purification fold (SA of purified enzyme / SA of crude enzyme) in the top phase

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme) in the top phase

7.26 – 7.29.

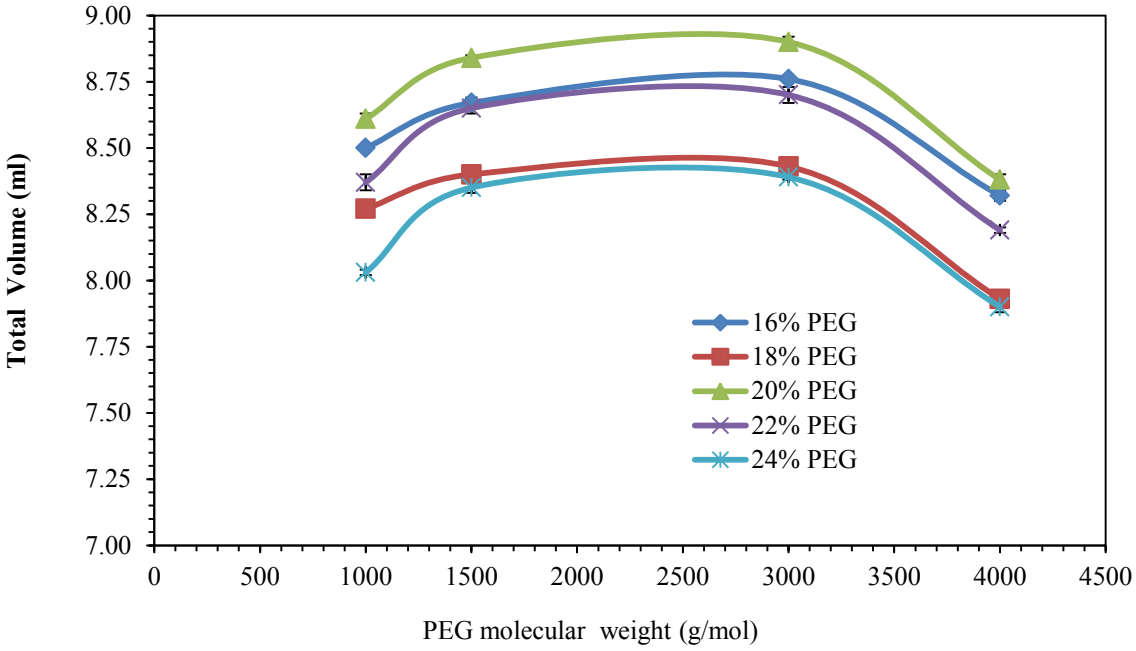
7.2.2.1 TV

The effects of PEG molecular weight and concentration on TV are presented in Figure 7.9. All TV increased when the PG molecular weight increased from 1000 to 3000 and then slightly decreased when the molecular weight further increased to 4000. All TV gave similarly shaped curves with increased PEG concentrations (decrease-increase-decrease). Based on the ANOVA and Duncan Multiple Range Test (Tables 7.30 and 7.31), PEG molecular weight and PEG concentration had significant effects on TV. There was also a significant interaction between PEG molecular weight and concentration. Grouping data showed that the TV of PEG 1000, PEG 4000 and one salt from PEG 3000 and 1500 were significantly different from one another while TV of PEGS 1500 and 3000 were not significantly different from each other. TV at PEG concentrations of 20%, 22% and one from 24% and 18% were significantly different while TV at 18% and 24% were not.

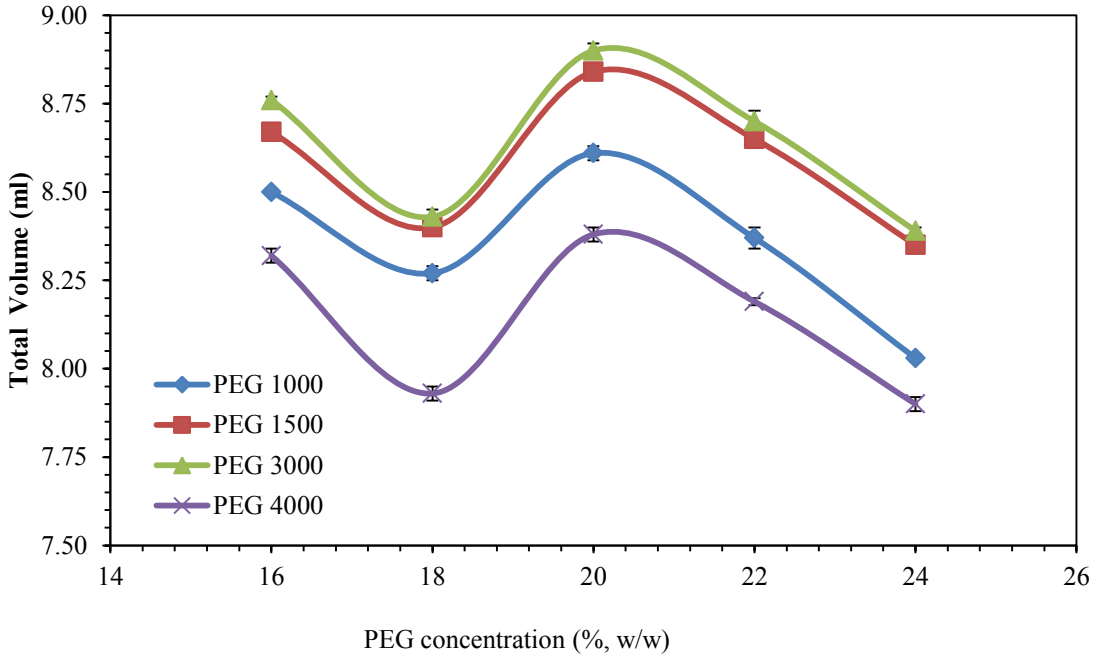
The TV increased from 8.50 to 8.76 ml (3.06%), 8.27 to 8.43ml (1.93%), 8.61 to 8.90ml (3.37%), 8.37 to 8.70ml (3.94%) and 8.03 to 8.39ml (4.48%) when the PEG molecular weight increased from 1000 to 3000 for the PEG concentrations of 16, 18, 20, 22 and 24%, respectively. It then decreased from 8.76 to 8.32 ml (5.02%), 8.43 to 7.93ml (5.93%), 8.90 to 8.38ml (5.84%), 8.70 to 8.19ml (5.86%) and 8.39 to 7.90ml (5.84%) when the PEG molecular weight increased from 3000 to 4000, respectively. The PEG 3000 with the 20% concentration gave the highest TV (8.90 ml).

7.2.2.2 V_R

The effects of PEG molecular weight and PEG concentration on V_R are shown in Figure 7.10. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.32 and 7.33), PEG molecular weight and PEG concentration had significant effects on V_R. There was also a significant interaction between PEG molecular weight and concentration. Grouping data indicated that V_R of PEG 1000, PEG 1500 and one PEG from 3000 and 4000 were significantly different at the 0.05 level while PEG 3000 and 4000 were not. V_R of PEG at concentrations of 20%, 24%, and one from 16% and 18% were significantly different from one another.



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.9: TV as a function of PEG (a) molecular weight and (b) concentration.

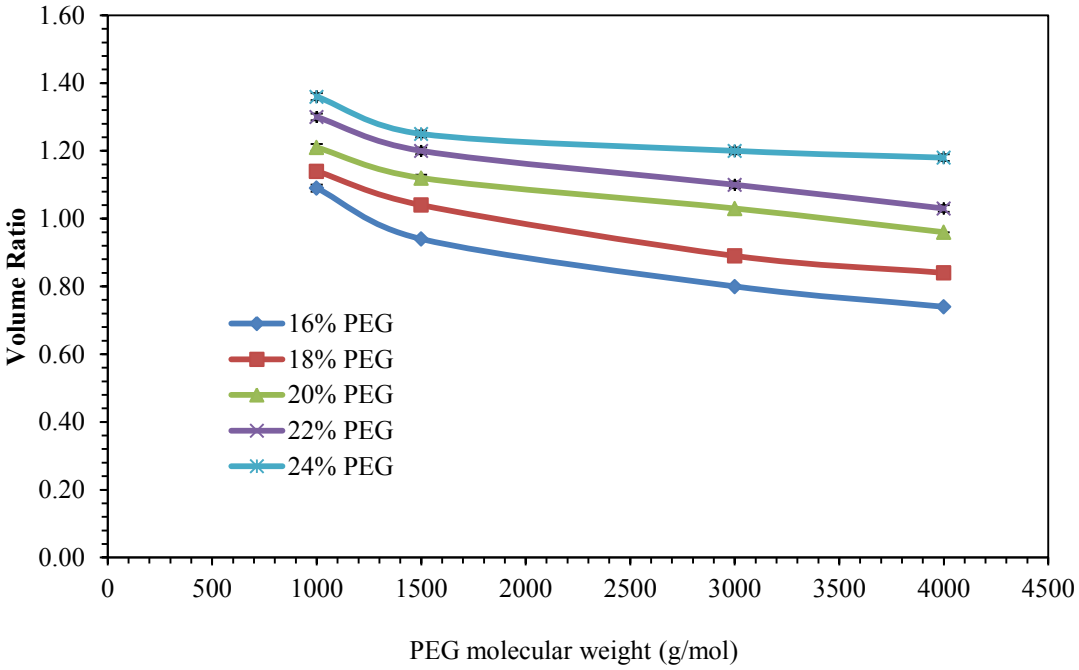
Table 7.30: ANOVA of TV (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	4.55702			
M	3	2.29326	0.764419	2002.85	0.0001
C	4	2.18153	0.545382	1428.95	0.0001
MC	12	0.0697	0.005581	14.62	0.0001
Error	40	0.01527	0.000382		

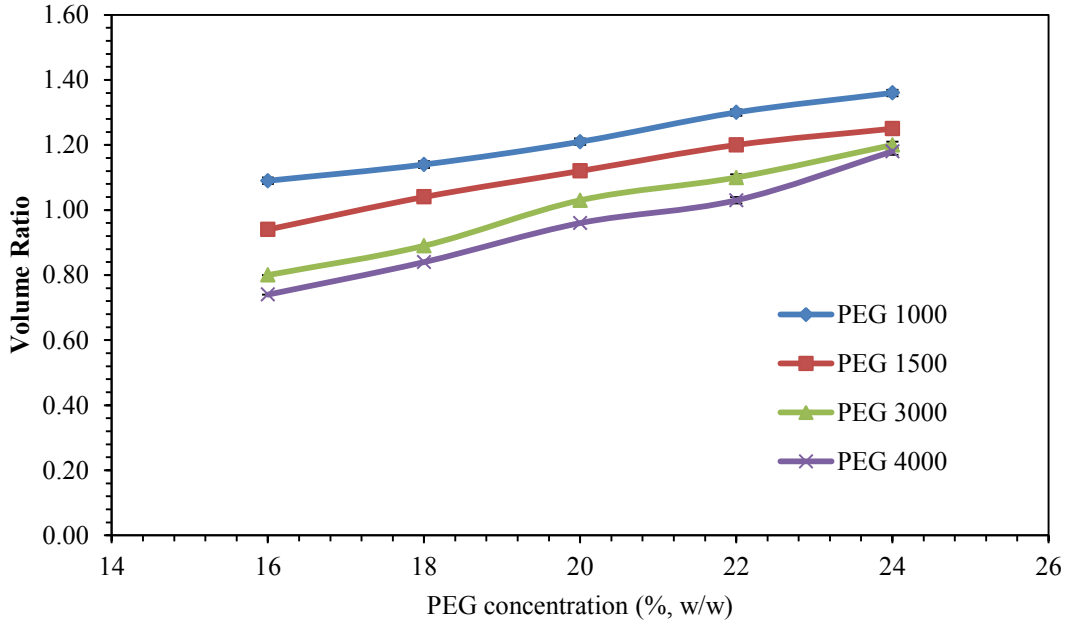
DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.997$,
CV = 3.30%

Table 7.31: The effects of PEG molecular weight and concentration on TV.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	8.36	B
1500	15	8.58	A
3000	15	8.64	A
4000	15	8.14	C
PEG concentration (%)			
16	12	8.56	AB
18	12	8.26	C
20	12	8.68	A
22	12	8.48	B
24	12	8.17	C



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.10: V_R as a function of PEG (a) molecular weight and (b) concentration.

Table 7.32: ANOVA of V_R (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	1.65279			
M	3	0.65469	0.218229	2785.90	0.0001
C	4	0.95694	0.239236	3054.07	0.0001
MC	12	0.03803	0.003169	40.46	0.0001
Error	40	0.00313	0.000078		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.998$,
CV = 15.64%

Table 7.33: The effects of PEG molecular weight and concentration on V_R.

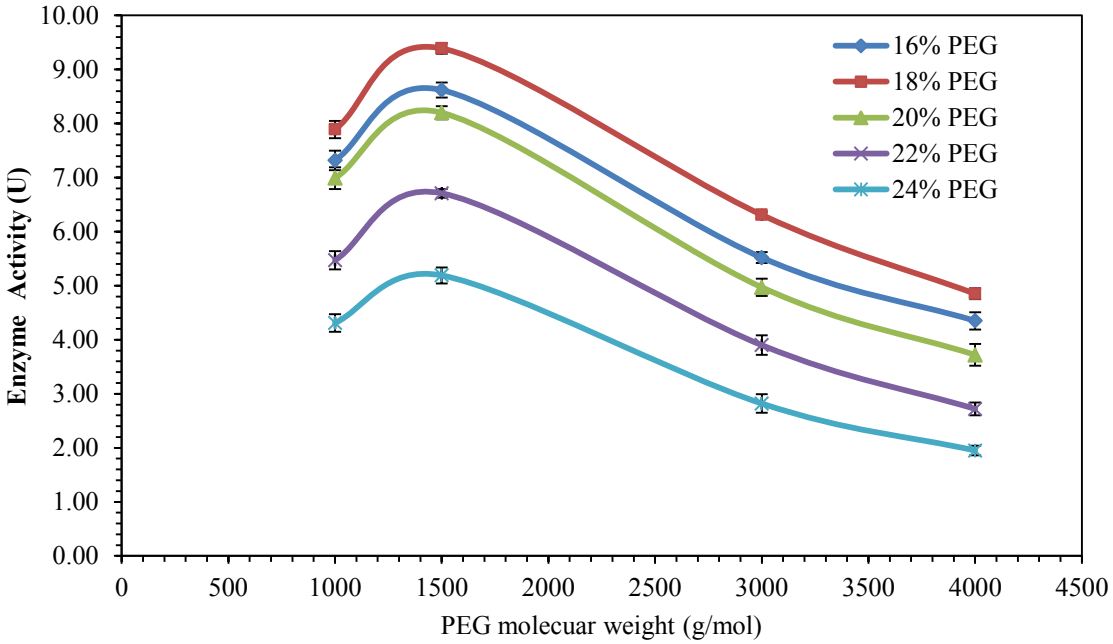
Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	1.22	A
1500	15	1.11	B
3000	15	1.00	C
4000	15	0.95	C
PEG concentration (%)			
16	12	0.89	C
18	12	0.98	C
20	12	1.08	B
22	12	1.16	AB
24	12	1.25	A

The V_R obtained with different PEG concentrations decreased with increasing molecular weight. When PEG molecular weight increased from 1000 to 4000, V_R obtained with PEG concentrations of 16, 18, 20, 22 and 24% decreased from 1.09 to 0.74 (32.11%), from 1.14 to 0.89 (21.93%), from 1.21 to 0.96 (20.66%), from 1.30 to 1.03 (20.77%) and from 1.36 to 1.18 (13.24%), respectively. The V_R obtained with different molecular weights increased linearly with increased PEG concentration. When PEG concentration increased from 16 to 18% (12.50%), the V_R obtained with PEG molecular weights of 1000, 1500, 3000 and 4000 increased from 1.09 to 1.36 (24.77%), from 0.94 to 1.23 (30.85%), from 0.80 to 1.20 (50.00%) and from 0.74 to 1.18 (59.46%), respectively. PEG 1000 at 24% concentration gave the highest V_R value (1.36).

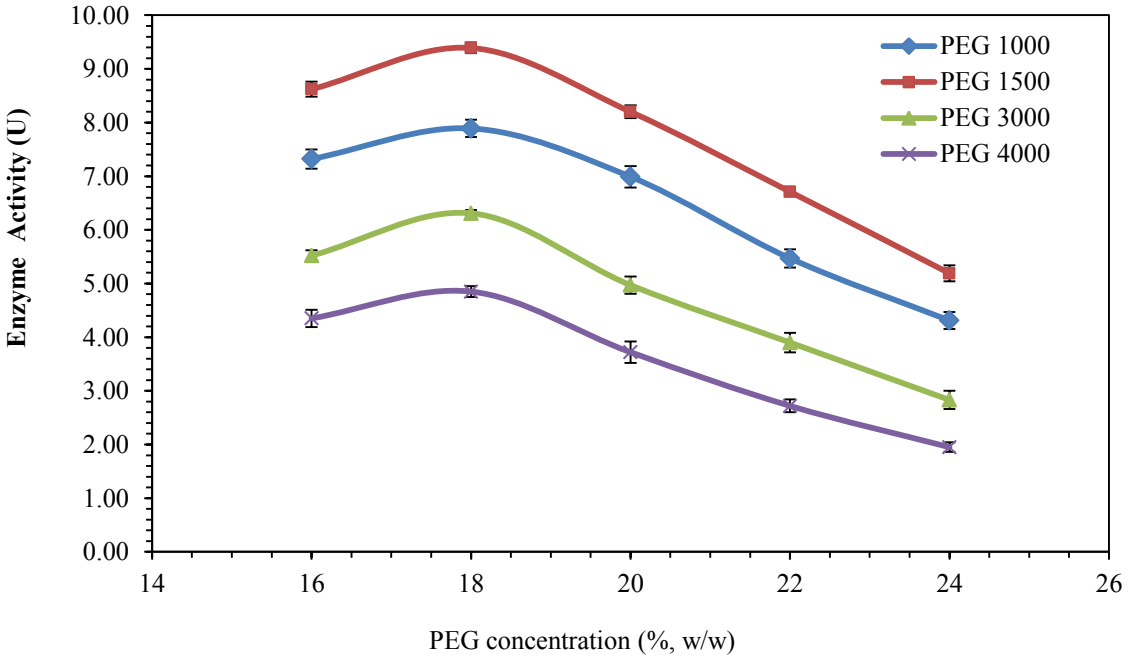
7.2.2.3 A_E

The effects of PEG molecular weight and concentration on A_E are shown in Figure 7.11. The A_E increased initially and then decreased with increased PEG molecular weight or concentration. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.34 – 7.35), PEG molecular weight and PEG concentration had significant effects on A_E. There was also a significant interaction between PEG molecular weight and PEG concentration. Grouping results indicated that the A_E of PEG 1000, 1500, 3000 and 4000 were significantly different from one another at the 0.05 level. A_E of PEG at concentrations of 16%, 18% and 24% were significantly different from one another.

For PEG concentrations of 16, 18, 20, 22 and 24%, the A_E increased from 7.32 to 8.62 U (17.76%), from 7.81 to 9.39 U (20.23%), from 6.99 to 8.20 U (17.31%), from 5.47 to 6.71 U (22.67%) and from 4.31 to 5.19 U (20.42%) when PEG molecular weight increased from 1000 to 1500 and then decreased from 8.62 to 4.35 U (49.54%), from 9.39 to 4.85 (48.35%), from 8.20 to 3.72 (54.63%), from 6.71 to 2.72 (59.46%) and from 5.19 to 1.95 (62.43%), when PEG molecular weight increased from 1500 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the A_E increased from 7.32 to 7.81 U (6.69%), from 8.62 to 9.39 U (8.93%), from 5.52 to 6.31 U (14.31%) and from 4.35 to 4.85 U (11.49%) when PEG concentration increased from 16% to 18% (12.50%) and then decreased from 7.81 to 4.31 U (44.81%), from 9.39 to 5.19 U (44.73%), from 6.31 to 2.83 U (55.15%) and from 4.85 to 1.95 U (59.79%) when the PEG concentration



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.11: A_E in the top phase as a function of PEG (a) molecular weight and (b) concentration.

Table 7.34: ANOVA of A_E (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	247.289			
M	3	147.699	49.2330	2285.66	0.0001
C	4	96.757	24.1892	1122.99	0.0001
MC	12	1.972	0.1643	7.63	0.0001
Error	40	0.862	0.0215		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.997$,
CV = 36.82%

Table 7.35: The effects of PEG molecular weight and concentration on A_E.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	6.40	B
1500	15	7.62	A
3000	15	4.71	C
4000	15	3.52	D
PEG concentration (%)			
16	12	6.45	A
18	12	7.11	A
20	12	5.97	AB
22	12	4.70	BC
24	12	3.57	C

increased from 18% to 24% (37.50%), respectively. For all PEG molecular weights, the highest A_E were achieved at 18% concentration. PEG 1500 with 18% concentration gave the highest A_E (9.39 U).

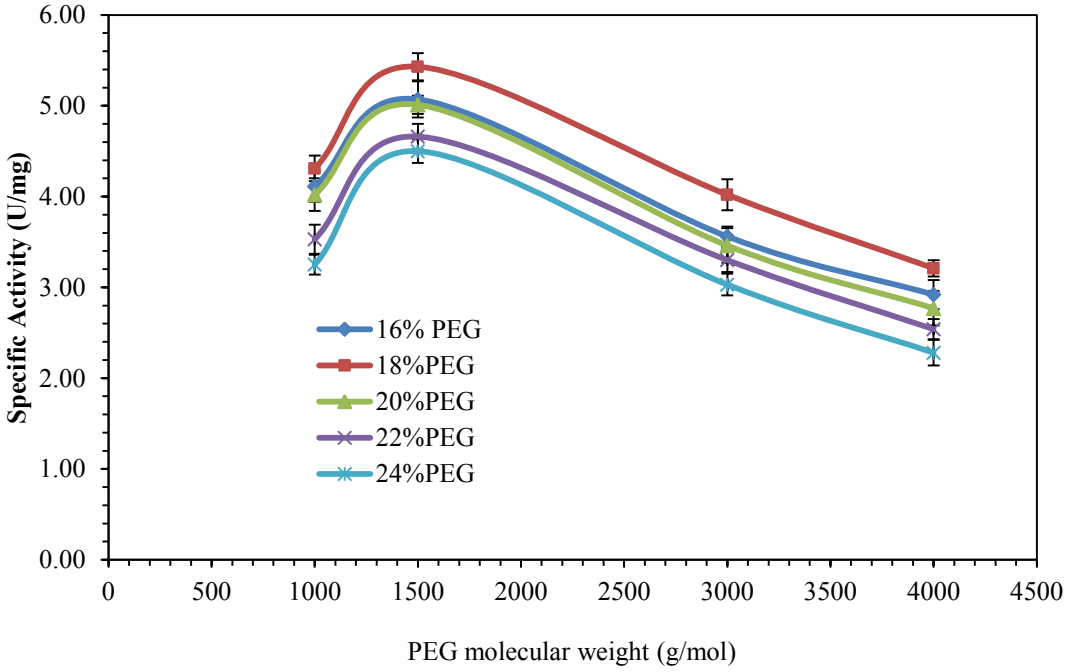
7.2.2.4 SA

The effects of PEG molecular weight and concentration on SA are shown in Figure 7.12. SA increased initially and then decreased with increased PEG molecular weight or concentration. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.36 and 7.37), PEG molecular weight and PEG concentration had significant effects on SA. Grouping results indicated that the SA of PEG 1000, 1500, 3000 and 4000 were significantly different from one another at the 0.05 level. The SA of PEG at concentrations of 18% and one from 22% and 24% were significantly different from one another.

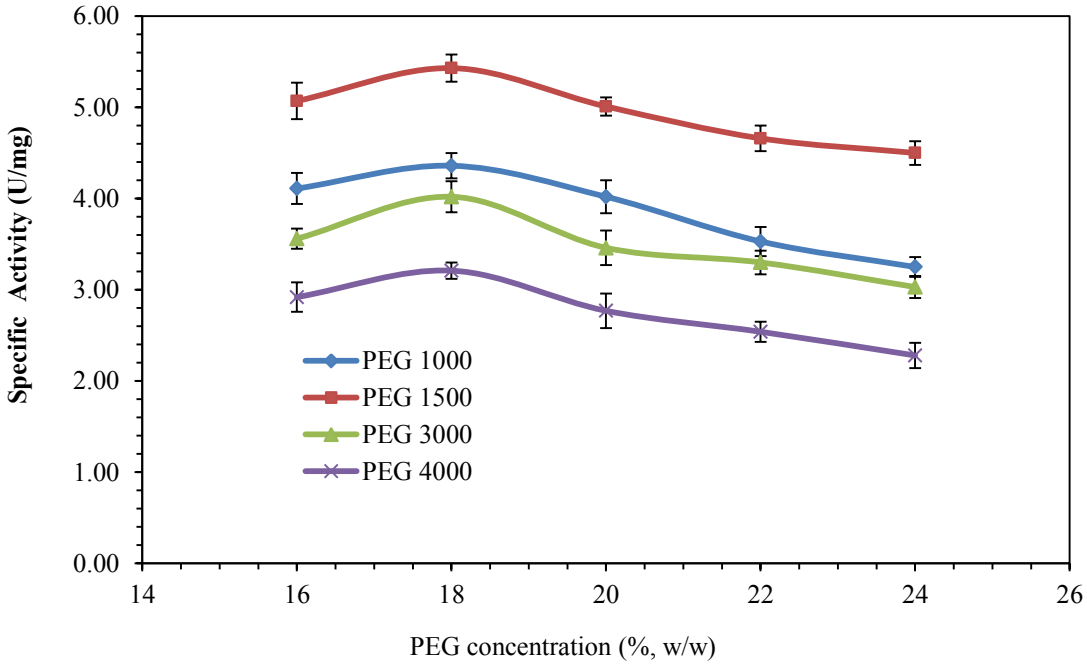
For PEG concentrations of 16, 18, 20, 22 and 24%, the SA increased from 4.11 to 5.07 U/mg (23.36%), from 4.36 to 5.43 U/mg (24.54%), from 4.02 to 5.01 U/mg (24.63%), from 3.53 to 4.66 U/mg (32.01%) and from 3.25 to 4.50 U/mg (38.46%) when PEG molecular weight increased from 1000 to 1500 and then decreased from 5.07 to 2.92 U/mg (42.41%), from 5.43 to 3.21 U/mg (40.88%), from 5.01 to 2.77 U/mg (44.71%), from 4.66 to 2.54 U/mg (45.49%) and from 4.50 to 2.28 U/mg (49.33%) when PEG molecular weight increased from 1500 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the SA increased from 4.11 to 4.36 U/mg (6.08%), from 5.07 to 5.43 U/mg (7.10%), from 3.56 to 4.02 U/mg (12.92%) and from 2.92 to 3.21 U/mg (9.93%) when PEG concentration increased from 16% to 18% (12.5%) and then decreased from 4.36 to 3.25 U/mg (25.5%), from 5.43 to 4.50 U/mg (17.1%), from 4.02 to 3.03 U/mg (24.6%) and from 3.21 to 2.28 U/mg (29.0%) when PEG concentration increased from 18% to 24% (37.50%), respectively. For all PEG molecular weights, the highest SA was achieved at 18% concentration. PEG 1500 at 18% concentration gave the highest SA.

7.2.2.5 C_P

The effects of PEG molecular weight and concentration on C_P in top, bottom and



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.12: SA in the top phase as a function of PEG (a) molecular weight and (b) concentration.

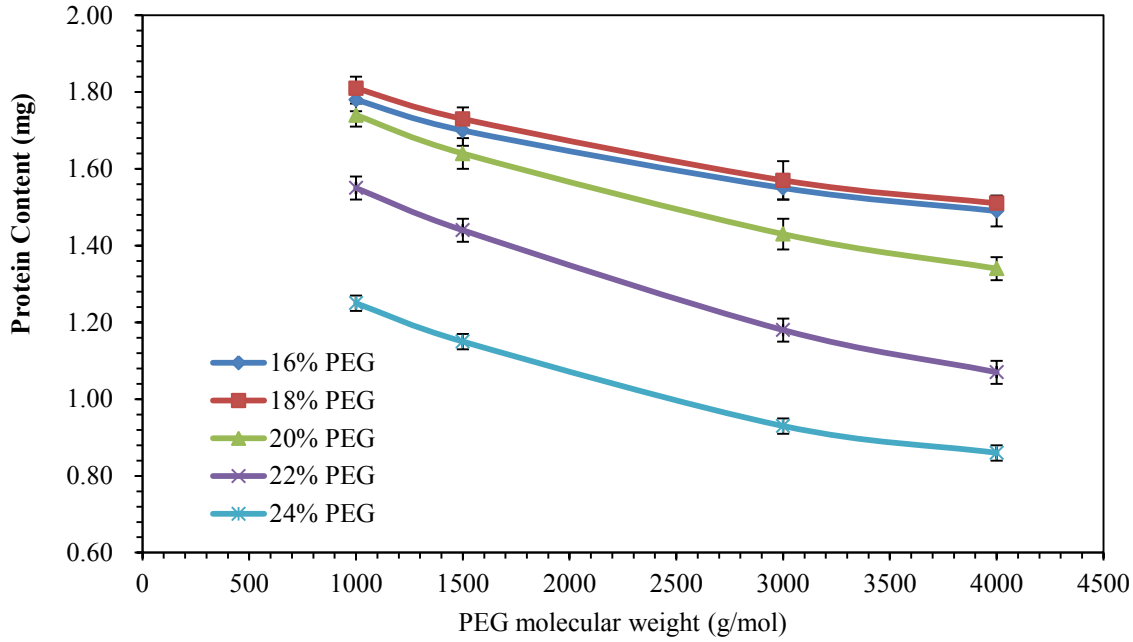
Table 7.36: ANOVA of SA (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	45.6056			
M	3	37.4403	12.4801	582.05	0.0001
C	4	7.0738	1.7684	82.48	0.0001
MC	12	0.2339	0.0195	0.91	0.5470
Error	40	0.8577	0.0214		

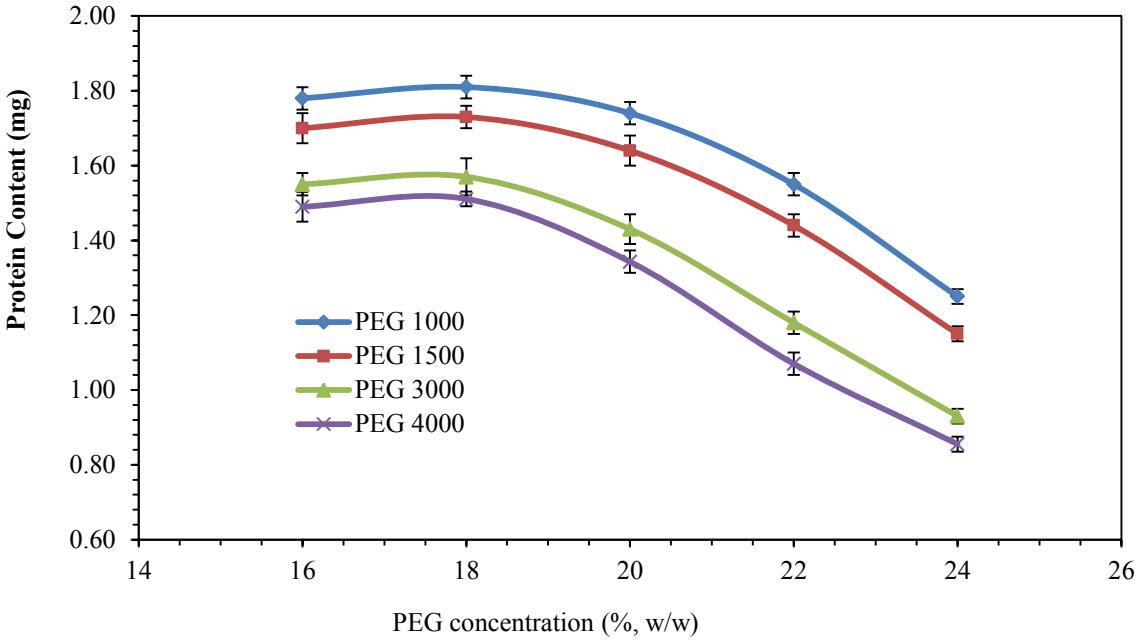
DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.98$,
CV = 23.45%

Table 7.37: The effects of PEG molecular weight and concentration on SA.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	3.85	B
1500	15	4.93	A
3000	15	3.47	C
4000	15	2.74	D
PEG concentration (%)			
16	12	3.92	AB
18	12	4.26	A
20	12	3.82	AB
22	12	3.51	B
24	12	3.26	B



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.13: C_P in the top phase as a function of PEG (a) molecular weight and (b) concentration.

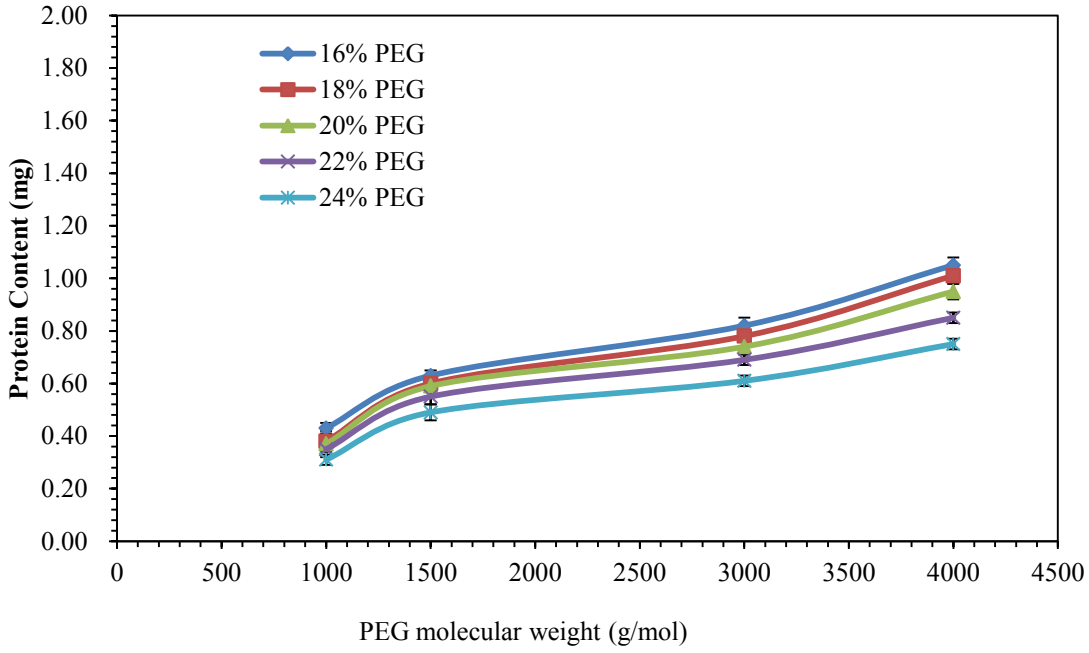
Table 7.38: ANOVA of C_P in the top phase (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	4.59766			
M	3	1.33692	0.445642	436.90	0.0001
C	4	3.17010	0.792525	776.99	0.0001
MC	12	0.04983	0.004153	4.07	0.0001
Error	40	0.04080	0.001020		

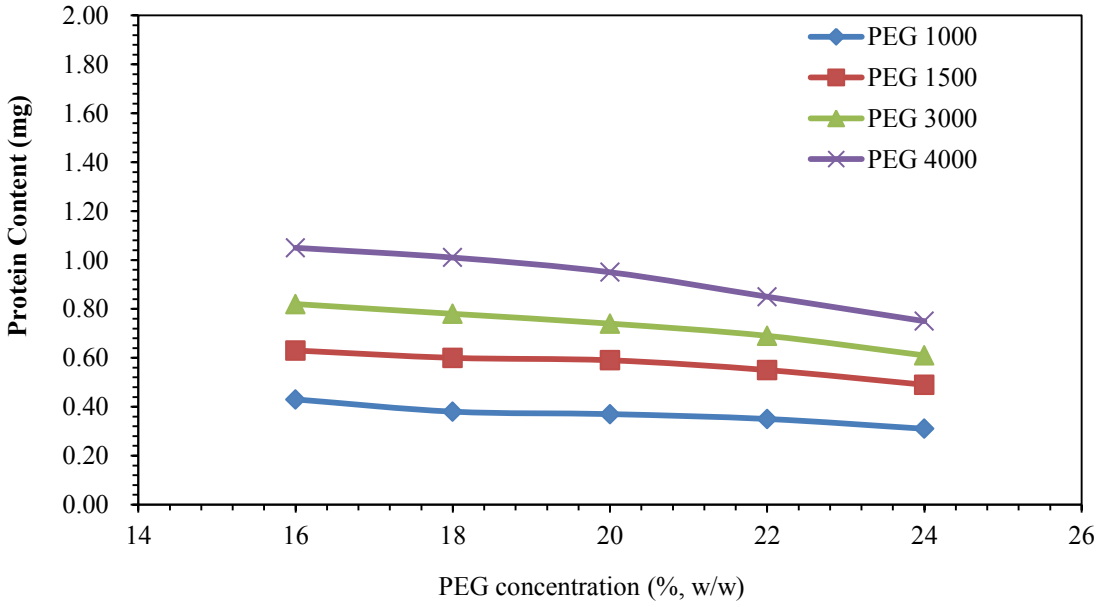
DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 19.44%

Table 7.39: The effects of PEG molecular weight and concentration on C_P in the top phase.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	1.63	A
1500	15	1.53	A
3000	15	1.33	B
4000	15	1.25	B
PEG concentration (%)			
16	12	1.63	A
18	12	1.66	A
20	12	1.54	A
22	12	1.31	B
24	12	1.05	C



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.14: C_P in the bottom phase as a function of PEG (a) molecular weight and (b) concentration.

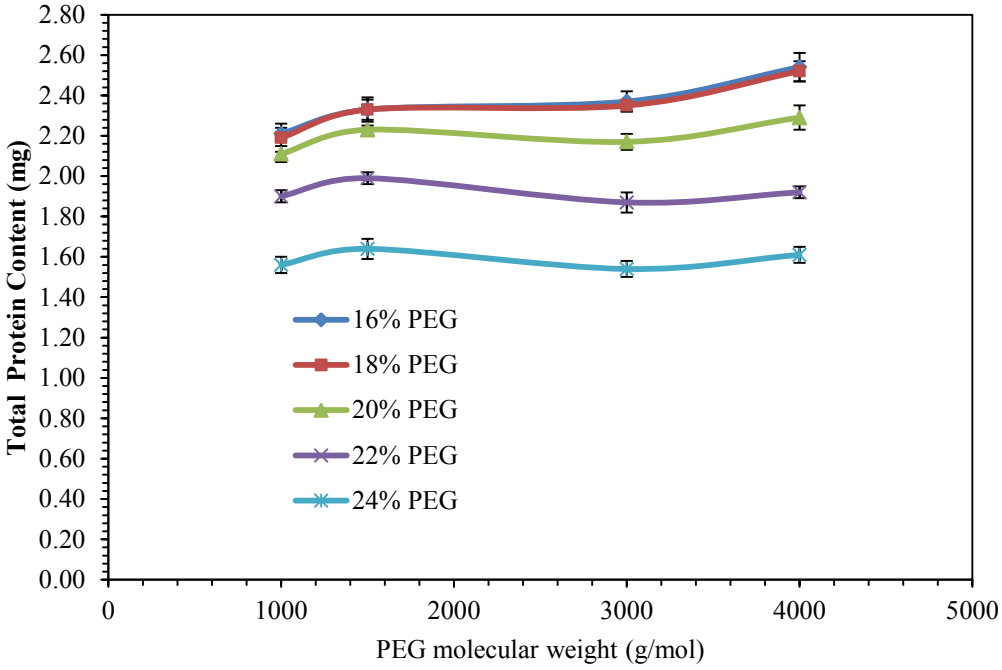
Table 7.40: ANOVA of C_P in the bottom phase (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	2.82162			
M	3	2.48079	0.826931	1246.63	0.0001
C	4	0.26726	0.066815	100.73	0.0001
MC	12	0.04703	0.003919	5.91	0.0001
Error	40	0.02653	0.000663		

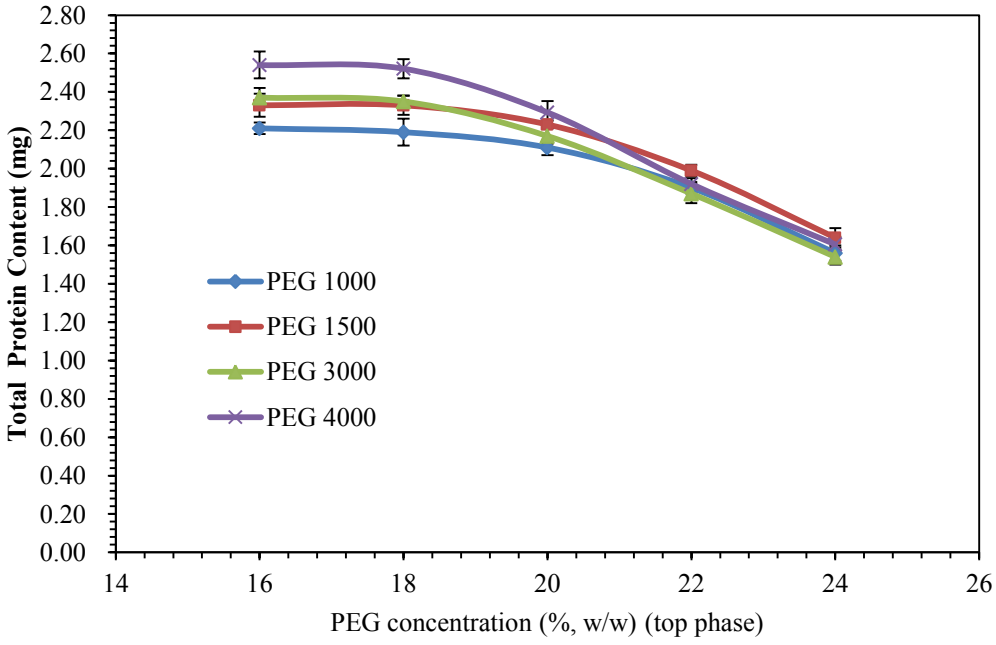
DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 33.79%

Table 7.41: The effects of PEG molecular weight and concentration on C_P in the bottom phase.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	0.37	D
1500	15	0.57	C
3000	15	0.73	B
4000	15	0.92	A
PEG concentration (%)			
16	12	0.73	A
18	12	0.69	AB
20	12	0.66	AB
22	12	0.61	AB
24	12	0.54	B



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.15: Total C_P as a function of PEG (a) molecular weight and (b) concentration.

Table 7.42: ANOVA of total C_P (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	5.73174			
M	3	0.25954	0.08651	41.25	0.0001
C	4	5.22232	1.30558	622.53	0.0001
MC	12	0.16599	0.01383	6.60	0.0001
Error	40	0.08389	0.00210		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 14.96%

Table 7.43: The effects of PEG molecular weight and concentration on total C_P.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	1.9944	A
1500	15	2.1053	A
3000	15	2.0610	A
4000	15	2.1751	A
PEG concentration (%)			
16	12	2.36250	A
18	12	2.34908	A
20	12	2.20083	B
22	12	1.92008	C
24	12	1.58717	D

total phases are shown in Figures 7.13, 7.14 and 7.15. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.38 – 7.43), PEG molecular weight and PEG concentration had significant effects and interactions on C_P in the top, bottom and total phases.

In the top phase, the C_P increased slightly first and then decreased with increased PEG concentration but decreased linearly with increased PEG molecular weight. For PEG concentrations of 16, 18, 20, 22 and 24%, the C_P decreased from 1.78 to 1.49 mg (16.29%), from 1.81 to 1.51 mg (16.57%), from 1.74 to 1.34 mg (22.99%), from 1.55 to 1.07 mg (30.97%) and from 1.25 to 0.86 mg (31.20%) when PEG molecular weight increased from 1000 to 4000, respectively. For the PEG molecular weights of 1000, 1500, 3000 and 4000, the C_P increased from 1.78 to 1.81 mg (1.69%), from 1.70 to 1.73 mg (1.76%), from 1.55 to 1.57 mg (1.29%) and from 1.49 to 1.51mg (1.34%) when PEG concentration increased from 16% to 18% (12.50%) and then decreased from 1.81 to 1.25 mg (30.94%), from 1.73 to 1.15 mg (33.53%), from 1.57 to 0.93 mg (40.76%) and from 1.51 to 0.86 mg (43.05%) when PEG concentration increased from 18% to 24% (37.50%), respectively. The highest C_P was observed at 18% concentration for all PEG molecular weights. PEG 1000 at 18% concentration gave the highest C_P (1.81 mg).

In the bottom phase, all C_P decreased with increased PEG concentration and increased with increased PEG molecular weight. For PEG concentrations of 16, 18, 20, 22 and 24%, the C_P increased from 0.43 to 1.05 mg (144.19%), from 0.38 to 1.01 mg (165.79%), from 0.37 to 0.95 mg (156.76%), from 0.35 to 0.85 mg (142.86%) and from 0.31 to 0.75 mg (141.94%) when PEG molecular weight increased from 1000 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the C_P decreased from 0.43 to 0.31 mg (27.91%), from 0.63 to 0.49 mg (22.22%), from 0.82 to 0.61mg (25.61%) and from 1.05 to 0.75 mg (28.57%) when PEG concentration increased from 16% to 24% (50.00%), respectively. The highest C_P values were observed at 16% concentration for all PEG molecular weights. PEG 4000 with the concentration of 16% gave the highest C_P (1.05 mg).

The total C_P decreased with increased PEG concentration. For PEG molecular weights of 1000, 1500, 3000 and 4000, total C_P decreased from 2.21 to 1.56 mg (%), from 2.33 to 1.64 mg (%), from 2.37 to 1.54 mg (%) and from 2.54 to 1.61 mg (%) when PEG concentration increased from 16 to 24%, respectively. All C_P obtained with PEGs of

various concentrations gave similarly shaped curves. The highest total C_P values were observed at 16% concentration for all PEG molecular weights. Overall, the PEG 4000 with the concentration of 16% gave the highest C_P (2.54 mg).

7.2.2.6 K_P

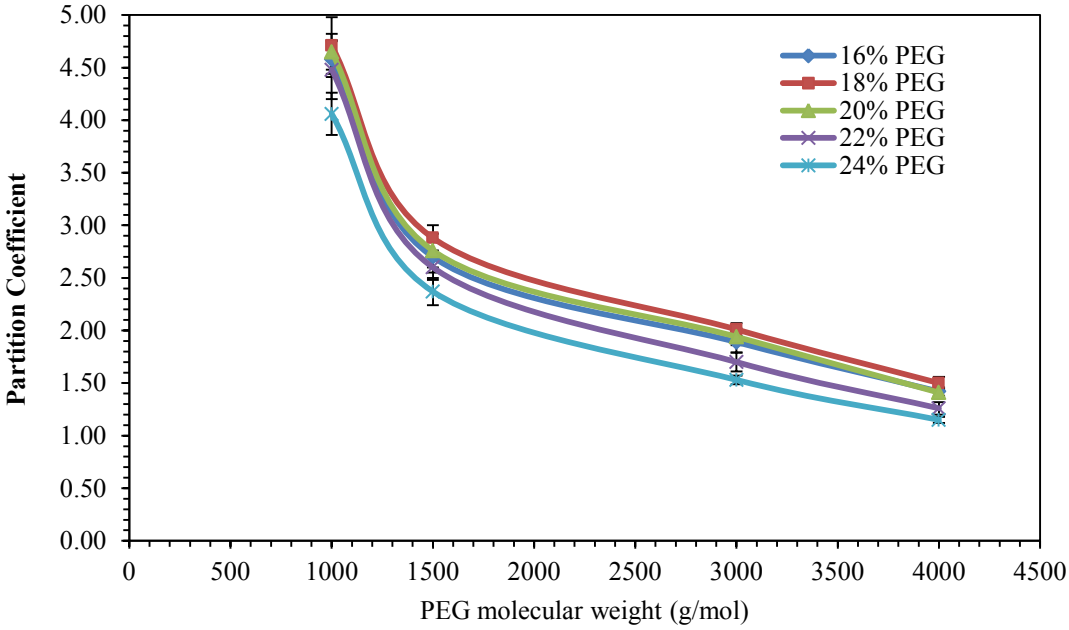
The effects of PEG molecular weight and PEG concentration on K_P are shown in Figure 7.16. All K_P decreased with increased PEG molecular weight but initially increased slightly and then decreased with increased PEG concentration. Based on ANOVA and Duncan Multiple Range Test (Tables 7.44 and 7.45), PEG molecular weight and PEG concentration had significant effects on K_P.

For PEG concentrations of 16, 18, 20, 22 and 24%, the K_P decreased from 4.58 to 1.42 (69.00%), from 4.71 to 1.50 (68.15%), from 4.65 to 1.41 (69.68%), from 4.48 to 1.26 (71.88%) and from 4.06 to 1.15 (71.67%) when PEG molecular weight increased from 1000 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the K_P increased from 4.58 to 4.71 (2.84%), from 2.70 to 2.88 (6.67%), from 1.89 to 2.01 (6.35%) and from 1.42 to 1.50 (5.63%) when PEG concentration increased from 16 to 18% (12.5%) and then decreased from 4.71 to 4.06 (13.8%), from 2.88 to 2.37 (17.7%), from 2.01 to 1.53 (23.9%) and from 1.50 to 1.15 (23.3%) when PEG concentration increased from 18 to 24% (37.50%), respectively. The highest K_P was observed at 18% concentration for all PEG molecular weights. PEG 1000 at 18% concentration gave the highest K_P (4.71).

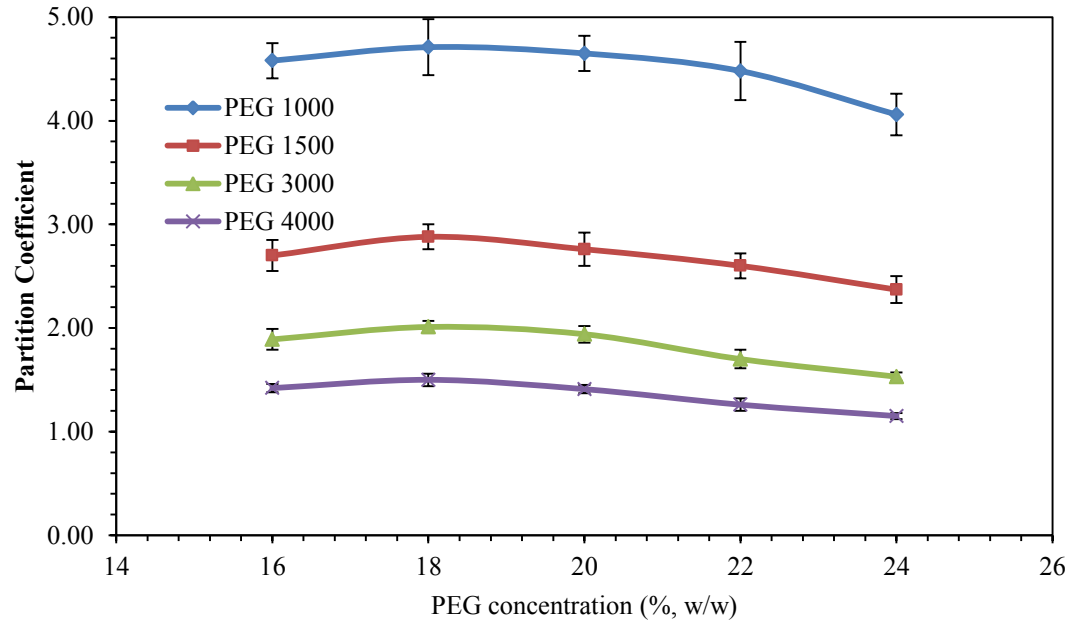
7.2.2.7 PF

The effects of PEG molecular weight and concentration on PF are shown in Figure 7.17. All PF increased initially and then decreased with increased PEG molecular weight and concentration. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.46 and 7.47), PEG molecular weight and PEG concentration had significant effects on PF.

For PEG concentrations of 16, 18, 20, 22 and 24%, the PF increased from 3.96 to 4.89 (23.48%), from 4.20 to 5.23 (24.52%), from 3.65 to 4.83 (32.33%), from 3.40 to 4.49 (32.06%) and from 3.13 to 4.35 (38.98%) when PEG molecular weight increased



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.16: K_P as a function of PEG (a) molecular weight and (b) concentration.

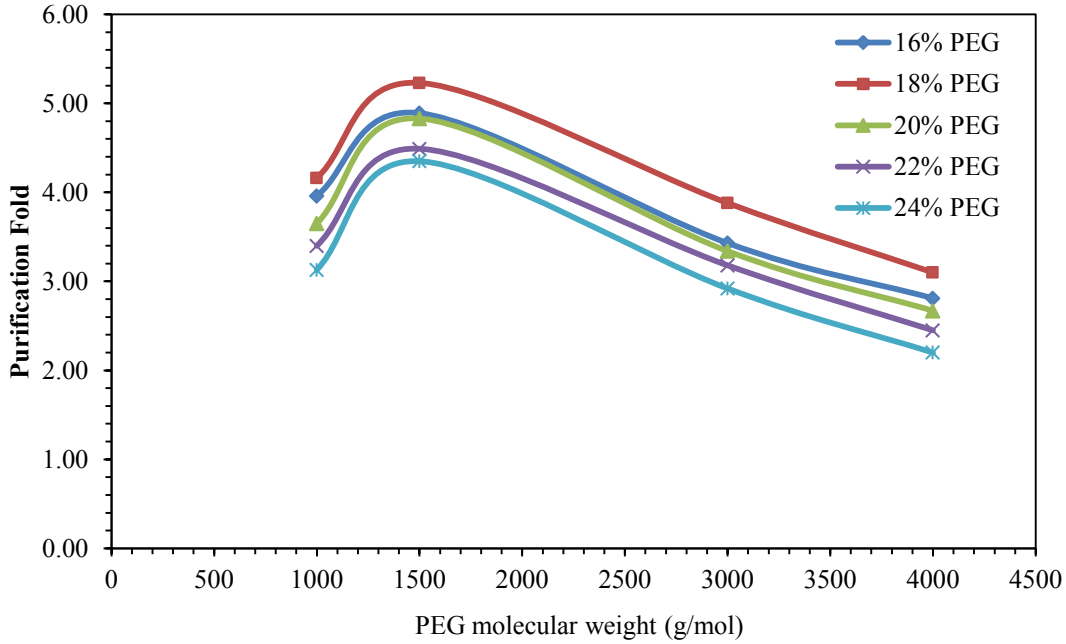
Table 7.44: ANOVA of K_P (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	89.4282			
M	3	86.7354	28.9118	1515.96	0.0001
C	4	1.7995	0.4499	23.59	0.0001
MC	12	0.1304	0.0109	0.57	0.8530
Error	40	0.7629	0.0191		

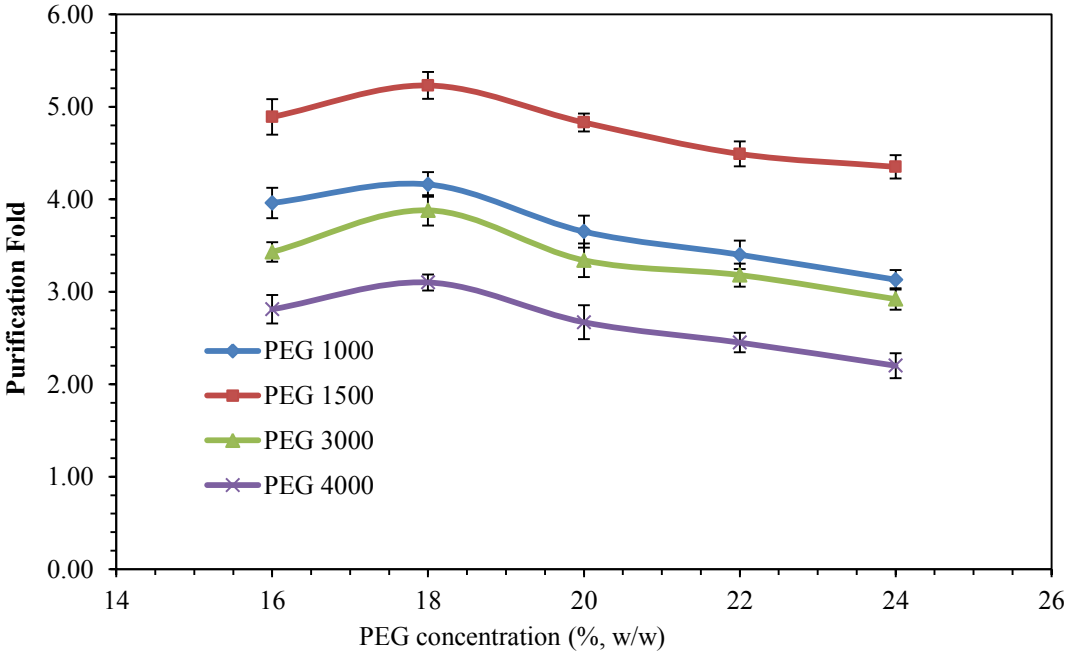
DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.99$,
CV = 47.72%

Table 7.45: The effects of PEG molecular weight and concentration on K_P.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	4.50	A
1500	15	2.66	B
3000	15	1.81	C
4000	15	1.35	D
PEG concentration (%)			
16	12	2.65	A
18	12	2.77	A
20	12	2.69	A
22	12	2.51	A
24	12	2.28	A



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.17: PF in the top phase as a function of PEG (a) molecular weight and (b) concentration.

Table 7.46: ANOVA of PF (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	42.0801			
M	3	34.7761	11.5920	532.19	0.0001
C	4	6.2835	1.5709	72.12	0.0001
MC	12	0.1492	0.0124	0.57	0.8520
Error	40	0.8713	0.0218		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.979$,
CV = 23.44%

Table 7.47: The effects of PEG molecular weight and concentration on PF.

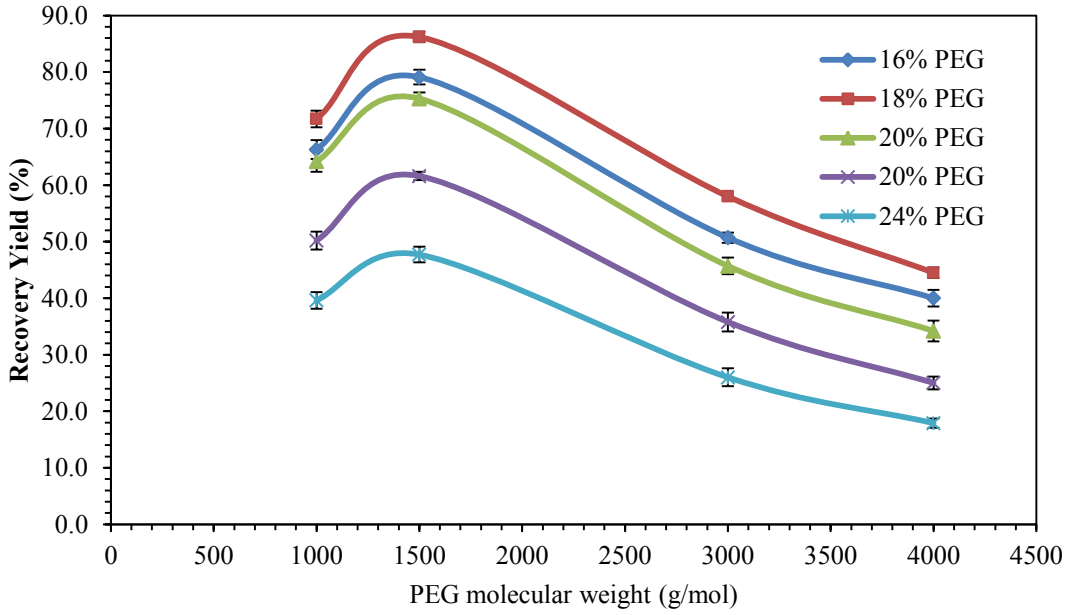
Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	3.66	B
1500	15	4.76	A
3000	15	3.35	C
4000	15	2.65	D
PEG concentration (%)			
16	12	3.77	AB
18	12	4.09	A
20	12	3.62	AB
22	12	3.38	B
24	12	3.15	B

from 1000 to 1500 and then decreased from 4.89 to 2.81 (42.54%), from 5.23 to 3.10 (40.73%), from 4.83 to 2.67 (44.72%), from 4.49 to 2.45 (45.43%) and from 4.35 to 2.20 (49.43%) when PEG molecular weight increased from 1500 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the PF increased from 3.96 to 4.20 (6.06%), from 4.89 to 5.23 (7.84%), from 3.16 to 3.88 (22.78%) and from 2.81 to 3.10 (10.32%) when PEG concentration increased from 16% to 18% (12.50%) and then decreased from 4.20 to 3.13 (25.48%), from 5.23 to 4.35 (16.83%), from 3.88 to 2.92 (24.74%) and from 3.10 to 2.20 (29.03%) when PEG concentration increased from 18% to 24% (37.50%), respectively. The highest PF were observed at 18% concentration for all PEG molecular weights. PEG 1500 with 18% concentration gave the highest PF (5.23).

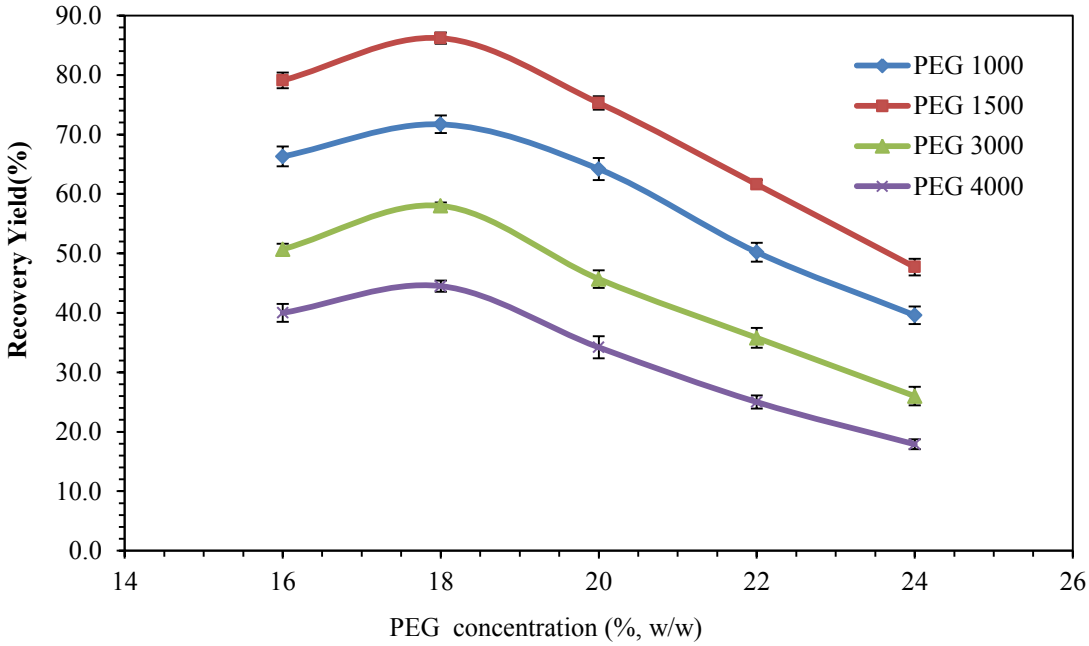
7.2.2.8 RY

The effects of PEG molecular weight and PEG concentrations on RY are shown in Figure 7.18. All the RY values increased initially and then decreased with increased PEG molecular weight and concentration. Based on the ANOVA and Duncan Multiple Range Test (Tables 7.48 and 7.49), PEG molecular weight and PEG concentration had significant effects on RY. There was also a significant interaction between PEG molecular weight and PEG concentration.

For PEG concentrations of 16, 18, 20, 22 and 24%, the RY increased from 66.3 to 79.1% (19.3%), from 71.7 to 86.2% (20.2%), from 64.2 to 75.3% (17.3%), from 50.2 to 61.6% (22.7%) and from 39.6 to 47.7% (20.5%) when PEG molecular weight increased from 1000 to 1500 and then decreased from 79.1 to 40.0% (49.4%), from 86.2 to 44.5% (48.4%), from 75.3 to 34.2% (54.6%), from 61.6 to 25.0% (59.4%) and from 47.7 to 17.9% (62.5%) when PEG molecular weight increased from 1500 to 4000, respectively. For PEG molecular weights of 1000, 1500, 3000 and 4000, the RY increased from 66.3 to 71.7% (8.1%), from 79.1 to 86.2% (9.0%), from 50.7 to 58.0% (14.4%) and from 40.0 to 44.5% (11.3%) when PEG concentration increased from 16 to 18% (12.5%) and then decreased from 71.1 to 39.6% (44.3%), from 86.2 to 47.7% (44.7%), from 58.0 to 26.0% (55.2%) and from 44.5 to 17.9% (59.8%) when PEG concentration increased from 18 to 24% (37.5%), respectively. The highest RY was observed at 18% concentration for all PEG molecular weights. PEG 1500 with 18% concentration gave the highest RY (86.2%).



(a) As a function of PEG molecular weight (mean ± S.D, n = 3).



(b) As a function of PEG concentration (mean ± S.D, n = 3).

Figure 7.18: RY in the top phase as a function of PEG (a) molecular weight and (b) concentration.

Table 7.48: ANOVA of RY (effects of PEG molecular weight and concentration).

Source	DF	SS	MS	F	P
Total	59	20653.1			
M	3	12362.4	4120.79	2267.28	0.0001
C	4	8050.8	2012.69	1107.39	0.0001
MC	12	167.2	13.93	7.67	0.0001
Error	40	72.7	1.82		

DF: Degrees of freedom,
SS: Sum of squares,
MS: Mean of squares,
M: PEG molecular weight,
C: PEG concentration,
MC: Interaction of PEG molecular weight and concentration,
CV: Coefficient of variation, ratio of the standard deviation to the mean,
$R^2 = 0.997$,
CV = 36.70%

Table 7.49: The effects of PEG molecular weight and concentration on RY.

Factor	Number of Observations	Mean (ml)	Duncan Grouping
PEG molecular weight			
1000	15	58.4	B
1500	15	70.0	A
3000	15	43.2	C
4000	15	32.3	D
PEG concentration (%)			
16	12	59.0	A
18	12	65.1	A
20	12	54.9	AB
22	12	43.2	BC
24	12	32.8	C

7.3 Comparing ATPS and ASF Methods

The ASF method was used for the purification of 4 g crude PG as described in Section 6.3. After analyzing the precipitates collected in a saturation range of 20–60%, the A_E, C_P, SA, PF and RY were determined (Table 7.50). The values of A_E, C_P, SA, PF and RY were 30.66 $\pm$ 1.84 U, 12.0 $\pm$ 0.32 mg, 2.55 $\pm$0.14 U/mg, 2.46 $\pm$ 0.14 and 70.4 $\pm$ 4.23%, respectively. The comparison between the ATPS and the ASF methods based on triplicates is shown in Table 7.51. All the parameters are significantly different at the 0.05 level.

Table 7.50: ASF purification of 4 g PG.

Parameters	ASF
Enzyme activity (U)	30.66 ± 1.84
Protein content (mg)	12.02 ± 0.32
Specific activity (U/mg)	2.55 ± 0.14
Purification fold	2.46 ± 0.14
RY (%)	70.4 ± 4.23

Conditions used: 20 – 60% saturation at 4°C.

Table 7.51: ASF purification of 4 g of PG and comparison to ATPS. (P-values were obtained from the t-test by comparing A_E, C_P, SA, PF and RY of the two methods, respectively.)

Purification Method	A_E (U)	C_P (mg)	SA (U/mg)	PF	RY (%)
ASF	30.66 ± 1.84	12.02 ± 0.32	2.55 ± 0.14	2.46 ± 0.14	70.4 ± 4.23
ATPS	37.67 ± 0.38	6.98 ± 0.12	5.40 ± 0.09	5.20 ± 0.08	86.6 ± 0.88
P-value	0.023	0.002	0.000	0.000	0.023

Optimum condition of ATPS: 15% $(NH_4)_2SO_4$ – 18% PEG 1500 at 4°C,

Conditions of ASF: 20 – 60% saturation at 4°C.

A_E: enzyme activity

C_P: protein content

SA: specific activity (A_E / C_P)

K_P: partition coefficient (C_P of top phase / C_P of bottom phase)

PF: purification factor (SA of purified enzyme / SA of crude enzyme)

RY: recovery yield (A_E of purified enzyme / A_E of crude enzyme)

CHAPTER 8. DISCUSSION

8.1 Extraction Profiles

ATPS and ASF purifications used the same PG extract and crude PG samples. A_E, C_P and RY decreased while SA and PF increased during PG extraction. This indicated that some of the PG was lost but the portion remaining was concentrated, resulting in a higher purity. The higher purity was due to the removal of proteins and small molecular peptides. Lower RY was due to the destruction of enzyme structure and denaturation of PG caused by homogenization, centrifugation and dialysis.

8.2 Effects of Salt Type and Concentration in ATPS

In this study, different initial salt concentrations were found for the formation of the two phases. These differences may be attributed to different ionic strengths of the different salts. Shang et al. (2007) observed a similar effect and suggested that it was controlled by the ionic radius of the negative ions. The theoretical fundamentals have not been well explained.

The formation of a biphasic system is based on the balancing of enthalpic and entropic effects involved in the aqueous hydration of the solutes (Huddleston et al., 1991). The enthalpic effect is repulsion, drived by the total energy of a thermodynamic system. The entropic effect is a thermodynamic force resulting from the entire system's statistical tendency to increase its state of disorder. The entropy increase upon mixing of molecules in a solution will favor the formation of a single phase. However, the mixing entropy is much reduced in solutions of macromolecules and, for long polymer chains, the entropy of mixing is a relatively small term in the total free energy of mixing. A weak repulsive enthalpic interaction between monomer units of the different polymers is sufficient to dominate the mixing entropy (Tjemeld and Johansson, 2000).

To form two phases, a critical salt concentration dependent on the temperature should be met and a higher salt concentration was required at a low temperature. A binodal curve which indicated the boundary curve of the single and biphasic phase regions based on liquid-liquid equilibrium can be used to determine the concentrations required to form two phases (Figure 8.1). The inside region shows the two phase

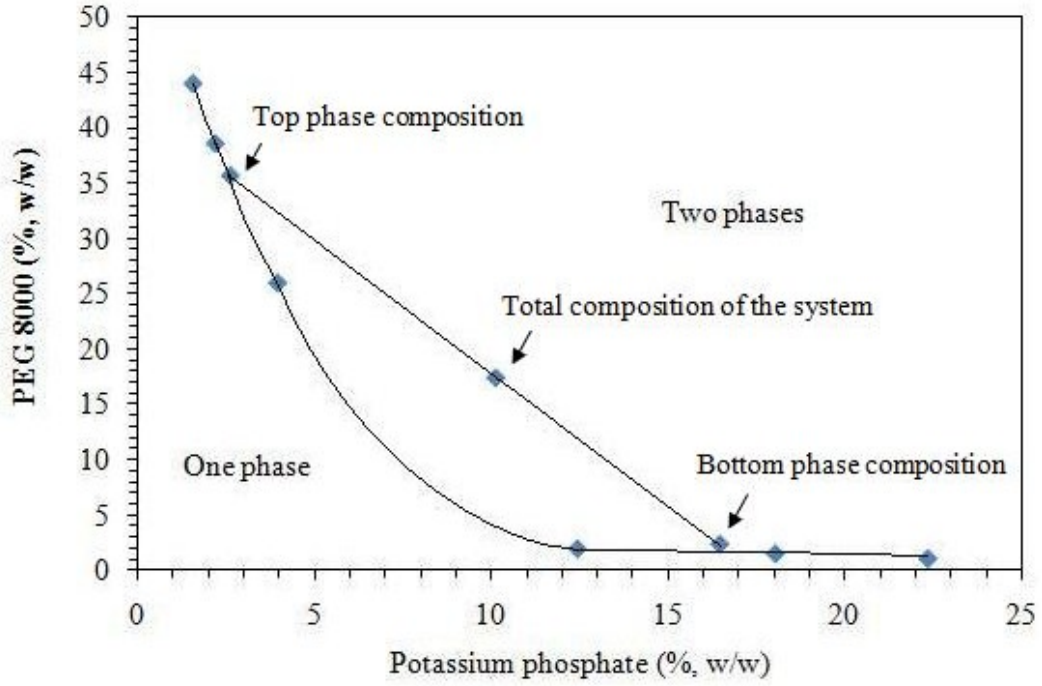


Figure 8.1: Binodal curve for PEG 8000 and potassium phosphate at 25°C and pH 7 (Silva et al., 1997).

formation while the outside region represents the single phase. From this curve the composition (weight percentage) of salt and PEG in each phase can be predicted. The binodal curves for commonly used salts ($MgSO_4$, $(NH_4)_2SO_4$, $NaSO_4$, $FeSO_4$, K_2HPO_4) at room temperature have been plotted, whereas data are quite limited at 4°C (best temperature for enzyme partition) (González-Tello et al., 1996; Huddleston et al., 2003). In our study, the critical concentrations for ATPS formation at 4°C were found to be 9, 12, 12 and 10% (w/w) for $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 at 18% PEG 1000, respectively.

TV and V_R decreased with increased salt concentration. Similar patterns were found by Nalinanon et al. (2009). Salt type also had a significant effect on TV and V_R. The decrease in TV may be attributed to the change of the density of the aqueous solution caused by different salt types or concentrations. Salt is more charge-polarized and capable of hydrogen bonding with water molecules than PEG (which is more hydrophobic than salt). As salt concentration increased, more salts migrated to the aqueous PEG phase and broke its original water structure. Water molecules underwent a rearrangement (Farruggia et al., 2004) and surrounded the salt to form a more ordered water layer. Therefore, a more compact water structure with a smaller volume of PEG molecules was formed (Nalinanon et al., 2009).

There were obvious similarities in the relationships of A_E and RY (as shown in Figures 7.3 and 7.8 (salt effect) and Figures 7.11 and 7.18 (PEG effect) respectively) and SA and PF (as shown in Figures 7.4 and 7.7 (salt effect) and Figures 7.12 and 7.17 (PEG effect) respectively) for salt and PEG. Because purified PG was derived from the same stomach samples, A_E of crude PG was a constant value in all experiments. RY is calculated from the ratio of A_E after purification to the constant value of A_E of crude extract; thus, RY only depended on A_E from purified PG and produced very similar trends. The same reasoning applied for the relationships for PF and SA.

It was reported by Nalinanon et al. (2009), Spelzini et al. (2005) and Imelio et al. (2008) that PG partitioned predominantly in the PEG-rich top phase but was negligible in the bottom phase. In this study, A_E was measured in all top phases. In the bottom phase A_E was only measured in ATPS composed of $MgSO_4$ and no activity was found, which

was consistent with other research (Nalinanon et al., 2009). Therefore, A_E in the bottom phase was not measured in ATPS composed of the other three salts.

The mechanism driving PG partition is not well-understood. One possible explanation is that the phase the protein partitioned to was determined by its hydrophobic properties and net charge. Protein tends to go to the PEG-rich top phase because its hydrophobic groups have electrostatic interactions with the oxygen molecules in the PEG chains. Another possible explanation is that negatively charged protein tends to go to the PEG-rich phase while positively charged protein tends to go to the salt-rich phase (Klomklao et al., 2005; Del-Val and Otero, 2003; Yang et al., 2008). It was reported that in the top phase, better partition can be achieved for proteins as their negative charge increases (Bandmann et al., 2000). With an isoelectric point (pI) of ~1-1.5, PG was negatively charged in all partition systems with a neutral or a slightly basic pH environment and was therefore only found in the PEG-rich top phase.

Johansson et al. (1998) reported that the partitioning of protein is influenced by the presence of salts and the effect is enhanced with increases in the net charge of the protein. The partition patterns are determined by the balancing of salting-in and salting-out effects in the top phase. At low concentrations, the salting-in effect occurs where salts stabilize proteins and other polyelectrolytes through nonspecific electrostatic interactions in the top phase which are dependent on the ionic strength of the medium and favor partition of PG and proteins (Arakawa and Timasheff, 1984). Salting in has a stronger effect than salting-out. At high concentrations, however, salts exert specific effects on proteins resulting in the destabilization or denaturation of proteins and a reduction in solubility (Arakawa and Timasheff, 1984), causing a salting out effect. The results of A_E indicate that the efficacy of the salts and preferential hydration of protein follow the lyotropic series, a classification of ions based upon salting-in or salting-out ability, where a stronger hydration favors PG partition in the top phase due to an increase in the solubility of PG (Huddleston et al., 1991; Rawdkuen et al., 2011). The anion has a greater effect on the effectiveness of salting-in than the cation. The most effective multi-charged anions are SO_4^{2-} >HPO_4^{2-} > $C_6H_5O_7^{3-}$ > $C_4H_4O_6^{2-}$ > CH_3COO^- > Cl^-, regardless of the cations used whereas the most effective cations or cation combination are Li^+ > Na^+ > K^+ > $(NH_4)^+$ >Mg^{2+} >Ca^{2+} (Carbonnaux et al., 1995; PrimerDigital, 2011). Higher hydration

and interaction means more enzymes in the top phase. Based on this, the partition effect of fours salts used in this study should follow the sequence $(NH_4)_2SO_4 > MgSO_4 > K_2HPO_4 > Na_3C_6H_5O_7$, which was consistent with our results for A_E and RY. For instance, at a concentration of 13%, A_E were 7.25 U $(NH_4)_2SO_4) > 5.99$ U $(MgSO_4) > 4.13$ U $(K_2HPO_4) > 3.72$ U $(Na_3C_6H_5O_7)$. Chaiwut et al. (2010) and Rawdkuen et al. (2011) used the same four salts to partition protease from *Calotropis procera* latex and reported a similar effect. However, C_P in the top phase did not follow this sequence. This may be due to the hydrophobic properties of other proteins present as impurities. Those proteins may have a weaker interaction with the anion and not be as affected by this lyotropic series.

K_E, the partition coefficicent of the enzyme, and K_P, the partition coefficicent of the protein, are used to characterize the distribution of enzyme and protein in the ATPS and are considered to be important parameters. K_E and K_P refer to the ratio of the enzyme activities and the ratio of protein contents in the top and bottom phases, respectively. When the enzyme is present in both phases, K_E can be calculated as with trypsin by Klomklao et al. (2005) and protease by Rawdkuen et al. (2011). In our study, K_E could not be calculated because PG was not present in the bottom phase. Alternatively, K_P can be used to characterize the partition. For PG, a low K_P usually gave a high PF and implies a higher purity of the interested enzyme (Nalinanon et al., 2009; Klomklao et al., 2005; Rawdkuen et al., 2011), which was found with K_2HPO_4, $MgSO_4$ and $(NH_4)_2SO_4$. For example, $MgSO_4$, with a $K_P < 1$, gave a high PF (> 6.2). K_2HPO_4, with a higher K_P (>9), gave a low PF ($1.2 < PF < 1.5$). Higher K_P implies that more impurities of proteins and peptides were partitioned in the top phase. However, $Na_3C_6H_5O_7$ gave a similar K_P to $(NH_4)_2SO_4$ but produced a much lower PF, which indicated a high C_P but low A_E in the top phase. This may be attributed to different salt properties, such as ionic charge and strength. For instance, $C_6H_5O_7^{3-}$, which carries more charges, may have a stronger effect of anionic denaturation and reduce the enzyme stability. Moreover, the weak interaction of $C_6H_5O_7^{3-}$ with PG as predicted by the lyotropic series also offers explanations for this effect.

RY is considered to be more important in this optimization. In this research, all the salt types gave a PF range of 1.17-6.26, much lower than that achieved with

chromatography. The ATPS method is only the first step (PF of 1-10) in PG purification; further steps such as chromatography (PF can be 10 – 400 at proper conditions) must be followed for higher purity. Although $MgSO_4$ at 11% gave the highest enzyme purity, $(NH_4)_2SO_4$ at 15%, giving the highest RY, was chosen as the best salt type and salt concentration. Although $(NH_4)_2SO_4$ at 15% was chosen, it did not provide a RY (71.7%) much greater than that of $MgSO_4$ at 10% (62.4%) (Figure 7.8). Because $(NH_4)_2SO_4$ at a higher concentration was used, the higher cost associated with the increased salt quantity as well as the disposal of waste salt should be taken into consideration in industrial applications.

8.3 Effects of PEG Molecular Weight and Concentration in ATPS

Biphasic systems occur at a critical PEG concentration and a higher concentration is required at a low temperature. The results showed that at 4°C the two phase separation was achieved with all PEG molecular weights at a concentration of 16% or higher. Raghavarao et al. (1998) reported two phase formation above 8 – 10% PEG at room temperature. Nitsawang et al. (2006) reported two phase formation above 4 – 12% concentration of PEG 6000 with 15% $(NH_4)_2SO_4$. The binodal curves with commonly used PEG (PEG 1000, 2000, 3000, 4000, 6000, 8000) at room temperature have been plotted as a reference (Silva et al., 1997; Huddleston et al., 2003; Johansson et al., 2011), whereas data are quite limited at 4°C as those for salts. The critical molecular weight of PEG must also be met. Tubío et al. (2007) suggested that for ATPS formation, a minimum molecular weight of 600 – 3350 is required, which is consistent with our results with PEG molecular weight $\geq$ 1000 used.

TV increased with increasing PEG molecular weight and then decreased. Eliassi and Modarress (1999) reported that the decrease in TV may be attributed to the changes of the density of the aqueous solution caused by PEG which resulted in a change of volume upon mixing. Intuitively, it would seem that the long chain of the large PEG molecule would occupy more space and increase the volume; however, a decrease would occur if the PEG chains coiled and twisted around each other, resulting in a decrease in the space occupied. With increasing PEG concentration, TV followed an unusual pattern of a decrease, followed by an increase and finally a last decrease (Fig 7.9b). This was

different from the trend expected – an increase, due to the enhanced space of more PEG molecules, followed by a decrease, resulting from the coiled PEG molecules at high concentration. The experimental curve found here showed an initial unexpected TV decrease. It may be that this decrease was derived from the formation of more ordered water structure by increased hydrogen bonds at low PEG concentration. The consistency of the result for all the PEG molecular weights suggested that this result was not simply due to experimental error.

V_R decreased with increased PEG molecular weight and increased with increased PEG concentration. These trends are consistent with data by Nalinanon et al. (2009). As with TV, the decrease in V_R as a function of molecular weight may be related to the reduced volume of the top phase resulting from the twisting of PEG chains. With an increased PEG concentration, more space was required to accommodate the PEG structures. Increasing PEG concentration may have caused a competition for water with the salt phase, resulting in increased volume in the top phase and decreased volume in the bottom phase (Wu et al., 2000).

A_E, SA, PF and RY increased with increased PEG molecular weight and PEG concentration and then decreased. Low PEG molecular weights (1000-1500) gave better partition than higher molecular weights. Similar results were reported by Nalinanon et al. (2009) and Chaiwut et al. (2010). Partition is determined by a balancing of electrostatic interaction and the excluded volume effect of PEG. Xia et al. (1993) reported that electrostatic interaction formed between the protonated carboxyl groups of PG and the oxygen ether of PEG (Figure 8.2), causing PG to transfer towards the PEG-rich phase. At low PEG molecular weight the dominant electrostatic interaction helps to stabilize the enzyme. Increasing PEG molecular weight increased this interaction and resulted in better hydration and higher solubility of PG in the PEG phase. However, PEG steric exclusion driven by an entropic force, known as the excluded volume effect, occurs at large PEG molecular weights (Nalinanon et al., 2009; Bhat and Timasheff, 1992) and excludes the protein from the top phase. Therefore, at large PEG molecular weights, the excluded volume effect dominates over the electrostatic effect, creating an overall repulsion effect (Knowles et al., 2011). At low PEG concentrations (16-18%), the

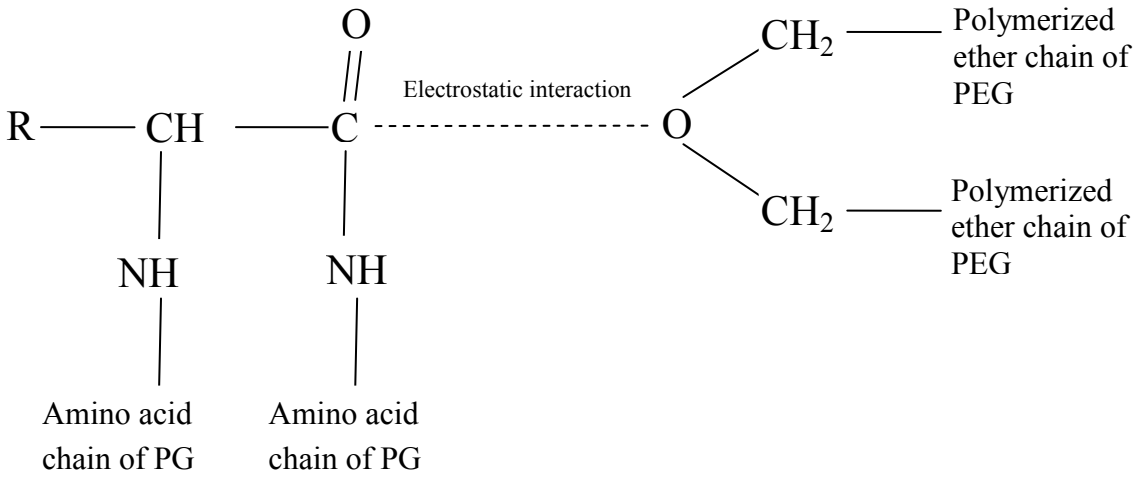


Figure 8.2: The electrostatic interaction formed between the protonated carboxyl groups of PG and the oxygen ether of PEG.

electrostatic interaction is enhanced and favors the partition of PG into PEG-top phase. However, high PEG concentrations lead to higher viscosities and make the partition difficult. In addition, high PEG concentrations can result in denaturation and possible precipitation of PG (Sharma and Kalonia, 2004).

K_P characterized the protein distribution and decreased with PEG molecular weight, similar to the work of Nalinanon et al. (2009) and Rodrigues et al. (2001). A sharp decrease was detected below PEG molecular weight of 1500. However, this decrease did not follow the rule that a lower K_P usually gave a higher PF of enzyme. It was found that PEG 4000 had the lowest K_P and PF while PEG 1500 gave the highest PF. This may be because of the excluded volume effect associated with the PG interaction. It is estimated that with increased molecular weight, more PG was partitioned away from the PEG phase. It also shows a similar trend to parameters mentioned above with increased PEG concentration.

In this study, 18% PEG 1500 gave the strongest electrostatic interaction and gave the best partition (highest RY). Highest PF was also achieved at this condition. Therefore, 15% $(NH_4)_2SO_4$ – 18% PEG 1500 was selected as the optimum salt-PEG combination for PG partition.

8.4 Comparing ATPS and ASF Methods

ASF was performed using the saturation range selected by Bougatef et al. (2008) and Feng et al. (2004) without optimization on the crude extract. ATPS and ASF were based on different separation principles. ATPS employs selective partition of the protein of interest in one aqueous phase while other proteins remain in the other phase. In contrast, ASF purified protein by selective precipitation of the protein of interest within one saturation range based on protein solubility while other proteins remain in the solution. A_E, C_P, SA, PF and RY were significantly different between the ATPS and ASF methods. The ATPS method gave a higher A_E than that of the ASF method while the ASF method gave a higher C_P than that of the ATPS method because ASF was found to be less selective in protein separation than ATPS and therefore it provided more proteins as impurities mixed with PG. The ATPS method gave much higher SA, PF and RY compared to those of the ASF. There is no literature on the comparison of two methods

but the ASF using similar saturations for PG purification gave a SA of ~0.88-3.0 U, PF of ~1.1-3.7 and RY of ~64-75% (Bougatef et al., 2008; Tanji et al., 2007; Zhou et al., 2007; Wu et al., 2009). Our data for SA, PF and RY were in these ranges. Therefore, ATPS showed better partition and higher effectiveness than ASF.

8.5 Industrial Applications

In our study, fish stomach represented only 1.58% of the whole fish of red perch and 8% PG was obtained from the stomach. In industrial applications, waste (flesh, heads, bones, fins, skin, tails and viscera) represent a large portion (30–80%) of whole fish. From this study, the waste composition of red perch is shown in Tables 8.1. Since PG represents only a small part of the total waste and whole fish, it is recommended that fish stomachs be separated from the other fish wastes for PG production. The stomachs should be stored at a low temperature (4^{o} C) to keep PG stable.

8.6 Summary of Investigation

Nalinanon et al. (2009) used similar salts at 15, 20 and 25% and PEG 1000, 2000, 4000 at 15, 20, 25 and 30% for ATPS at room temperature to partition PG from albacore tuna and reported the best PF was achieved at 20% $MgSO_4$ – 20% PEG 1000 with SA of 23.5 U/mg, PF of 6.34 and RY of 87.5%. They obtained a much higher SA value that ours since the unit of A_E that they used was different from that in this research (units for C_P are the same). They employed the unit of activity defined as an increase of releasing 1 mmol of tyrosine per min whereas we used the unit defined as an increase of 1.0 in absorbance at 280 nm per minute. The best RY by Nalinanon et al. (2009) was achieved at 15% $(NH_4)_2SO_4$ – 20% PEG 2000; these conditions were very similar to the conclusions in this study. Although units of A_E were different, the RY (because it is a ratio that can eliminate the unit effect) can still be compared. They gave a higher RY (98.6%) than ours (86.6%). Spelzini et al. (2006) and Imelio et al. (2008) used ATPS derived from PEG 600 and 4000 with potassium phosphate to purify commercial pepsin at room temperature and both obtained higher RY (>90%) than this work. Similar work has been carried out with trypsin (Klomklao et al., 2005), xylanase (Yang et al., 2008), chymosin (Spelzini et al., 2006), α-chymotrypsin (Tubío et al., 2007), *Bacillus subtilis* neutral protease (Han and

Table 8.1: Waste composition in red perch.

Part	Weight (g)	Percentage of whole fish (%)	Percentage of total waste (%)
Whole	587.0	100	-
Total waste	279.5	49.60	100
Head	189.7	32.32	67.9
Tail	42.2	7.18	15.08
Fins	17.5	2.47	5.19
Viscera	33.1	5.63	11.83
Stomach	9.29	1.58	3.19

Lee, 1997) and *Mucor bacilliformis* acid protease (Fernández Lahore et al., 1995). Most research used room temperature for purification of commercial enzymes while in this study 4°C was used in the phase formation and PG partition because our crude PG extract did not have good stability and quickly showed a drop in A_E at room temperature. At room temperature, fast autolysis of the crude PG occurred and made it quite difficult to maintain stable PG (Nguyen et al., 1998). Compared to room temperature, low temperature required narrower ranges of salt and PEG concentrations to form biphasic phases. To summarize, good partitioning of PG was achieved by our optimized ATPS method and the purified PG had higher PF and RY than those by ASF.

8.7 Recommendations

Future work should be performed to determine the optimum concentration (w/w) of crude PG to extract. It is reported that the crude enzyme concentration has an important influence on the partition (Asenjo and Andrews, 2011). PEG 1000 was used and 10% PG was selected according to Nalinanon et al. (2009). Higher levels of crude PG, as found by Imelio et al. (2008) may be optimal.

Temperature can be optimized for best RY. Low temperature gave a higher stability of enzyme by minimizing autolysis while high temperature gave a higher A_E because pepsin is more active and hydrolyzes substrate faster at high temperture. An optimal temperature was reported at 7°C (Harkker et al., 2008). A water bath could be used to control the temperature.

Our experiment showed that it was impossible to use buffer to maintain a constant pH for all salt types because only the limited amount of water in the system was not sufficient to maintain the same pH. A better technique for pH control during purification is needed.

The Bradford method was used for measuring protein content after ATPS purification. A small reduction in absorbance was found upon the mixing of PEG with protein and may have resulted in lower C_P than actual. Therefore, an alternative method for protein determination should be used in the future.

The ASF method should be optimized. The optimal saturation range for protein collection can be determined and a better comparison with the optimized ATPS can be made.

Compared with the pure PG sample which was quite stable at 4°C for 14 days, a reduced stability of PG (loss of A_E) was detected when mixed with PEG (Nalinanon et al., 2009). Since ATPS purification may change the properties of proteases, the storage stability of extracted PG should be tested. If the PEG was responsible for a reduction in A_E, a proper way to separate PG from PEG should be developed.

The potential to recycle materials should also be investigated. For instance, after purification, PEG can be recovered from the top phase by back extraction. Highly concentrated salt is added and this helps to achieve a new equilibrium and form a new ATPS, in which the PG transfers to the bottom salt phase from the PEG phase. The salt can be separated by dialysis or ultrafiltration to obtain the PG.

CHAPTER 9. CONCLUSIONS

The partition of PG from red perch using ATPS at 4°C was investigated. The effects of salt type ($MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4) and concentration (6, 7, 8, 9, 10, 11, 12, 13, 15, 17, 19%) and PEG molecular weight (PEG 1000, 1500, 3000 and 4000) and concentration (16, 18, 20, 22 and 24%) on the partitioning of PG were studied. Several parameters including total volume (V_T), volume ratio (V_R), enzyme activity (A_E), specific activity (SA), partition coefficient (K_p), purification fold (PF) and recovery yield (RY) were evaluated.

Salt type and salt concentration had significant effects on each parameter. $MgSO_4$, $(NH_4)_2SO_4$, $Na_3C_6H_5O_7$ and K_2HPO_4 required different critical salt concentrations (9, 12, 12 and 10%, respectively) to form biphasic systems. TV and V_R decreased with increased salt concentration since salt formed hydrogen bonds with water molecules and formed a more compact and ordered water structure. PG partitioned predominantly in the PEG-rich top phase due to its negative charge. A_E, C_P, SA, PF and RY increased with increased salt concentration and then decreased, while K_P had an opposite pattern. Salt partition effects were determined by the balancing of salting-in and salting-out effects. Low salt concentrations favoured salting-in and stabilized the PG while high concentrations produced salting-out and caused an adverse partition effect. Salt efficacy and preferential hydration followed the lyotropic series and higher interaction gave a higher A_E and RY. A low K_P usually gave a high purity of PG while $Na_3C_6H_5O_7$ produced a low purity, likely due to anionic denaturation and weak hydration. $(NH_4)_2SO_4$ at 15% which gave the highest RY (71.7%) was selected as the optimum salt type and salt concentration.

PEG molecular weight and PEG concentration also had significant effects on each parameter. To form two phases, a critical PEG molecular weight and concentration were required. TV increased with increased PEG molecular weight and PEG concentration while different patterns were found for V_R, which was due to the volume change upon mixing and the competition of PEG with salt for water. A_E, SA, PF and RY increased with increased PEG molecular weight and concentration and then decreased. The PEG partition effect was determined by the balancing of electrostatic interaction and the excluded volume effect. Low PEG molecular weight favored electrostatic interaction to

yield a better stabilization while high PEG molecular weight produced steric exclusion and brought a weakened partition effect. Low PEG concentration enhanced the hydrophobic interaction and helped partition PG while high PEG concentration increased the viscosity and surface tension and obstructed partition. A low K_P no longer gave a high purity of PG due to the excluded volume effect. PEG 1500 at 18% concentration gave the highest RY (86.2%) and was selected as the optimum PEG molecular weight and PEG concentration.

15% $(NH_4)_2SO_4$ – 18% PEG 1500 was the optimal ATPS combination and presented a better partition (SA of 5.40 U/mg, PF of 5.20 and RY of 86.6%) than ASF (SA of 2.55 U/mg, PF of 2.46, RY of 70.4%). ATPS was proven as a feasible, effective and gentle way to purify PG.

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