# CHAPTER IV

# RESULTS AND DISCUSSION

## 4.1 Physicochemical properties of TBSP

TBSP was prepared as described in section 3.1.1 and freeze-dried for storage in a water-proof plastic bag and stored in a refrigerator (4°C) until needed for study.

### 4.1.1 Proximate analysis and amino acid profile of TBSP

Table 4.1 shows the proximate analysis results for TBSP. TBSP contained 44.20% of crude protein (N x 6.25) determined by Dumas combustion method. The value for ash measures the total mineral or salt content of 47.65% in the sample. The fat content determined by solvent extraction was only 1.98% and the total calories content was 195 Calories/100 g sample.

Table 4.1 Proximate analysis of TBSP (wet basis)

| Description | Unit |
|---|---|
| Moisture | 5.97±0.12% |
| Fat | 1.98±0.08% |
| Ash | 47.65±1.43% |
| Protein* | 44.20±0.44% |
| Carbohydrates** | 0.20% |
| Calories | 195 Calories/100 g sample |

Note: - * By Dumas's method (F = 6.25), ** Calculated by subtraction

- Proximate analysis shows means for n = 2 replicates (± sd).

- Analysis by Medallion Laboratories, 9000 Plymouth Avenue North, Minneapolis, MN 55427 www.medallionlabs.com

In comparison to the results for TBSP, the proximate composition of herring soluble protein powder has been reported to be 62% protein, 20.5% fat, 3.5% moisture and 13.7% ash (Liceaga-Cesuaida and Li-Chen, 1999). Arrowtooth flounder had a composition of 79.1% protein, 14.9% fat, 2.4% moisture and 3.6% ash (Sathivel et al., 2005). These proximate analysis results were different because of the difference in type of fish and methods for extraction of fish proteins. The protein content of the threadfin bream powder 44.20%, is lower than other fish powder because of the high ash content of 47.65% that may be attributed to the use of 0.1 M sodium phosphate buffer pH 7 for extraction of sarcoplasmic proteins.

Marti et al. (1994) studied waste water form fishmeal industries processing. They found that waste water had average concentration of soluble proteins 2.73 g/L, however the concentration range was 0.3-7.5 g/L. Although, Montero and Gomez-Guillen (1998) reported that the crude protein content in muscle wash-water from Antarctic krill (*Euphasia superba dana*) was 2.02%. Bourtoom et al. (2006) determined proximate composition of protein recovered from the first stage of surimi wash-water, which was reconstituted in the laboratory from threadfin bream fish *N. hexodon* and was freeze-dried. They found 80.88, 2.94, 6.26 and 9.92 g/100 g of crude protein, crude fat, ash and other, respectively. However, Chawla, Venugopal, and Nair (1996) investigated protein content of unwashed and washed minced of threadfin bream *N. japonicus*. They found that protein content of unwashed and after washing washed minced fish meat were 14.9% and 6.9%, respectively. So the lost of protein during washing was 8%. All of these researches show that fish soluble proteins were lost in fish processing. If they could be recovered for utilization, this will increase their value and also reduce the cost of waste water treatment.

The moisture content for TBSP prepared in this study was low. The value of 5.97% moisture agrees with the values reported by other researchers for dried protein powders (Sathivel et al., 2004; Sathivel and Bechtel, 2006). Low moisture content is necessary to preserve food samples and to obtain a long shelf-life. When water is limiting, the growth of microorganisms will be inhibited. Chemical changes and other deterioration will also be limited in a low moisture system (Fennema, 1996). In addition to drying, TBSP was stored at low temperatures in order to avoid sample deterioration. The fat content of TBSP is low which is good for consumers who may

be concerned about high-fat foods. A low fat food will also be protected from spoilage due to fat oxidation. The presence of lipids can also influence the protein functionality, e.g. foaming (section 4.3.3).

Other investigators (Liceaga-Cesualda and Li-Chan, 1999; Sathivel et al., 2004; Sathivel et al., 2005) have reported the ash content of 3% to 18% for fish protein isolate or powders. The ash value for TBSP (47.65%) is unexpectedly high. The ash content for a sample gives an estimate of the total salt content. The high ash in the TBSP sample may be attributed to the use of 0.1 M sodium phosphate buffer pH 7 for extraction of sarcoplasmic proteins. It would be possible to reduce the ash content of TBSP samples by dialyzing against distilled water before freeze-drying. This treatment increased the protein content of the TBSP. Some of the functional properties of TBSP may be improved and TBSP could be widely used without limitation of ash content.

The amino acid profile of TBSP is shown in Table 4.2. The amino acid analysis was determined by using acid hydrolysis and followed by HPLC. The analysis of amino acid content was performed by a contracted laboratory (Medallion Laboratories, 9000 Plymouth Avenue North, Minneapolis MN 55427 www.medallionlabs.com). The data does not include results for several amino acids including, cysteine which was not determined in this analysis. The cysteine content for TBSP was determined separately as described in Table 4.3. Two other amino acids (glutamine and asparagine) are also not reported because these amides are hydrolyzed to form aspartic acid and glutamic acid and ammonia (as shown in Table 4.2) during acid hydrolysis of proteins.

The amino acid data for TBSP (Table 4.2) shows that this ingredient contains most of the essential amino acids (phenylalanine, methionine, threonine, leucine, isoleucine, valine and lysine), which is important for high nutritional quality. There is also a high lysine content in TBSP (10%). Lysine content in processed foods can be lost due to reaction with sugars in the Maillard reaction. The concentration of branched chain amino acid (isoleucine, leucine, valine) is also quite high for TBSP. These amino acids are thought to be important in helping athletes repair and build muscle tissue (Guthrie and Picciano, 1995). In summary, the amino acid profile for

TBSP would tend to suggest that ingredient is nutritionally sound. However, more research is needed to assess the nutritional properties of TBSP directly.

Table 4.2 Amino acid profile of TBSP

| Amino acids | Amount (%) |
|---|---|
| Aspartic acid | 11.8 |
| Threonine | 4.7 |
| Serine | 4.6 |
| Glutamic acid | 11.8 |
| Proline | 4.1 |
| Glycine | 6.3 |
| Alanine | 6.9 |
| Valine | 5.7 |
| Methionine | 2.3 |
| Isoleucine | 3.9 |
| Leucine | 8.0 |
| Tyrosine | 3.6 |
| Phenylalanine | 5.3 |
| Histidine | 3.3 |
| Lysine | 10.0 |
| Arginine | 5.3 |

Note: Proximate analysis shows means for n = 2 replicates (± sd).
The standard deviation for amino acid profile is ± 5% (relative deviation).
Analysis by Medallion Laboratories, 9000 Plymouth Avenue North, Minneapolis, MN 55427
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The total protein and total soluble protein of TBSP are shown in Table 4.3 together with the contents of sulfhydryl group and the surface hydrophobicity. The data shows that the total crude protein in TBSP determined by Kjeldahl analysis was 41.97% total solid (F = 6.25), which agrees with the 44.20% value determined by

Dumas method by an external laboratory. The total soluble protein was 47.99% total protein, determined by modified Lowry's method. TBSP also contained free sulfhydryl group (not including S-S bonds) at a level of 72 μmoles/g protein and the surface hydrophobicity was 69.36. Literature results for mackerel and pollock freeze-dried protein showed 72.3 and 93.9% protein (dry basis), and 19.7% and 26.4% soluble protein, respectively. The sulfhydryl group content was 65 μmoles/g protein for mackerel and 80 μmoles/g protein for pollock (Opstvedt et al., 1984).

Table 4.3 Compositions of TBSP

| Content | Quantity |
|---|---|
| Total protein* | 41.97±0.31% total solid |
| Total soluble protein** | 47.99±0.64% total protein |
| Sulfhydryl group | 72±2.4 μmoles/g protein |
| Surface hydrophobicity | 69.36±0.31 |

Note: * By Kjeldahl's method (F = 6.25), ** By modified Lowry's method

### 4.1.2 Determination of molecular weight of TBSP by SDS-PAGE

Samples of freeze-dried TBSP were analyzed for molecular weight using SDS-PAGE (12.5% gel) as described in section 3.1.7. The results showed in Figure 4.1. The SDS-PAGE gel was photographed and scanned using computerized software to find the protein band staining intensity, peak area, and other parameters. To determine the molecular weight for each protein band, a calibration graph was drawn between logarithmic molecular weight versus peak position (Figure B1 in Appendix B) resulting in straight-line segments.

When the fish sarcoplasmic proteins were separated by electrophoresis, they gave profiles that were unique to the species. By comparing this profile with the reference profiles of authentic samples, the species can be specified (Mackie, 1997). Liceaga-Gesualdo and Li-Chan (1999) reported that the electrophoretic patterns of the herring soluble fraction powder have various bands in the range of 45 to 14.2 kDa.

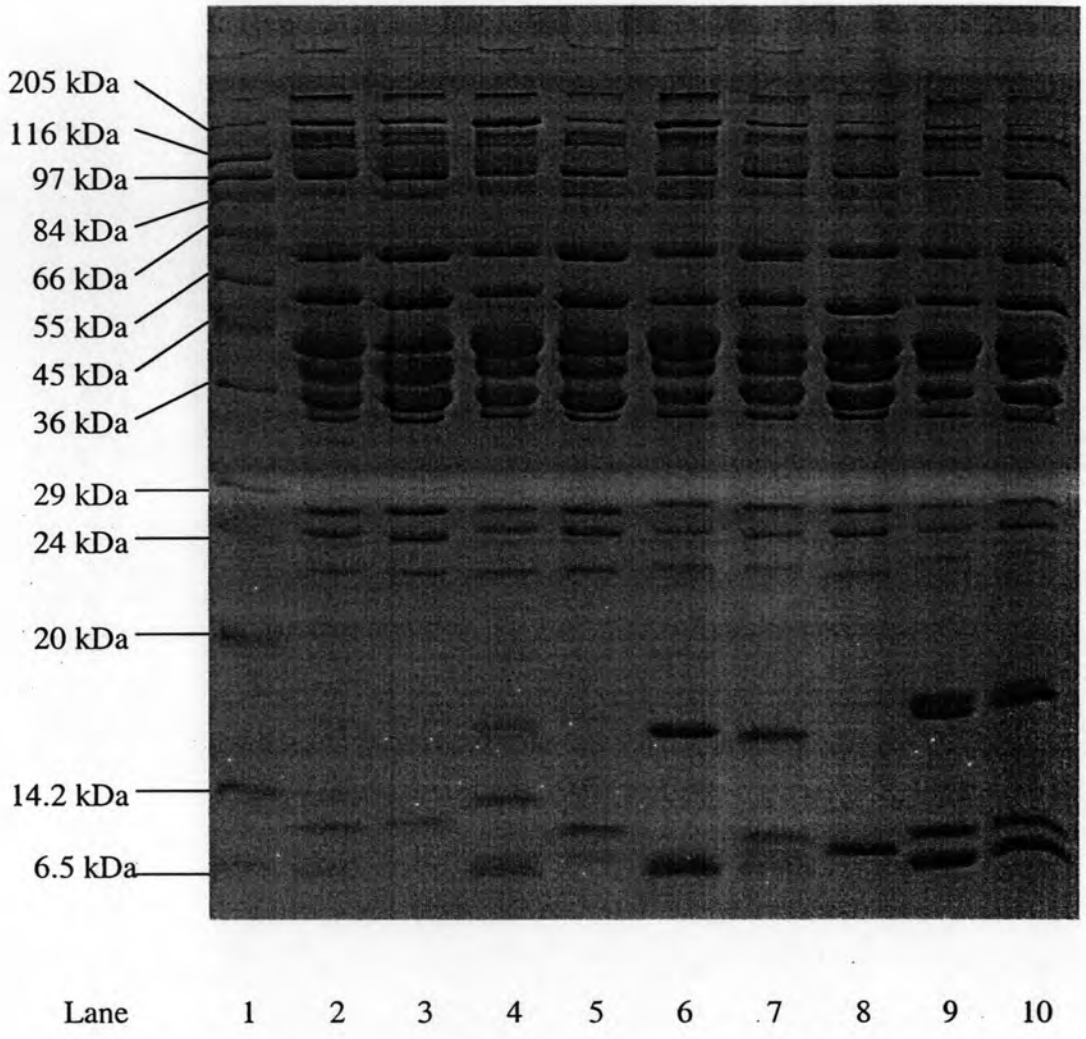


Figure 4.1 Separation of fish sarcoplasmic proteins on SDS-PAGE. Lane 1 represents marker proteins (wide length 6.5-205 kDa, Sigma). Lane 2-10 are fish sarcoplasmic proteins that were extacted from fishes; Lane 2, Ornate threadfin bream *N. hexodon*; Lane3, Japanese threadfin bream *N. japonicus*; Lane4, Fork-tailed threadfin bream *N. fergosus*; Lane5, Lattice monocle bream *Scolopsis taeniopterus*; Lane6, Purple-spotted bigeye *Priacanthus tayenus*; Lane7, Tiger-toothed croaker *Otolithes ruber*; Lane8, Brushtooth lizardfish *Saurida undosquamis*; Lane9, Nile tilapia *Oreochromis niloticus* and Lane10, Ruby tilapia *O. niloticus x O. placidus*. Fish sarcoplasmic proteins samples containing 6 μg of protein were applied to each lane.

Nakagawa, Watabe, and Hashimoto (1988a) used the PAGE technique to establish quantitatively the sarcoplasmic protein patterns of various fish species. Polyacrylamide slab gel electrophoresis (10% gel) in the presence of SDS made it possible to categorize those fishes into three groups by patterns: (a) marine white-fleshed, demersal or bottom fish (e.g. red sea bream, equal amounts of 43 kDa, 40 kDa and 35 kDa components), (b) marine red-fleshed, pelagic fish (e.g., Pacific mackerel rich in 40 kDa compared to 45 and 35 kDa components), and (c) freshwater fish (rich in 43 kDa compared to 40 and 35 kDa components). Therefore, red sea bream showed an approximately equal distribution (12.6%, 13.7% and 13.5%) for 43 kDa, 40 kDa and 35 kDa components. Pacific mackerel showed 43 kDa, 40 kDa and 35 kDa distributions of 13.7, 18.7 and 11.6% and Carp showed 19.5, 12.3 and 10.7% distribution for the 43 kDa, 40 kDa and 35 kDa components (Nakagawa, Watabe, and Hashimoto, 1988a).

Figure 4.1 shows the SDS-PAGE (12.5% gel) results for demersal fish and some fresh water fish. The protein profile for ornate threadfin bream *N. hoxodon* studied in this work is shown in lane 2. The other fish was studied for comparison including Japanese threadfin bream, Fork-tailed threadfin bream, Lattice monocle bream, Purple-spotted bigeye, Tiger-toothed croaker, and Brushtooth lizardfish. Nakagawa, Watabe, and Hashimoto (1988a) studied on demersal fish that had the electrophoresis patterns of 42 kDa, 40 kDa and 35 kDa components. All of these fishes were the same as their report. Nile tilapia and Ruby tilapia, freshwater fish, had rich in 42 kDa component, compared to 40 and 35 kDa components, as expected for fresh water fish. The major component at 42 kDa might be actin (Haard, 1995). The Figure 4.1 also showed the band as 205 kDa which might be myosin (Benjakul et al., 1998). Therefore, sarcoplasmic proteins of ornate threadfin bream also had myofibrillar proteins as component. Finally, in all the extracted fish sarcoplasmic proteins there were some high molecular weight proteins (MW $\geq$ 205 kDa, Figure 4.1) which are possibly from the myofibril fraction.

As with all structural and contractile muscle, fish flesh contains three main groups of proteins, namely: sarcoplasmic or water-soluble proteins, myofibrillar proteins, and connective tissue proteins. These are readily separated by fractional extraction techniques. Muscle aqueous extracts consist mainly of water-soluble

proteins which account for 20-35% of total muscle proteins (Mackie, 1996). Most of these proteins are enzymes involved in metabolic processes. Every metabolism has its specific pathway, and accompanying associated enzyme systems. Therefore, differences in amino acid composition of proteins should be expected in different types: marine and freshwater fish (Nakagawa, Watabe, and Hashimoto, 1988a), also in different species (An et al., 1989; Scobbie and Mackie, 1988), and in closely related species (Chen and Hwang, 2002; Huang, Marshall, and Wei, 1995). The unique nature of the sarcoplasmic proteins of each species is a reflection of the unique nature of metabolic pathways present in that species. Therefore, the separation patterns or profiles obtained on electrophoresis gel can be used for the unequivocal identification of the species (Mackie, 1990, 1996, 1997; Rehbein et al., 1995; Sotelo et al., 1993)

The SDS-PAGE molecular weight of proteins extracted from TBSP was calculated from a calibration graph. The logarithmic molecular weight versus migration distance was ploted, resulting in straight-line graph, using wide range molecular weight marker proteins as standard (Figure B1). The computer programe was used to perform linear regression of the data and the equation of the linear regression line was used to estimate the size of the unknown proteins. The curve is linear only over a limited range of molecular weights of standard marker, i.e. between 14.2-66 kDa. So, the calculations from equation, for the unknowns which locate out of the range of linear correlation might not be the correct values. The calculated values of the molecular weight of threadfin bream proteins on SDS-PAGE showed 4-distinct molecular weight size ranges: 10-12 kDa (5%), 25-29 kDa (12%), 40-62 kDa (53%) and 80-100 kDa (30%) (Calibration from QuantiScan version 2.1 for Windows (Biosoft, UK)). The similarity of all fishes is that examined had about 70% of the protein with molecular weight lower than 62 kDa.

### 4.1.3 Determination of molecular weight of TBSP by gel filtration

The molecular weight of polypeptides present in TBSP was determined using gel filtration analysis as described in section 3.1.8. Samples of standard proteins were injected to calibrate the gel filtration column. Each protein was injected to the

column and the elution volume ($V_e$) was collected and measured at 280 nm. The column void volume ($V_o$) was determined by injection of blue dextran. To determine the molecular weight values, $V_e/V_o$ (defined in Table C1 in Appendix C) were compared with values determined using five standard proteins. The resulting calibration graph was then fitted with the straight-line graph in Figure B2 (in Appendix B) which shows a plot between logarithmic molecular weight against $V_e/V_o$ for each standard protein. The results are summarized by a straight-line graph having a negative slope (-1.8303) and intercept (4.3634) and correlation coefficient ($R^2$) value of 0.9986.

The analysis was performed on the freeze-dried powder sample (TBSP and not fresh protein extract), which was a light yellow color with the composition described in section 4.1. The TBSP powder, was first dissolved in deionized water (25 mg/mL), adjusted to pH 7 and then centrifuged to remove the undissolved residues. The profile showed approximately one peak out of separation range. The position for the highest peak is shown in Figure 4.2 with arrow and molecular weight estimated in Table C1 (in Appendix C). This gel filtration with Sephacryl S-200 HR can not separate proteins in TBSP because the peak lies outside the molecular size range of Sephacryl S-200 HR (i.e. 5 – 250 kDa).

Under the conditions used for gel filtration analysis, TBSP sample contained protein material with a high molecular weight. As described, the Sephacryl S-200 HR used in this study has a fractionation range for globular protein of 5-250 kDa. So, molecular weight of TBSP may be higher than 250 kDa (Figure 4.2). The results from a gel filtration experiment depend on many factors including (a) column choice, (b) type of solvent used and its salt content, (c) method of protein isolation, and (d) processing effects on the extracted protein. In this work, the chosen column choice (Sephacryl S-200 HR) is not appropriate to use in separating the proteins of TBSP powder.

The component subunits of a protein are held together by bonds, these linkages are broken prior to electophoresis in SDS-PAGE while gel filtration separates proteins as native components. Therefore, the studies of molecular weight of proteins by SDS-PAGE (section 4.1.2) and gel filtration (section 4.1.3) are different.

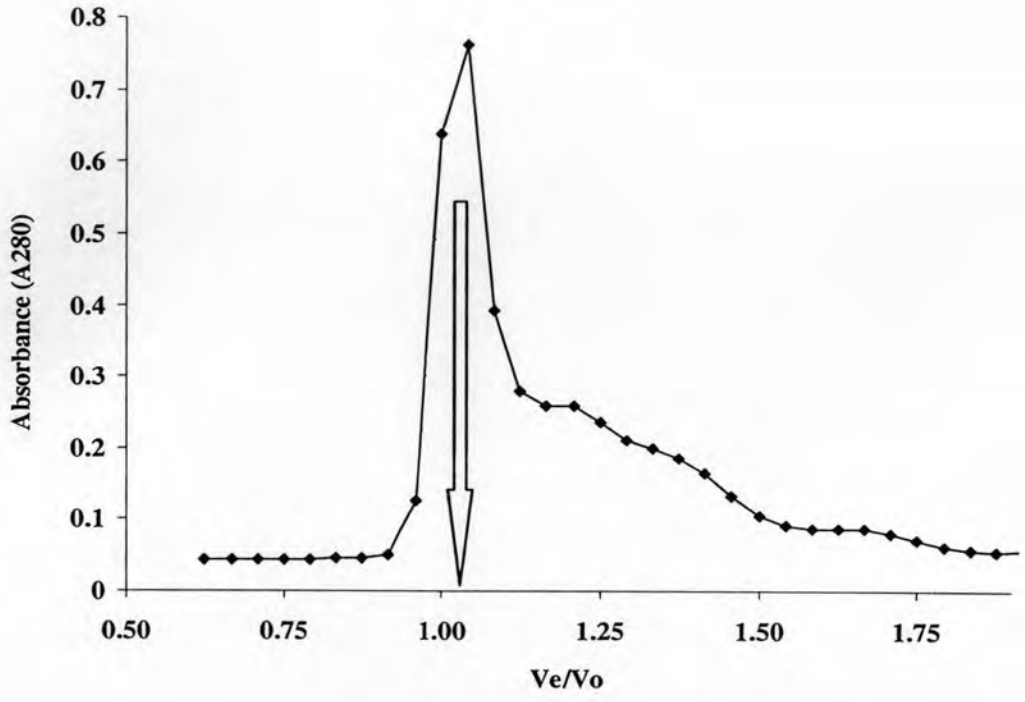


Figure 4.2 The elution profile of the 2.2% TBSP (in deionized water, pH 7) from a column (16/70 cm) of Sephacryl S-200 HR, flow rate 0.5 mL/min, fraction volume 2.5 mL. The elution was performed by 0.05 M phosphate buffer, pH 7.0, containing 0.15 M NaCl in refrigerator (4°C). The position for the highest peak is shown with arrow.

### 4.1.4 Differential scanning calorimetry of TBSP

Differential scanning calorimetry (DSC) is a useful method for analyzing protein structure (Biliaderis, 1983; Wright and Wilding, 1984). In a DSC experiment, the protein sample is sealed in a small aluminum pan and then slowly heated. A heat absorption peak can be seen when the protein denatures. If a protein sample is already denatured before DSC analysis, then no peak will be seen (Hohne, Hemminger, and Flammersheim, 1996). From the DSC experiment it is possible to measure the sample "melting" temperature (Tm) and the peak area which is the heat absorbed for protein denaturation (watts/g protein). This is also called enthalpy of denaturation.

Figure 4.3a shows the DSC curve of soluble TBSP determined as described in section 3.1.9. To make these measurement, TBSP (10%) was dissolved in deionized water, adjusted to pH 7 and stirred 1 h. The solution was centrifuged and supernatant was pipetted (15 $\mu$L) into a DSC pan. Experiments were also run with beta-lactoglobulin and casein as calibration proteins; Figure 4.3b and Figure 4.3c, respectively. The sealed samples were heated with a linear heating rate of 5°C/min over a temperature range of 40-110°C.

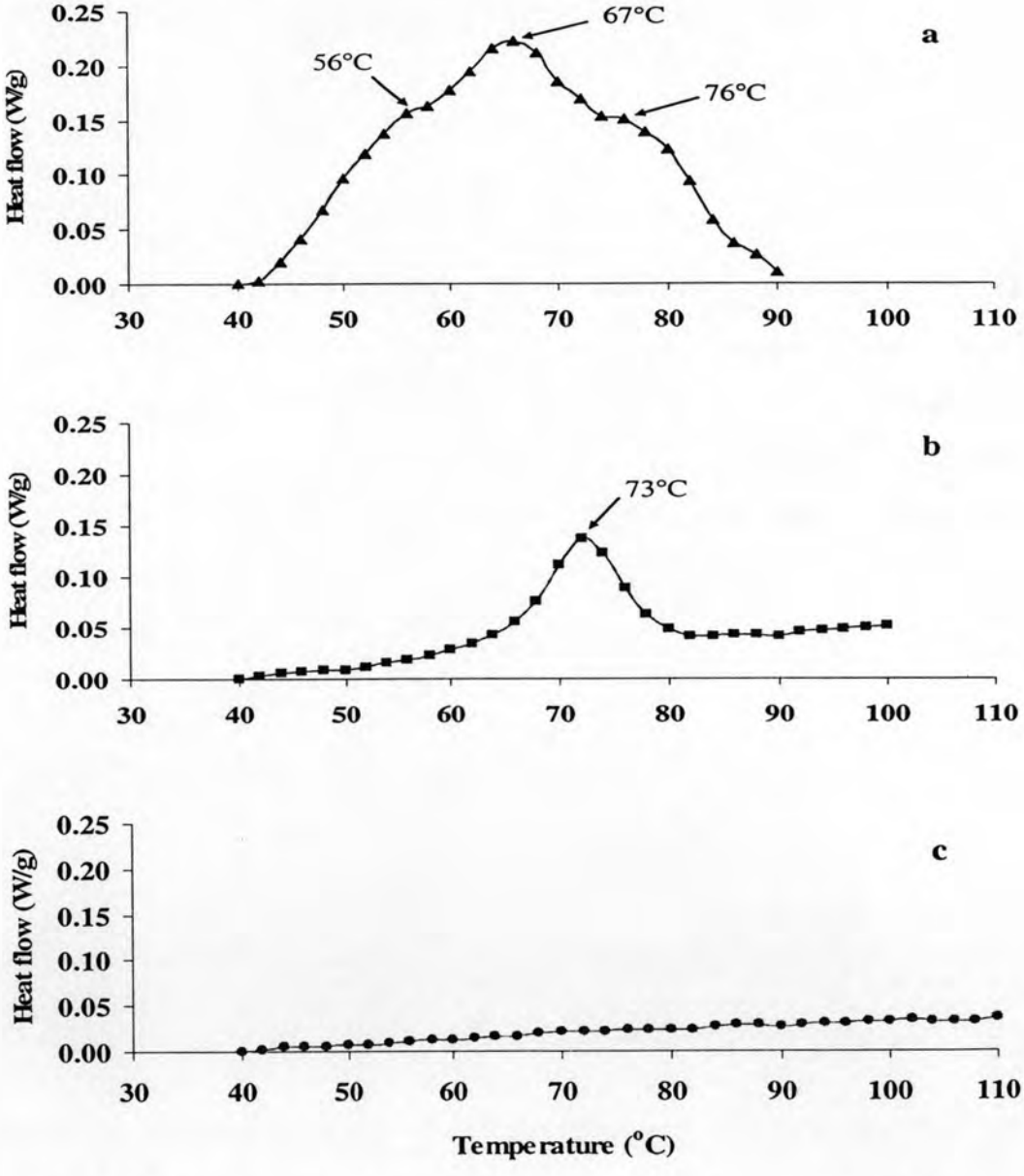


Figure 4.3 DSC thermograms of (a) soluble TBSP, (b) beta-lactoglobulin and (c) casein

The DSC profile for TBSP showed three endothermic transitions with Tm values at 56°C, 67°C and 76°C (Figure 4.3a). Because of the small amount of connective tissue in fish muscle, these transitions can be assigned to denaturation of myofibrillar and sarcoplasmic proteins (Brown, 1986). So, these Tm attributed to sarcoplasmic proteins, myosin and actin, respectively. Therefore, sarcoplasmic proteins was denatured easier than others because it had lowest Tm. By comparison, the DSC thermogram for beta-lactoglobulin had one peak with a Tm value of 73°C (Figure 4.3b). Beta-lactoglobulin is suitable for calibrating DSC experiments because it gives a clear peak with well-known Tm value. Results for casein showed no peak as expected because casein has no tertiary structure (Figure 4.3c). Swaisgood (1996) explained that casein has unique primary and tertiary structures unlike other proteins, which may explain the lack of DSC peak.

The exudative sarcoplasmic fraction from hake showed three transitions with peak temperature $T_{max}$ values of 45.2, 59.0 and 75.5°C and $\Delta H$ of 3.92 cal/g (Beas et al., 1990). $T_{max}$ of hake muscle transitions were lower than those of TBSP. Similar results were reported with other fish species (Akahane et al., 1985; Poulter et al., 1985).

## 4.2 Biochemical characteristics of proteases from TBSP

### 4.2.1 Effect of substrates choice on crude protease of TBSP

Fish muscle contains several proteases including, calcium activated proteases (calpains), cathepsins and some metallo-proteases (Kolodziejska and Sikorski, 1996). The activity of muscle proteases has also been shown to affect the quality of stored fish. However, few papers have been published on proteases from threadfin bream tissue. The amount of protease activity associated with protein concentrate or isolates from most fish has not been well characterized. The studies of protease detected from TBSP samples are described in this part of the thesis.

Table 4.4 shows the enzymatic specific activity (ESA) for crude proteases from TBSP or soluble protein from cod which were prepared by methods described in section 3.1.1. Enzyme substrates used for protease assay were azocasein, sodium caseinate or endogenous protein substrate (native muscle protein from TBSP), for

studying the suitable substrate for crude protease of TBSP. The ESA was determined by methods described in section 3.2.1.1-3.2.1.3. In these experiments, performed at Pennsylvania State University, State College, PA, USA, cod fish sarcoplasmic proteins extract was chosen as an internal standard. It is well known that cod muscle has proteolytic activity (Gudmundsdottir and Palsdottir, 2005; Stoknes, Walde and Synnes, 2005). A solution of TBSP showed a high proteolytic activity toward natural fish protein (endogenous substrate activity assay) and casein, but no activity was detected with azocasein.

Table 4.4 Enzymatic specific activity of crude protease from threadfin bream and cod

| Fish | Protein content (mg/mL) | Substrate | Enzyme Specific Activity (ESA)[A] |
|---|---|---|---|
| TBSP (freeze-dried) (1 or 5% w/v solution) | 3.17±0.31 | Azocasein | -[B,C] |
| | | Na- Caseinate | 0.38±0.01 |
| | | Endogenous protein substrate | 0.45±0.03 |
| Cod protein extract (1% w/v) freeze-dried | 2.07±0.19 | Azocasein | -[B,C] |
| | | Na-caseinate | 0.38±0.01 |
| | | Endogenous protein substrate | 0.27±0.06 |

Note: The results for enzyme specific activity show means for n = 3 replicates (± sd). ESA was measured at A450 for azocasein, and A280 for casein and endogenous substrate.

[A] the unit of ESA is A280/mg/h = A280 x h/mg except for the azocasein assay, is A450/mg/h,

[B] No activity detected, [C] Increasing to 5% TBSP and 2% azocasein still showed no activity

Protease activity could not be detected in the TBSP solution if azocasein was used as substrate (Table 4.4). Similarly no activity was detected for cod muscle extract with azocasein substrate. Both TBSP solution and cod muscle extract showed protease activity when assayed with sodium caseinate or the endogenous fish protein substrate. The specific activity of 1% cod freeze-dried solution was 0.27 and 0.38 ($A_{280}$/mg/h) by endogenous substrate assay and Na-caseinate, respectively. The ESA from the TBSP solution was 0.45 and 0.38 for the endogenous substrate assay and Na-caseinate, respectively. A protein substrate must be selected so that good results are obtained with the detection methodology being used (Sarath, De La Motte and Wagner, 1989). Therefore, sodium caseinate is suitable for crude protease of cod, whereas endogenous substrate is suitable for crude protease of TBSP. Cod and threadfin bream are different in species, so the protein components and also the amount of protease are different.

An et al. (1994) compared the azocasein assay to other assays using casein, hemoglobin (Hb) or BSA as substrate. The source of protease was Pacific whiting crude muscle homogenates at pH 3, 5 and 7. All four substrates showed maximum activity at pH 5. At pH 5, azocasein and casein were better substrates than Hb or BSA, but at pH 7 casein was better than the others.

In the work described in this thesis, the crude muscle extract used as a source of enzyme contained a large amount of protein, as shown by the protein content, approximately 2-3 mg/mL (Table 4.4). Occasionally, the native substrate for the protease is easily obtained and can be used for assays (Sarath, De La Motte and Wagner, 1989). Among the components, myosin was shown to be the most preferred substrate and is the first protein hydrolyzed by the protease, thus showing the minimal effect of added exogenous substrate (Hurtado et al., 1999).

Azocasein is often used as good substrate for analyzing protease activity in fish muscle (An et al., 1994), but was not suitable for TBSP and cod crude protease and resulted in no activity being detected. The presence of high levels of myofibrillar proteins in the crude extract could have interfered with the assay by the competitive inhibition of the protease, resulting in substantial reduction in hydrolysis of azocasein (Hurtado et al., 1999). An et al. (1994) reported that high levels of proteins included in the extract may compete with azocasein for the active site of protease, thus

underestimating the enzyme activity. From SDS-PAGE data TBSP extract has many proteins, such as actin and myosin (Figure 4.1) which are suitable substrates for fish muscle protease. The mechanism by which azocasein inhibits fish muscle proteases is not certain. It is possible that the azo-chromophore, which gives azocasein its yellow color (Sarath, De La Motte and Wagner, 1989), binds metal ions such as zinc, i.e. azocasein could inhibit metallo-proteases from fish.

In this research, endogenous protein substrate (section 3.2.1.3) in TBSP provided additional advantages for the assay because it is less time consuming and easier handling. Therefore, the endogenouse protein substrate of TBSP was used in every testings of crude protease activity later on in this study.

### 4.2.2 Determination of the pH-activity profile for crude protease of TBSP

The pH-activity profile for protease enzymes can provide information about the groups in the active site and help to identify the proteases from TBSP samples. The pH-activity profile for crude protease of TBSP was assayed using the endogenous substrate assay method (section 3.2.1.3). The assay was run at 55°C with incubation time for 1 h. The results were shown in Figure 4.4. From this graph, crude protease of TBSP has low activity between pH 2–5. The crude protease of TBSP showed activity at pH 6-9 with optimum activity at pH 7. Therefore, the crude protease of TBSP can be considered to be a neutral (and/or alkaline) protease similar to fish protease reported by Osatomi et al. (1997), Choi et al. (1999) and Cao et al. (2000).

Since a crude protein mixture was used in this study, it is not certain whether the sample contains one type of alkaline protease or a mixture of different alkaline proteases. These results do not allow us to pinpoint whether the sample contains small amounts of acid proteases. To get more information about the type of enzymes present, inhibitor studies were then conducted.

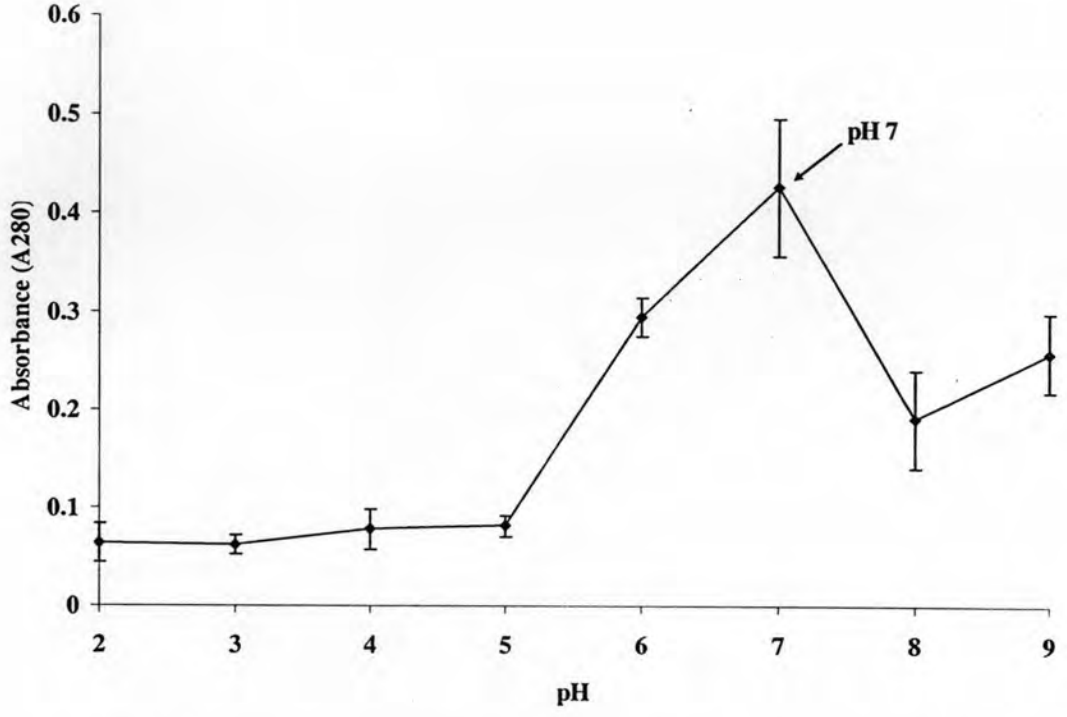


Figure 4.4 Crude protease activities (Absorbance at 280 nm) of TBSP at pH 2-9 by endogenous substrate activity assay

In Figure 4.4 the acitivity of crude protease of TBSP was highest at pH 7. The ability of the amino group at the active site of an enzyme to interact with the substrate depends on their electrostatic state. If the pH is not appropiate (strong acid or alkaline pH), this introduces a total positive or negative charges in the hydrophobic interior of the enzyme and ultimately leads to enzyme inactivation (Mathewson, 1998). However, the charge on one or all of the required amino acids is such that the substrate can neither bind nor react to produce product. The optimum pH can vary, depending upon protein substrates (Seymour et al., 1994). Differences in optimum pH have been attributed to the accessibility of the substrate to the active site at the particular pH environment.

### 4.2.3 Determination of the temperature-activity profile for crude protease of TBSP

Information related to the effect of temperature on the protease activity from TBSP is necessary in order to develop accurate assays for this enzyme. The temperature-activity profiles for crude protease of TBSP were determined as described section 3.2.1.3. Enzyme solutions were adjusted to pH 7 and incubated at different temperatures for 1 h according to the endogenous substrate activity assay. The results of temperature-activity profiles are shown in Figure 4.5.

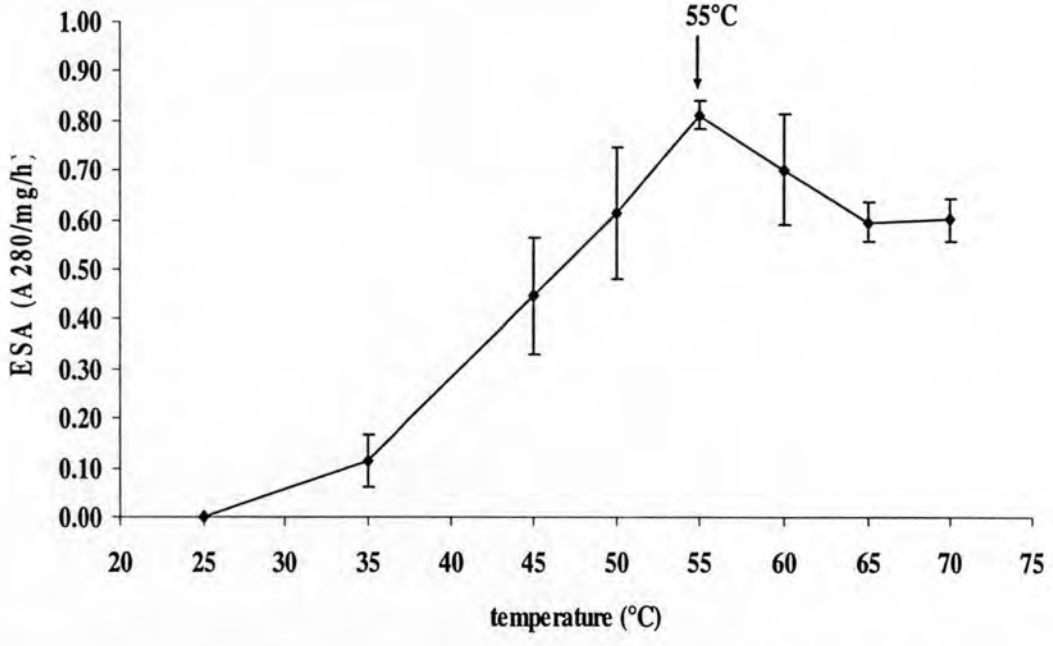


Figure 4.5 Temperature enzymatic specific activity profile of 1% TBSP in 0.1 M phosphate buffer pH 7 using endogenous substrate assay

At near physiological temperatures (around 30-40°C) there was little change in enzyme specific activity, but highest activity was observed at 55°C. The crude protease of TBSP had an optimum activity at a temperature of 55°C with endogenous substrate. By comparison, a heat-stable alkaline protease from bigeye snapper muscle had an optimum temperature of 60°C using a caseinolytic assay (Benjakul, Visessanguan, and Leelapongwattana, 2003). The optimum temperature of purified

protease from threadfin bream muscle showed a temperature optimum of 60°C (Kinoshita, Toyohara, and Shimizu, 1990). Osatomi et al., (1997) and Choi et al. (1999) studied proteases from fish. They found optimum temperature of serine protease was 55°C. From the results of this study, the optimum temperature of the crude protease of TBSP was 55°C, so it may be a serine protease.

### 4.2.4 Effect of inhibitors on crude protease of TBSP

Protease inhibitors are used to identify protease. This approach is effective even when a sample contains several different enzymes. The effect of four inhibitors on crude proteases of TBSP was investigated using endogenous substrate and caseinolytic activity assays described in section 3.2.4. Samples were allowed to stand for 60 min before measuring the absorbance at 420 nm. Before incubation with inhibitors, the TBSP solution was preheated in water bath at 60°C for 3 min and centrifuged to precipitate large protein components that can interfere with the results.

The inhibitors selected for this study included, iodoacetic acid (IAA), phenylmethylsulfonylfluoride (PMSF), 1,10-phenanthroline and pepstatin A. The list of compounds includes inhibitors for sulfhydryl proteases, serine proteases, Zn-metallo proteases and aspartic acid protease (Beynon and Salvesen, 1989).

The study of enzyme inhibition employed two different protease assays: the endogenous substrate assay and caseinolytic assay. The concentration of each inhibitor was selected from the literature and the level is high enough to ensure 100% inhibition of the proteases – if this is present at typical concentrations found in animal tissues (Beynon and Salvesen, 1989).

The results of the inhibition studies for threadfin bream protease are shown in Table 4.5 and Figure 4.6. With the endogenous substrate activity assay, PMSF completely inhibited protease activity from TBSP. However, 1,10-phenantroline and iodoacetic acid (IAA) partially inhibited the observed enzyme activity. Pepstatin A did not affect crude enzyme activity. With the caseinolytic activity assay, the data are the same as with the endogenous substrate activity assay except that both of IAA and pepstatin A did not affect crude enzyme activity from TBSP.

Table 4.5 Effect of inhibitors on enzymatic specific activity of crude crude protease from TBSP

| Inhibitor (and solvent) | Conc. (mM) | Enzymatic Specific Activity ($A_{420}$/h/mg protein) | | % Protease Inhibition | |
|---|---|---|---|---|---|
| | | Endogenous substrate assay | Caseinolytic assay | Endogenous substrate assay | Caseinolytic assay |
| Control/ water | 0 | 2.36 ± 0.21 | 4.67 ± 0.56 | - | - |
| Control/ MeOH | 0 | 2.40 ± 0.71 | 3.77 ± 0.36 | - | - |
| Iodoacetic acid/ Water | 1.0 | 1.53 ± 0.12 | 6.02 ± 1.48 | 35.3 | 0.00 |
| PMSF/ MeOH | 1.0 | 0 ± 0 | 1.98 ± 0.67 | 100.0 | 47.5 |
| 1,10-Phenanthroline/ MeOH | 1.0 | 1.39 ± 0.25 | 3.38 ± 1.83 | 42.0 | 10.2 |
| Pepstatin A/ MeOH | 1$\mu$M | 2.43 ±0.79 | 6.08 ± 1.15 | 0.0 | 0.0 |

Note: ESA was measured from A420 (TNBS method) with standing for 60 min before measuring
The results for ESA show means for n = 3 replicates (± sd).
% Inhibition = $((ESA_{control} - ESA_{inhibitor})/ESA_{control}) \times 100$
MeOH = methanol

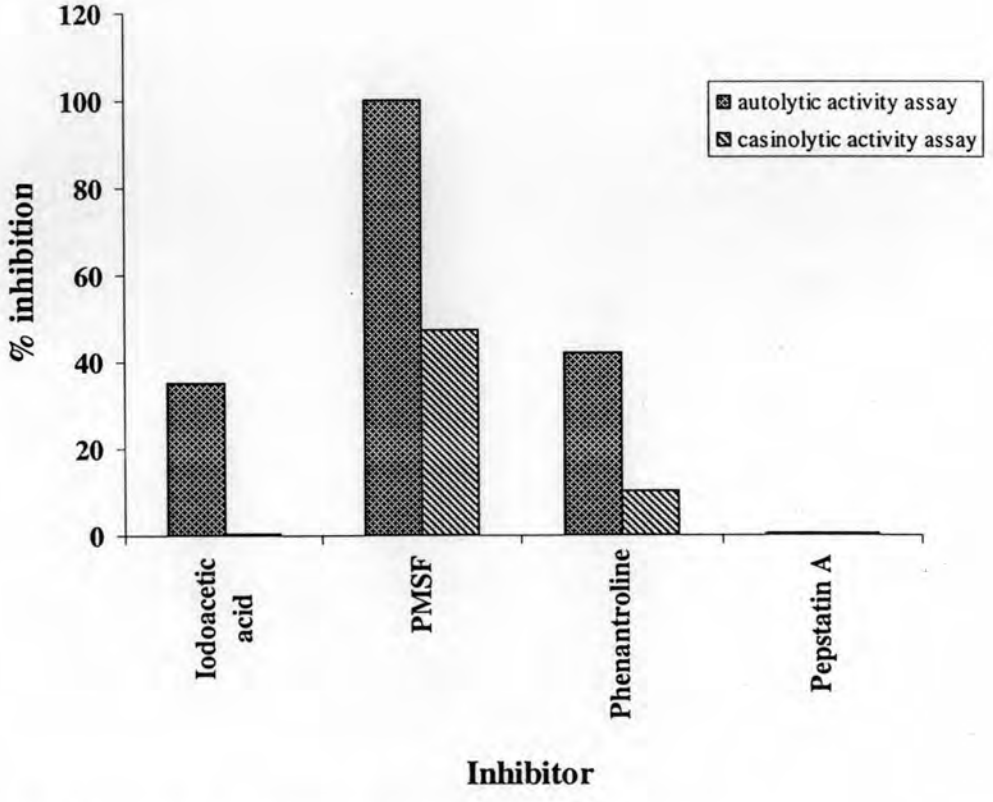


Figure 4.6 Effect of inhibitors on crude protease of TBSP

Two control experiments were performed with water as solvent or methanol (2%) as solvent because some inhibitors require methanol to dissolve. The results from Table 4.5 and Figure 4.6 show clearly that crude protease of TBSP was 48-100% inhibited by PMSF, which is a specific inhibitor for serine proteases. The next strongest inhibitor for TBSP proteolytic activity was 1,10-phenanthroline, which inhibits zinc metallo-proteases closely followed by iodoacetate. In all cases the percentage of inhibition was lower when sodium caseinate was used as the enzyme substrate compared with the endogenous substrate assay.

IAA affects thiol (cysteine) groups of proteins and its inhibition indicates the presence of thiol protease(s). Thiol proteases are sometime called cysteine proteases or sulfhydryl proteases because the active site has an SH group (Boneto et al., 1984). As described above, PMSF inhibits serine proteases, phenantroline inhibits zinc-metallo proteases and pepstatin A inhibits acid proteases with aspartic acid at the active site. Therefore, the enzyme activity inhibition pattern shown in Table 4.5

indicates the presence of a mixture of serine, thiol and metallo-proteases. However, the major enzyme in crude enzyme in TBSP was a serine protease (Table 4.5).

### 4.2.5 Effect of activators on crude protease of TBSP

The effect of activators on proteases can provide important information about enzyme biochemical characteristics. Therefore, the effect of activation of protease in TBSP was determined as described in section 3.2.5. One hundred milligram of TBSP was dissolved with 5 mM concentrations of various metal ions solutions (2% TBSP): LiCl, NaCl, KCl, $MgCl_2$, $CaCl_2$, $MnCl_2$, $FeCl_2$, $ZnCl_2$, $FeCl_3$ and $AlCl_3$ with magnetic stirrer at room temperature (25°C) for 1 h. These solutions were incubated at 55°C for 30 min. The remaining activities of the resulting samples are measured according to the endogenous substrate activity assay as mentioned before (section 3.2.1.3, protease assay with native substrate). The relative activity was expressed as a percentage ratio of the enzymatic specific activity of samples with metals and without metal. The relative activity is expressed as a percentage ratio of the enzymatic specific activity of samples.

The effect of ions on the activity of crude protease of TBSP is shown in Table 4.6. $Ca^{2+}$ enhanced the TBSP protease. $Al^{3+}$, $Mn^{2+}$ moderately enhanced while $Zn^{2+}$and $Fe^{3+}$ had slight effect on TBSP protease. The metal ions: $K^+$, $Li^+$, $Na^+$ and $Fe^{2+}$ were moderately inhibiting and $Mg^{2+}$ showed the most inhibition of crude protease of TBSP. From Table 4.6 the different activators can be classified into five groups according to their effects on threadfin bream muscle proteolytic activity: $^{a}$ (strongly activator ions - $Ca^{2+}$), $^{bc}$ (mildly activating ions - $Mn^{2+}$, $Al^{3+}$), $^{bcdef}$ ( no effect ions - $Fe^{3+}$, $Zn^{2+}$, and control ), $^{efg}$ (mildly inhibitory ions - $Fe^{2+}$, $Na^+$, $Li^+$, $K^+$) and $^{g}$ (strongly inhibiting ions - $Mg^{2+}$). $Ca^{2+}$ ions enhanced the TBSP proteolytic activity by 83% whilst $Mg^{2+}$ had the most inhibition of the crude protease of TBSP. Such results show clearly that activation by calcium and inhibition by $Mg^{2+}$ are clear features of crude protease of TBSP. The study of crude protease from TBSP also shows that $Mg^{2+}$ is the strongest inhibitor – probably because it has nearly the same ionic size as the $Ca^{2+}$ and could compete for the same binding site as calcium. In the

past research showed that calpain, which is an intracellular cysteine protease, is controlled by calcium ions and calpastatin (Wang, Su, and Jiang, 1992).

Table 4.6 Effect of ions on activity of crude protease from TBSP

| Ion | Relative activity (%) |
|---|---|
| $Ca^{2+}$ | $183.30^{a} \pm 4.85$ |
| $Al^{3+}$ | $112.95^{b} \pm 2.33$ |
| $Mn^{2+}$ | $110.95^{bc} \pm 0.93$ |
| $Zn^{2+}$ | $106.51^{bcd} \pm 8.43$ |
| control | $100.00^{cde} \pm 0.00$ |
| $Fe^{3+}$ | $99.39^{def} \pm 6.63$ |
| $K^{+}$ | $94.45^{efg} \pm 5.97$ |
| $Li^{+}$ | $91.36^{efg} \pm 2.57$ |
| $Na^{+}$ | $90.44^{efg} \pm 3.45$ |
| $Fe^{2+}$ | $88.11^{fg} \pm 6.75$ |
| $Mg^{2+}$ | $86.43^{g} \pm 3.17$ |

Note: (1) a-g; mean values with different letters are significantly different at $p<0.05$ using SAS (2000) program

(2) % Relative activity = (ESA of effected ions/ ESA of control) x 100

In this study, crude protease of TBSP is probably a mixture of serine, cysteine and metallo-protease. This may explain how threadfin bream proteolytic activity might be enhanced and inhibited with many ions. The $Ca^{2+}$ ion is a classical trypsin activator and also activator for calpains. There is likelihood also the presence of calpain-like protease in threadfin bream tissue. The crude enzyme activity of TBSP has optimum pH 7 and temperature 55°C, which was completely inhibited by PMSF which is serine protease inhibitor and activated by $Ca^{2+}$. Therefore, the major enzyme protease in TBSP was trypsin-like protease (serine protease).

### 4.2.6 Effect of frozen storage and freeze-drying on crude protease of TBSP

In this research, some samples of threadfin bream were stored frozen in a freezer at -18°C for 1.5 years. During the study, sarcoplasmic protein extracts were also freeze-dried to produce TBSP powder. The purpose of the study described here was to evaluate the effect of 1.5-year frozen storage as well as freeze-drying on the protease activity in TBSP.

The effects of processing method (form of storage and freeze-drying) on protease activity in threadfin bream were studied (section 3.2.6). The endogenous substrate assay had good sensitivity and was able to differentiate enzymatic specific activity of crude protease between fresh extracted and freeze-dried fish proteins. Crude proteases extract was prepared in two ways using threadfin bream samples stored frozen for 1.5-years or purchased freshly from a market in Thailand (fresh frozen fish). The S1, S3 and S5 were enzyme extracts (fresh) prepared directly from fish tissue by homogenizing fish tissue with a Waring blender as described in section 3.1.1. For preparing protease extract from freeze-dried TBSP; S2, S4 and S6 were prepared by fresh sample from S1, S3 and S5 was freeze-dried, respectively. They were reconstituted with deionized water and assayed for protease activity.

The result of these studies is summarized in Table 4.7. ESA of freeze-dried fish proteins both of fresh frozen fish (S2) and frozen storage fish (S4) is lower than fresh extract (S1 and S3), by 43% and 44.2%, respectively. In this study, the control was the cod fish muscle extract. Freeze-drying produced 29.4% loss of protease activity from cod fish muscle extract (Table 4.7). Within the margin of experimental error, the loss of protease activity due to freeze-drying was the same (43-44%) if protease activity was prepared from freshly purchased fish (fresh frozen fish) or from the fish stored at -18°C for 1.5-years (frozen storage fish). The enzyme from fresh extract of threadfin bream (frozen 1.5 years) (S3) had % relative ESA of 111.5% because the fish in S3 was from different batch from S1. Another important result is that, freezing threadfin bream for 1.5-years at -18°C resulted in no loss of protease activity. This indicates that there should have no significant effect on other.

These results mean that fish wash-water (also has sarcoplasmic proteins) intended to be used for surimi or TBSP manufacture could be stored frozen for a long period of time before use. In this way, more fish wash-water could be collected

before processing to give TBSP. This means that the protein powder manufacturer has more flexibility. Nonetheless, some researchers reported that freezing can cause some denaturation and aggregation of fish muscle proteins (Park, 2000; Saeed and Howell, 2004). For this reason, sarcoplasmic proteins may be later on denatured by freezing which may affect the functional properties of this protein.

Table 4.7 Effect of freeze-drying on crude protease activity of threadfin bream

| Sample | Source of fish | Enzyme preparation method | Enzymatic Specific Activity (% Relative) | Effect of freeze-drying |
|---|---|---|---|---|
| S1 | Threadfin bream (fresh frozen) | Enzyme direct from fish tissue | 100 ± 3.3 | - |
| S2 | Threadfin bream (fresh frozen) | Enzyme from freeze- dried TBSP powder | 56.9 ± 9.1 | 43% loss |
| S3 | Threadfin Bream (Frozen storage 1.5 years) | Enzyme direct from fish tissue | 111.5 ± 0.7 | 11.5% increase |
| S4 | Threadfin Bream (Frozen 1.5 years) | Enzyme from freeze-dried TBSP powder | 67.3 ± 5.8 | 44.2% loss |
| S5 | Cod (fresh) | Enzyme from fish tissue | 100 | - |
| S6 | Cod (fresh) | Enzyme from freeze- dried powder | 70.5 | 29.4% loss |

* Fresh value is taken as 100%

### 4.2.7 Determination of enzyme transglutaminase in TBSP

Transglutaminase (TGase) is an enzyme that catalyses protein cross-linking reaction between the lysine ($NH_2$) group and the amide group of glutamine. Past studies have shown that many fish species contain TGase in their tissues. Protein cross-linking catalyzed by addition of TGase has been shown by others to affect protein functionality especially gelation (Seki, Nozawa, and Ni, 1998; Kuraishi, Yamazaki, and Susa, 2001; De Jong and Koppelman, 2002). The study described in this section initially was done to assess whether TBSP had significant TGase activity. Transglutaminase activity was determined according to the method in section 3.2.7. The result of this study is summarized in Table 4.8.

Table 4.8 Transglutaminase activity in TGase sources and TBSP

| TGase type / Supplier | Enzyme protein concentration | Enzymatic Specific Activity |
|---|---|---|
| Sigma | 2 units/mL | 7.26 ± 0.18 |
| Ajinomoto, Activa®-TGase | 1% w/w | 3.73 ± 0.16 |
| TBSP | 5% w/w | 0.00 ± 0.04 |

In this study, two commercial samples of TGase were used as internal standards to check the validity of the assay. Transglutaminase activity was found in the TGase standard form Sigma and the commercial TGase (Activa®-TGase) from Ajinomoto. The results show that the assay used in this study was valid because it could detect TGase activity in commercial samples. However, transglutaminase activity was absent in TBSP even when a concentrated 5% (w/w) sample of TBSP was tested. It was concluded that at the concentrations of TBSP used in functionality testing (section 4.3-4.4) there was no TGase activity in the protein samples.

## 4.3 Functional properties of TBSP

### 4.3.1 Solubility

Solubility is the important functional property of the protein because it provides an index of native structure and it is a critical prerequisite for using a protein in foods (Kinsella, 1982). Solubility is the result of the surface active properties of proteins. Many functional properties of food proteins have been related to solubility, such as foaming, emulsification and gelation.

The pH-solubility profile for TBSP was determined using the protein analysis method as described in section 3.3.1. The protein concentration of the supernatant was determined using the modified Lowry's method (Peterson, 1977). The Lowry calibration graph (Figure B3 in Appendix B) was used to determine the concentration of protein dissolved after 1 h stirring.

Figure 4.7 shows that TBSP had moderate solubility (42-46%) at pH 2, 3, 4, 7, 8 and 9 and the lowest solubility at pH 5 (16.09%), while at pH 6 the solubility of TBSP was 27.28%. The solubility of TBSP was high in both acid and alkaline pH regions. Because of the preparation method in section 3.1.1, the threadfin bream fish was extracted with 0.1 M phosphate buffer (i = 0.05), so the content of ash in TBSP is very high (47.65%). For such reasons, some myofibrillar proteins were probably extracted out in TBSP. These may affect the functional properties of TBSP. Most of the sarcoplasmic proteins have the molecular weight of 40-62 kDa. However, in the data given in Figure 4.1 (SDS-PAGE of ornate threadfin bream) some proteins have molecular weight more than 65 kDa, such as myosin (205 kDa). Therefore, some myofibrillar components were extracted in this study.

The solubility of food protein reflects the balance between solvent-protein interactions and protein-protein interactions. When pH value of the solvent is far from the isoelectric point (pI) of protein, protein-protein attractive interactions are low and then protein-solvent interactions are strongest. When the solvent pH matches the protein pI then protein net charge is zero and the tendency for protein-protein interaction increase leading to aggregation. However, from the solubility results the mixture of proteins in TBSP has the lowest solubility at pH 5. Most proteins in the "sarcoplasmic proteins" family are globular structures and relatively small in size

with the molecular weights between 30 and 65 kDa (Xiong, 1997) which is the same as in this study (Figure 4.1). However, data from Figure 4.1 also show high molecular weights which might be myofibrillar proteins. The data of DSC in Figure 4.3a, also demonstrate that TBSP had myofibrillar proteins in it.

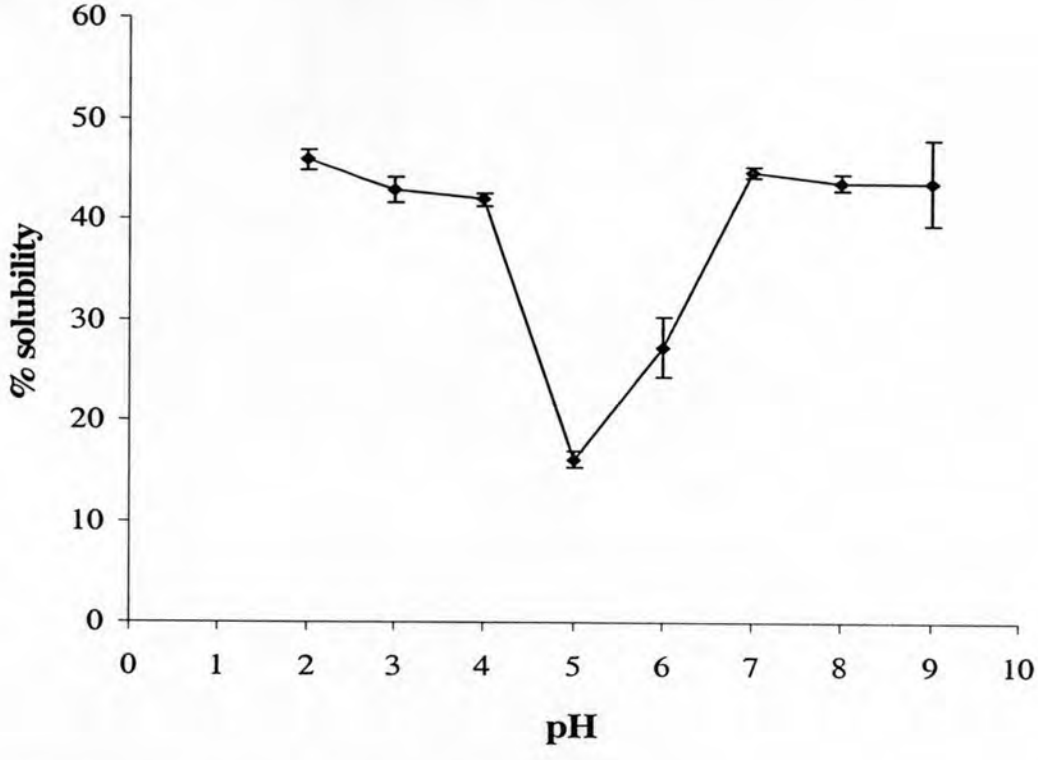


Figure 4.7 Solubility property of TBSP at pH 2-9

It was not clear until recently that, in fact, some myofibrillar proteins are also soluble in extremely dilute salt solutions (Stanley, Stone, and Hultin, 1994; Stefansson and Hultin, 1994). They reported that the solubility is extremely sensitive to salt concentration between ionic strength 0.0003 and 0.001. This is because a minute quantity of ionic compounds is sufficient to increase protein surface charges, thereby increasing protein-water interactions. For cod muscle myofibrillar proteins, minimal solubility is established between an ionic strength of 0.01-0.2. Protein solubility increases rapidly as ionic strength increases from 0.2 to 1.0, and as the ionic strength further increases, the solubility declines presumably due to the "salting out"

effect (Stefansson and Hultin, 1994). The solubility of other fish protein powders have been determined by Petursson, Decker, and McClements (2004), which agrees with the data reported in this study.

### 4.3.2 Water and oil holding capacity

Water holding capacity (WHC) refers to the ability of the protein to adsorb water and retain it against centrifugal force within a protein matrix (Barbut, 1996). Several protein ingredients are used as water-holding additives in muscle foods; however, fish proteins are not widely used as water-adsorption agents. The WHC of TBSP is shown in Table 4.9. Its WHC is 0.73±0.03 g water/g protein. The method for WHC and oil holding capacity (OHC) testing was described in section 3.3.2.

Table 4.9 Water and Oil holding capacity of TBSP

| Property | Unit |
|---|---|
| Water holding capacity | 0.73 ± 0.03 g water/g protein |
| Oil holding capacity | 5.26 ± 0.04 g oil/g protein |

There are no literature values for WHC and OHC of TBSP. The WHC of red salmon head hydrolysate ranged from 1.0-3.3 mL/g protein (Sathivel, Smiley, Prinyawiwatkul, and Bechtel, 2005). The TBSP had a WHC value lower than that reported for red salmon head hydrolysate. Protein composition and conformation have significant effects on WHC and OHC (Barbut, 1996).

OHC is an important functional characteristic of food ingredients used in the meat and confectionery industries (Shahidi, Han, and Synowiecki, 1995). The OHC of TBSP is 5.26±0.04 g oil/g protein

Sathivel, Smiley, Prinyawiwatkul and Bechtel (2005) reported the OHC of red salmon head hydrolysates to range from 3.7 to 7.8 mL oil/g protein. Other reports of OHC range from 3.9 to 11.5 mL oil/g protein for herring protein powder (Sathivel et al., 2004), 3.7 to 7.3 mL oil/g protein for hydrolyzed herring by product proteins

(Sathivel et al., 2003), and 2.86 to 7.07 mL oil/g protein for Atlantic salmon protein hydrolysates (Kristinsson and Rasco, 2000a). The mechanism of OHC is mainly due to physical entrapment of the oil. Protein powders with higher bulk density bind greater amounts of fat (Kinsella, 1982). In comparison on OHC between TBSP and fish hydrolysates, OHC of TBSP is lower than the other. Fat absorption is affected by protein source, processing conditions, composition of additive, particle size and temperature (Zayas, 1997). OHC for a protein-based food is governed by the protein's matrix structure (strand size, pore size). In protein powder foods fat binding is influenced by the size of powder particles and their density. Protein powders with a low-density and a small particle size adsorb and entrap more oil than high-density protein powders do. Protein food characteristics such as WHC, OHC and other functional properties can be altered by chemical and physical modification (Hall and Ahmad, 1992). Studies of chemically modified TBSP are reported in section 4.4 of this thesis.

### 4.3.3 Foaming properties

Foaming characteristics of protein require good surface activity. In the case of foaming, a protein helps to form dispersed air droplets within a continuous water phase. The protein also stabilizes the newly formed air droplets by covering the water: air interface. The study of foaming properties of TBSP is reported in this section.

It is important that foam capacity and foam stability are considered separately. A protein may have excellent foam capacity, but it may not produce stable foam, and vice versa (Wilde and Clark, 1996). Proteins are polymers of amino acids which have hydrophilic, apolar or hydrophobic side chains. The amphipathic character that these side chains confer to proteins is responsible for their adsorption at interfaces. To form a foam efficiently (i.e. to possess high foam capacity), a protein needs to adsorb rapidly during the transient stage of foam formation (Closs, Courthaudon, and Lorient, 1990). The principles underlying the efficient formation of a protein-stabilized foam, rely therefore mainly on the intrinsic properties of the protein itself; that is, the size, surface hydrophobicity and structural flexibility of the protein (Wilde and Clark, 1996).

#### 4.3.3.1 Foaming capacity and stability: effect of pH

The foaming ability of TBSP was determined using a standard method to determine the percent foam overrun (% overrun) and foam stability as described in details in section 3.3.3.1 of the experimental section. In the study reported here, sodium caseinate and beta-lactoglobulin were used for method development and also to provide reference results. The two proteins were chosen also because casein is thought to have little organized folded (tertiary) structure and beta-lactoglobulin has tightly folded structure. The concentration of TBSP used was 2.2% protein (5% sample; 44.20% total protein content). The results for TBSP summarized in Figures 4.8 and 4.9.

To determine the foam capacity, 5% TBSP solution (2.2% protein w/w; pH 7) was blended under standard conditions and volume increased due to air incorporation was measured. The degree of air incorporation was 150% (Figure 4.8). This % overrun value for TBSP was about 60% of the value obtained with beta-lactoglobulin and casein. The protein concentrate in beta-lactoglobulin and casein were nearly 5% i.e. the last two proteins were used at 2 times higher concentration. Increasing concentration of protein in TBSP to 5% protein (double the amount of TBSP sample) is expected to produce 1.1-1.4 increase foaming characteristics (Lawal, 2005). When the effect of pH on protein foaming capacity was studied (section 3.3.3.2), the % overrun value for TBSP was found to be constant over the range of pH 2 – 9. However, the overrun values for casein and beta-lactoglobulin decreased slightly at pH 5 compared to values at pH 2 and pH 7-9 (Figure 4.8). The high % overrun at pH 2 and pH 7-9, over the % overrun at pI value of casein (4.6) and beta-lactoglobulin (5.3) may be due to higher solubility of proteins at extream acid and basic pH. The pH level, by influencing the net charge, affects the rate of adsorption and extent of protein-protein interaction in the interfacial film (Mahajan and Dua, 2002). Therefore, higher solubility of protein could result in more foam capacity. These results are the same as Damodaran (1996a) who revealed that at pH other than pI, foamability of proteins is often good, but foam stability is poor.

Foaming stability is a measure of the foam volume remains after a fixed time. From Figure 4.9, foam stability measured for TBSP was slightly higher than values for beta-lactoglobulin and casein at all studied pH values except at pH 5 where the

casein value was noticeably higher. The solution pH affects the properties of foams by affecting the net charge of the protein and resulting film formation and film properties. The rate and extent of surface pressure development, protein structure, protein-protein interactions, film thickness, and viscoelastic properties are all affected by the net charge on the protein molecule (German and Phillips, 1994). Generally, more rapidly formed stronger films are obtained at pH values close to the isoelectric pH of most proteins. The enhanced foam stability of many proteins is pronounced in the isoelectric pH range (Phillips, Schulman, and Kinsella, 1990; Adebowale and Lawal, 2003). The basis for proteins forming a viscoelastic film is that of protein-protein interactions. Electrostatic processes probably play an important role in reducing interactions. Proteins have a distribution of both positive and negative charges throughout the molecule, and will therefore possess a specific net charge at a given pH. Thus, neighboring molecules will have a similar net charge and will likely repel each other. This explains why protein foams are generally more stable around

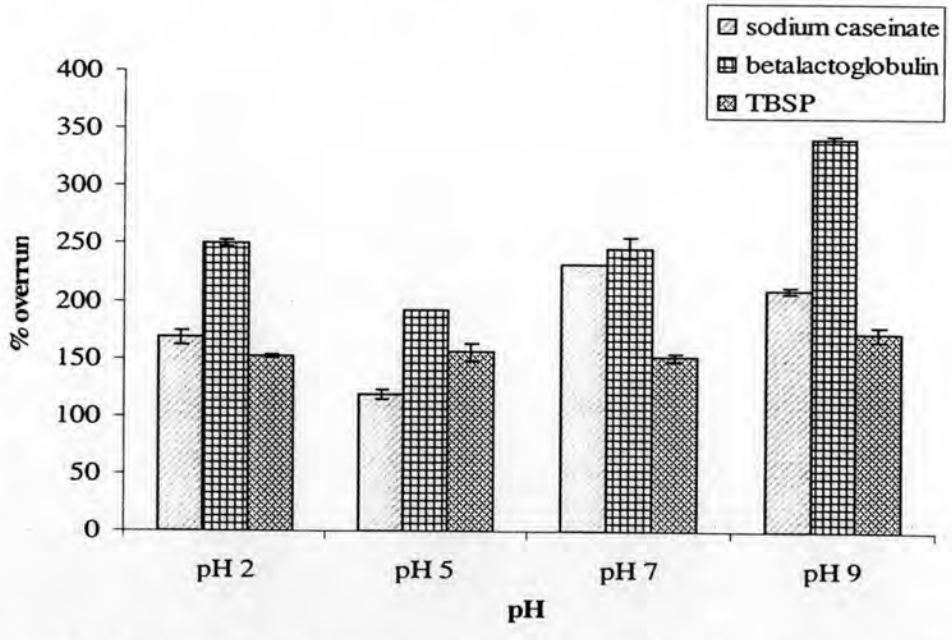


Figure 4.8 Effect of pH on foam capacity of TBSP. Sodium caseinate and beta-lactoglobulin were used for calibration. The concentration of TBSP used was 2.2% (w/w) and 5% w/w casein or beta-lactoglobulin dissolved in water.

their isoelectric point, where their net charge is zero and repulsion is minimized (Wilde and Clark, 1996).

In this study, foam capacity tended to be lower at pH 5 near the pI value for beta-lactoglobulin (pI = pH 5.3) and casein (pI = 4.6). By contrast, foam stability was highest for casein at pH 5 which is close to the pI value (pI = 4.6) for this protein. Closs, Courthaudon, and Lorient (1990) reported optimum foam stability at pH 4 for casein (pI is 4.6). In this study, the foam stability of beta-lactoglobulin between pH 5- pH 9 was nearly the same. These results do not exactly parallel with the results of Phillips et al. (1990) and Phillips (1992) who observed optimum foam stability for whey protein isolate at pH 5. Whey protein concentrate consists of a mixture of proteins including beta-lactoglobulin, alpha-lactalbumin and bovine serum albumin.

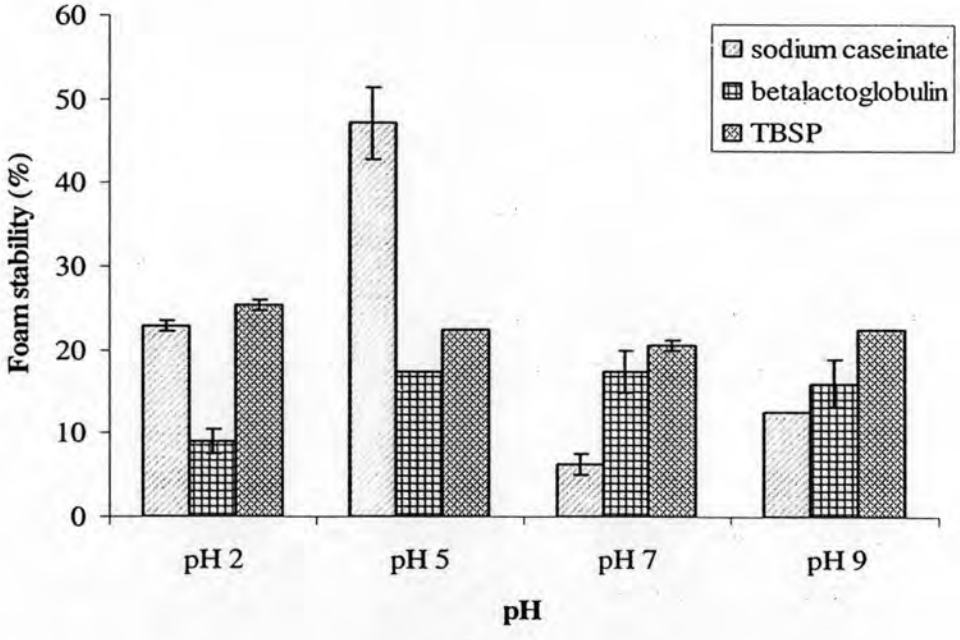


Figure 4.9 Effect of pH on foam stability of TBSP. The concentration of TBSP used was 2.2% (w/w) and 5% w/w casein or beta-lactoglobulin dissolved in water.

The current study uses pure beta-lactoglobulin and casein as models. High foam stability is generally caused by the enhanced interfacial behavior of beta-lactoglobulin at pH close to the isoelectric point. The result in Figure 4.9 shows that

TBSP has higher foam stability than beta-lactoglobulin and casein except at pH 5 when the foam stability for casein is higher than TBSP. However, foam stability of TBSP was not different between pH 2-9 which is similar to the response of beta-lactoglobulin between pH 5-9 (Figure 4.9).

#### 4.3.3.2 Foaming capacity and stability: effect of salts

Salts affect the physicochemical properties and interactions between proteins either in three major ways, (a) by ionic strength effects, (b) by specific binding to the protein charged groups, or (c) at high concentrations by altering water structure and thereby changing hydrophobic effects. Salts can be effective probes for studying the effects of altering hydrophobicity on foaming (German and Phillips, 1994).

The presence of salt may increase the total water content of the protein system at specific water activity values, although it may reduce the preferential binding of water to the protein. These effects are markedly dependent on the nature of the anion and cation components (Sathe and Salunkhe, 1981). Effect of salts on foaming properties was studied (Phillips, Yang, and Kinsella, 1991; German and Phillips, 1994; Oshodi and Ojokan, 1997). The effect of salt is important because salts are constituents of food product formulations. Salt concentrations are approximately 0.2-0.3 M in most food products (Oshodi and Ojokan, 1997). The study of effect of cation and anion salts on foaming properties of TBSP is reported in this section.

##### 4.3.3.2.1 Effect of cations and anions on foam capacity

The effect of cations and anions on foaming capacity was tested as described in section 3.3.3.3.1 and section 3.3.3.3.2, respectively. To test the effect of different cations, each protein material (5% w/w) was dissolved in 0.2 M NaCl, $NH_4Cl$, $MgCl_2$ and $CaCl_2$ solution, adjusted to pH 7 and stirred for 1 h. To test the effect of different anions, each protein material (5% w/w) was dissolved in NaSCN, NaCl and $Na_2SO_4$ solution (0.2 M), adjusted to pH 7 and stirred for 1 h. Foaming c haracteristics were then determined as described in section 3.3.3.1.

The effect of cations on foam capacity was as shown in Figure 4.10. The foam capacity for TBSP was 150% due to air incorporation to produce a foam. Upon addition of 0.2 M NaCl, $NH_4Cl$, $MgCl_2$ and $CaCl_2$, the % overrun for TBSP was not

different from the control (Figure 4.10). In the same way, the overrun values for casein (230-240%) and beta-lactoglobulin (250-270%) did not vary with the type of cation added (Figure 4.10) except with $CaCl_2$ that the foam capacity for casein decreased. In summary, all the cations tested had no effect on foam capacity, except that $Ca^{2+}$ reduced the foam capacity of casein.

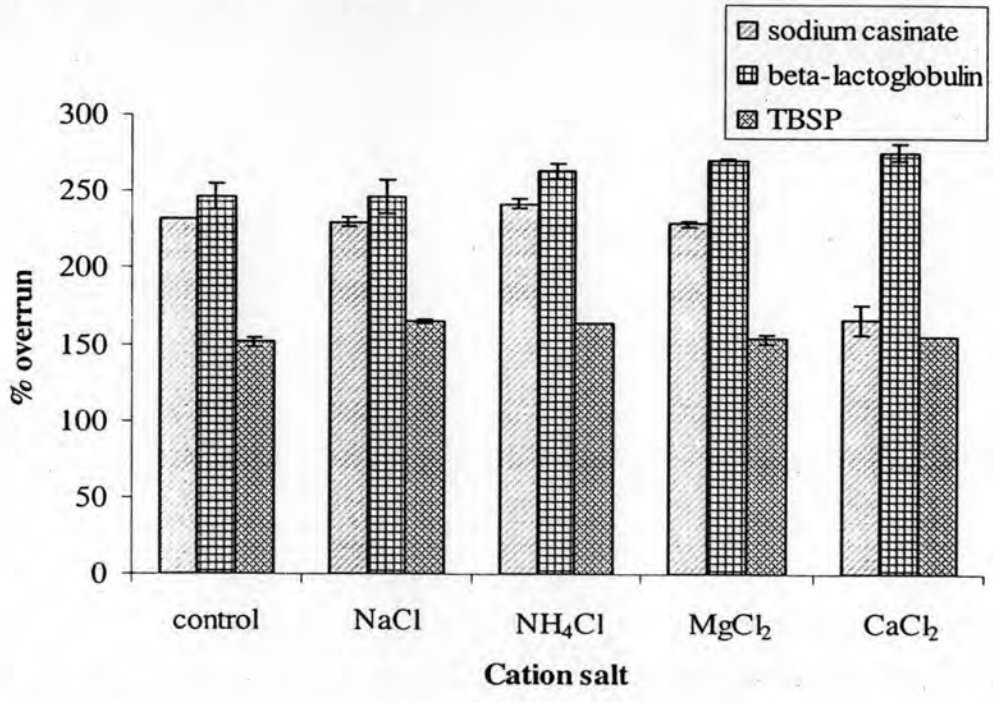


Figure 4.10 Effect of cations on foam capacity of 5% TBSP compare with 5% sodium caseinate and 5% beta-lactoglobulin. Each salt concentration was 0.2 M.

The effect of anions on foam capacity is shown in Figure 4.11. Upon addition of 0.2 M NaSCN, NaCl and $Na_2SO_4$, the % overrun was only slightly higher than control (Figure 4.11). The overrun values for casein and beta-lactoglobulin were higher than TBSP for all anion salts (230-250%). In conclusion, results in Figure 4.11 show that all the anions ($SCN^-$, $Cl^-$, $SO_4^{-2}$) tested had no effect on protein foam capacity compared to the control (water).

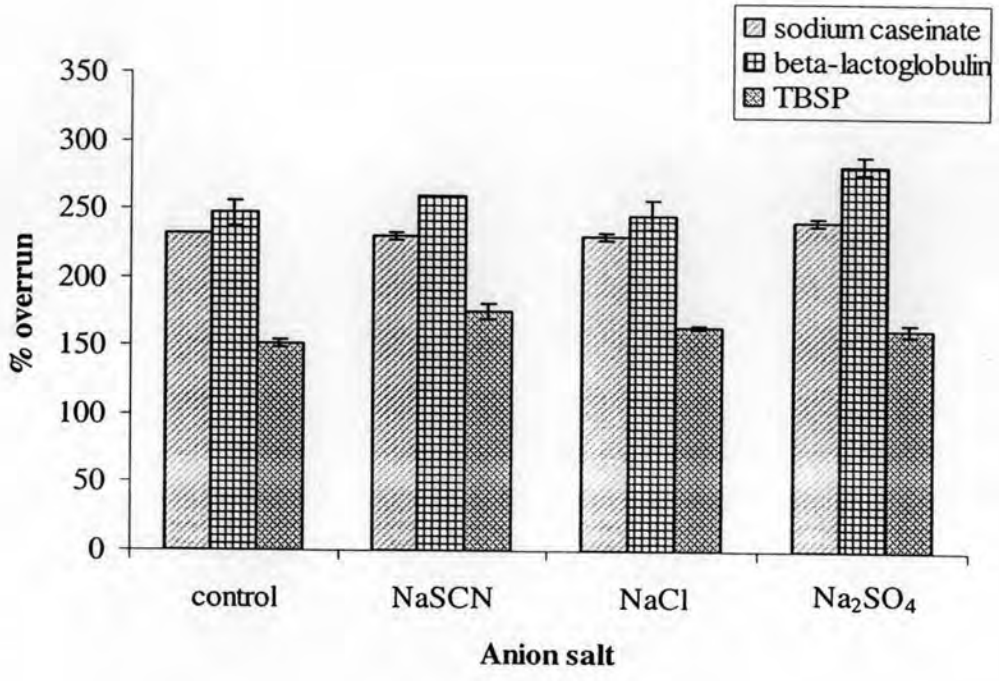


Figure 4.11 Effect of anions on foam capacity of 5% TBSP compare with 5% sodium caseinate and 5% beta-lactoglobulin. Each salt concentration was 0.2 M.

Phillips, Yang, and Kinsella (1991) reported addition different anions (0.1 M $Na_2SO_4$, NaCl or NaSCN) reduced foam capacity of whey protein isolate. This was attributable to an ionic effect, i.e., the ions interacted with counter-charges on the proteins thereby reducing electrostatic interactions. In this study, there was little or no effect of different cations or anions on foam overrun (Figure 4.10 and 4.11).

#### 4.3.3.2.2 Effect of cations and anions on foam stability

For TBSP in 0.2 M $MgCl_2$, foam stability was equal to control. So, $Mg^{2+}$ has no effect on TBSP foam stability (Figure 4.12). In contrast, 0.2 M NaCl, $NH_4Cl$, and $CaCl_2$ caused decreased in foam stability by 8-10% compared to the TBSP sample without salt (control). Therefore, $Ca^{2+}$, $Na^+$ and $NH_4^+$ reduced the TBSP foam stability compared to the control (salt free) solution and $Mg^{2+}$ had no effect. The beta-lactoglobulin and casein foams were stabilized by divalent ions ($Ca^{2+}$, $Mg^{2+}$) and not affected by $Na^+$ and $NH_4^+$ as shown below (Figure 4.12).

In this study, the stability for TBSP foam was decreased by $SCN^-$ and $Cl^-$, and not affected by anion $SO_4^{-2}$ present in solution (Figure 4.13).

Phillips, Yang, and Kinsella (1991) reported that the relative effectiveness of anions at improving foam stability followed the Hofmeister series ($SO_4^{2-} < Cl^- < SCN^-$). In agreement, the relative effectiveness of anion salts at decreasing foam stability of TBSP in this study was $SO_4^{2-} < Cl^- < SCN^-$. It was also found that the relative effectiveness of cation salts at decreasing foam stability of TBSP was $Mg^{2+} < NH_4^+ < Ca^+ < Na^+$. In comparing the results in this thesis, with literature data, it must be noted that TBSP has very high ash content (47.65%, Table 4.1), where most of the salt ions in the "control" sample are $Na^+$ and phosphate ion. These ions may affect on foaming properties.

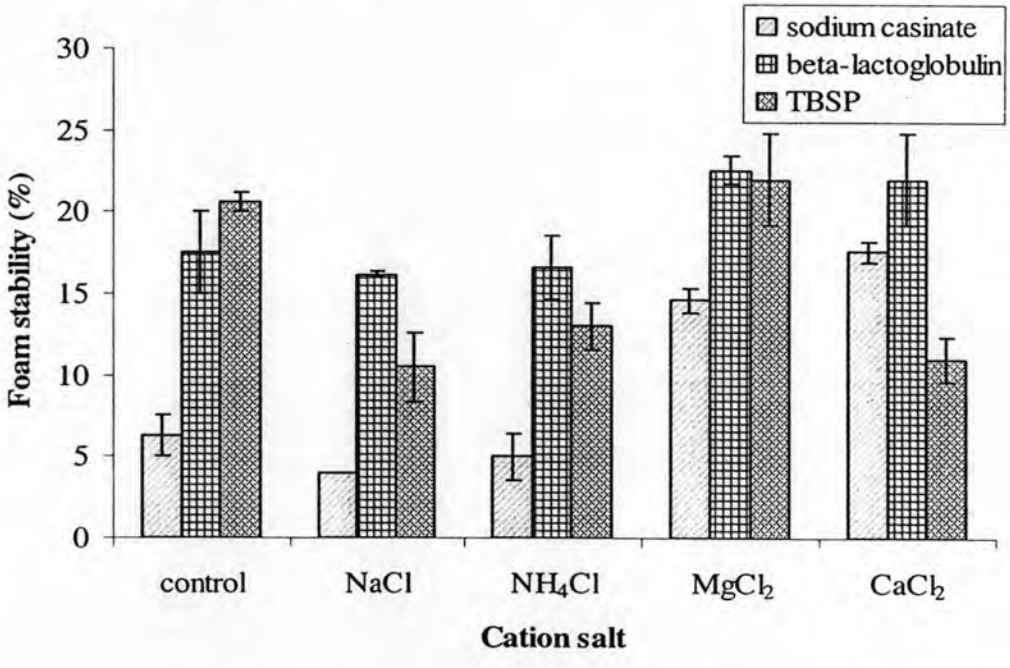


Figure 4.12 Effect of cations on foam stability of 5% TBSP compare with 5% sodium caseinate and 5% beta-lactoglobulin. Each salt concentration was 0.2 M.

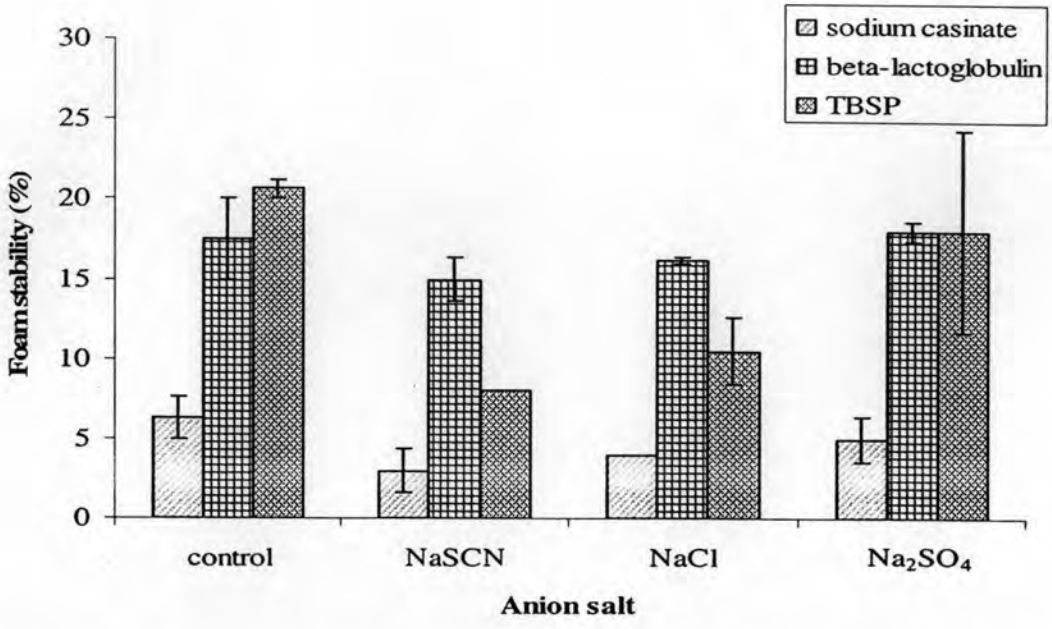


Figure 4.13 Effect of anions on foam stability of 5% TBSP compare with 5% sodium caseinate and 5% beta-lactoglobulin. Each salt concentration was 0.2 M.

### 4.3.4 Emulsifying properties

Emulsifying characteristics of protein require good surface activity. In emulsification, the protein stabilizes the dispersed oil droplets within the water continuous phase. The emulsions droplets are stabilized by proteins adsorbed at the water: oil interface. The study of foaming and emulsifying properties of TBSP is reported in this section.

Beta-lactoglobulin, the major globular protein of whey, is the most extensively characterized and best described of all food proteins (Kinsella and Whitehead, 1989). In this study, beta-lactoglobulin was used as reference protein to compare emulsifying properties with TBSP.

Emulsifying activity index (EAI) and emulsion stability index (ESI) were determined by the turbidimetric technique of Pearce and Kinsella (1978) as described in section 3.3.4.1 and section 3.3.4.2, respectively. The index for emulsifying properties (EAI and ESI) of the emulsions of 0.5% beta-lactoglobulin and TBSP are compared in Table 4.10.

Table 4.10 Emulsifying activity index and emulsion stability index of beta-lactoglobulin and TBSP

| Proteins | EAI ($m^2$/g sample) | ESI (min) |
|---|---|---|
| Beta-lactoglobulin | 77 ± 0.13 | * |
| TBSP | 46 ± 3.13 | 60.45 ± 2.05 |

Note: * Indicates stable emulsion within study time (10 min)

The emulsifying capacity of proteins depends on the shape, charge, and hydrophobicity of the protein molecules, neutrality of dipoles, hydration of polar groups. Emulsion stability depends on the magnitude of these interactions to the ability to adsorb rapidly at the interface, has well-distributed charged groups, and has the ability to form a strong cohesive film (Zayas, 1997).

From the data as shown in Table 4.10, TBSP had an EAI value of 46 ± 3.13 $m^2$/g sample and ESI value of 60.45 ± 2.05 min. Both values are very low when compared with beta-lactoglobulin. From such data, it may be predicted that the average charge and hydrophobicity of many different proteins molecules present in TBSP is difference from beta-lactoglobulin. Therefore, the rate of absorption of TBSP at the O/W interface is slower. Also the distribution of charged grouping in TBSP may lead it to form a weak cohesive film than beta-lactoglobulin.

As evidence for this interpretation, Dickinson et al. (1993) and Dickinson (1997) have compared the emulsification properties of beta-lactoglobulin and casein. According to this work, casein has good EAI because of its open structure which allow rapid adsorption at the O/W interface. Beta-lactoglobulin which is a tightly folded protein, adsorbs slowly at O/W interface but forms cohesive film, once it unfolds. The strong interfacial film formed by beta-lactoglobulin involves covalent bonds via SH/SS exchange reactions. Therefore, the content of SH groups in TBSP may also be another important factor.

### 4.3.5 Gelation

The gelation of proteins is an important functional attribute for food production. Heat-set gelation normally occurs at or above the denaturation temperatures of proteins; therefore data on the denaturation temperature are necessary as a first stage in studies of heat-set gelation. DSC results for TBSP are reported in section 4.1.4 of this thesis. Figure 4.3a (section 4.1.4) shows that TBSP heat denaturation has three endothermic transitions with Tm values at 56°C, 67°C and 76°C. The minimum temperature for TBSP heat denaturation is therefore expected to be $\geq$ 55°C.

The gelation of TBSP (5% w/w) was examined by oscillatory rheology at temperatures of 20-90°C with a scan at a rate of 1°C/min (Ramp rate) using 100 mM NaCl with 5 mM $CaCl_2$ solution at pH 7 as solvent (section 3.3.5.1) and using dynamic temperature ramp testing (parallel plate diameter 50 mm, Gap = 1.000 mm, strain = 1%, and frequency = 0.1 rad/s). G', G" and tan delta were obtained of TBSP are shown in Figure 4.14.

The data in Figure 4.14 also shows that TBSP undergoes gelation transitions in two stages, first at temperatures of 50-80°C and also at 80-90°C. At temperature of 50-55°C, The G' started to increase gradually until 80°C. This data shows TBSP was denatured at $\geq$ 50°C and slow increased until to 80°C. Therefore, TBSP is a heat induced gel. The result agrees with denaturation result obtained using the differential scanning calorimeter (section 4.1.4), which revealed that TBSP has three endothermic transitions with Tm values at 56°C, 67°C and 76°C. These transitions were assigned to denaturation of myofibrillar and sarcoplasmic protein.

The reaction begins at the temperature of 50°C. The second stage of the reaction of TBSP is at temperature 80-90°C. The conclusion temperature of TBSP denaturation was at 90°C. At this temperature, G' was the highest (Figure 4.3a). Proteins aggregate and the cross-linking polymer make stronger gel. Moreover, increasing of G' indicates the development of an elastic gel net work that is increasing gelation (Joseph, Lanier, and Hamann, 1994).

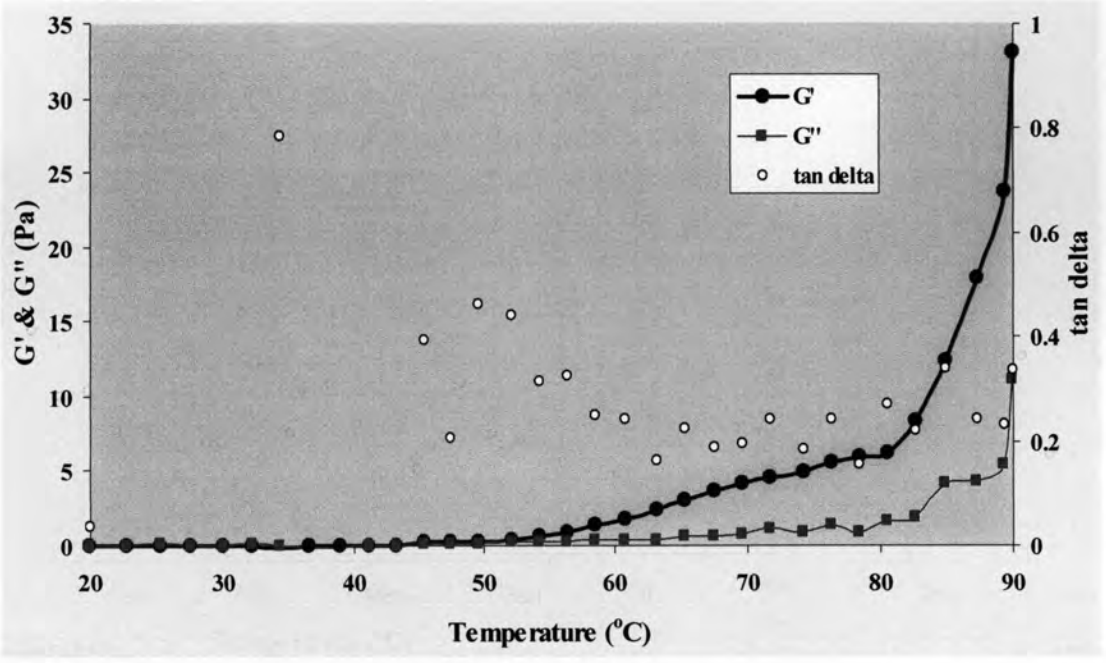


Figure 4.14 Rheology of 5% TBSP examined by oscillatory rheology. The temperature range was 20-90°C with a scan rate of 1°C/min (Ramp rate). Other conditions are: parallel plate diameter 50 mm, Gap = 1.000 mm, strain = 1% and frequency = 0.1 rad/s.

#### 4.3.5.1 Effect of salts on TBSP gelation

The effect of salts on the gelation of TBSP was studied similarly to the approach described above except that different salts were added to the protein solution before heating (section 3.3.5.2). For each salt, gelation extent was measured from the maximum G' value at 90°C. A summary the results are shown Table 4.11. The gel of TBSP increased in strength in the presence of calcium chloride but only moderately in the presence of sodium chloride. The gel strength of beta-lactoglobulin increased in the presence both of NaCl and $CaCl_2$.

However, Grabowska, and Sikorski (1976) reported that the sarcoplasmic (albumin) fraction possessed no gelation ability, as the fraction only coagulated upon heating to 80°C. This was different from TBSP, because TBSP also had some myofibrillar proteins (Figure 4.1 and 4.3a) that can affect the gelation of TBSP. The myofibrillar fraction exhibited excellent gel-forming ability. Even extensively

"washed" myofibrillar fractions formed weak gels, with the loss of gel strength possibly arising from loss of some of the minor protein components present in the initial myofibrillar preparations. This illustrates the difference in gel-forming ability between fibrous (myofibrillar) and globular (sarcoplasmic) muscle proteins (Ziegler and Acton, 1984).

Table 4.11 Effect of salts on G' (Pa)** of beta-lactoglobulin and TBSP

| Additives | beta-lactoglobulin* | TBSP* |
|---|---|---|
| No additive | ( _ ) | 4.2 |
| $CaCl_2$ (5 mM) | ( _ ) | 8.7 |
| NaCl (100 mM) | ( _ ) | 3.3 |
| NaCl (10 mM) & $CaCl_2$ (5 mM) | 11.4 | 20.1(80°C) |
| NaCl (100 mM) | 22.2 | 3.6 |
| NaCl(100 mM) & $CaCl_2$ (5 mM) | 68.7 | 33.1 |
| NaCl (200 mM) | ( _ ) | 10.2 |

Note: * Oscillatory rheology data was measured for 5% (w/w) TBSP and beta-lactoglobulin.

** Storage modulus (G') at 90°C unless otherwise indicated.

( _ ) Indicates no gelation and G' values not above instrument noise.

The storage modulus (G') of 5% TBSP with and without $CaCl_2$ and NaCl significantly increased with temperature at ≥ 50°C (Figure D1 in Appendix D) In the presence of NaCl, G' decreased at 10 and 100 mM NaCl, but increased at 200 mM NaCl. The presence of $CaCl_2$ caused an increase in G'. The increase in G' with temperature indicated the increase in protein-protein interactions. From DSC result, TBSP denatured at temperature ≥ 40°C (Figure 4.3a). This suggested that protein denaturation enhanced protein-protein interaction resulting in heat induced protein gelation. The higher the value of G' indicates the higher the gelation (Joseph, Lanier, and Hamann, 1994). Salt-bridges between denatured protein molecules were formed in the presence of $CaCl_2$, which gave the stronger gel strength.

Beta-lactoglobulin was denatured at temperature ≥ 40°C (Figure 4.3b) even though G' did not change (increase) with temperature for 5% beta-lactoglobulin with and without 5 mM $CaCl_2$ or 10 mM NaCl. However, in the presence of 100 mM NaCl and 5 mM $CaCl_2$ with 10 or 100 mM NaCl, G' increased with temperature at ≥ 80, 75 and 73 °C, respectively (Figure D2 in Appendix D). G' increased in the presence of 5 mM $CaCl_2$ and also increased with NaCl concentration. These results imply that no heat induced gelation occurred even if beta-lactoglobulin was denatured for 5% beta-lactoglobulin with and without 5 mM $CaCl_2$ or 10 mM NaCl. Heat induced gelation of 5% beta-lactoglobulin happened in the presence of 100 mM NaCl and 5 mM $CaCl_2$ with 10 or 100 mM NaCl. The gel strength (G') of 5% beta-lactoglobulin in the presence of 100 mM NaCl and 5 mM $CaCl_2$ with 10 mM NaCl was lower than that of all 5% TBSP samples. But 5% beta-lactoglobulin in the presence of 5 mM $CaCl_2$ with 100 mM NaCl gave the highest gelation.

The presence of neutral salts affects the rheological properties of heat-induced actomyosin gels and the relative effects of the cations on the heat-induced gelling properties of actomyosin are in the order: $K^+ \approx Na^+ > NH_4^+ > Mg^{2+} > Ca^{2+}$ (Zayas, 1997), but the TBSP opposite of actomyosin. In this study, TBSP with 5 mM $Ca^{2+}$ has G' higher than 10 mM $Na^+$. However, the combination of NaCl and $CaCl_2$ gave the G' higher than one salt. For TBSP, the gelation of TBSP with 200 mM NaCl was lower than the 100 mM or 10 mM NaCl that combined with 5 mM $CaCl_2$.

#### 4.3.5.2 Effect of pH on TBSP gelation

The gelation of TBSP (5% w/w) was examined by oscillatory rheology at the temperatures of 20-90°C, rate of 1°C/min (Ramp rate) using distilled water as solvent. The pH was adjusted to 5, 7 and 8.5 with 0.1 M HCl and NaOH as described in section 3.3.5.3. Values for the storage modulus (G') showed that TBSP started to denature at temperatures ≥ 50°C (Figure D3 in Appendix D). The order of decreasing G' for TBSP was pH 7 > pH 8.5 > pH 5, but the G' of beta-lactoglobulin decreased in the order, pH 5 > pH 8.5 ≈ pH 7, as shown in Table 4.12.

Table 4.12 Effect of pH on G' (Pa)**of beta-lactoglobulin and TBSP

| pH | beta-lactoglobulin* | TBSP* |
|---|---|---|
| pH 5 | 77.7 | 0.64 |
| pH 7 | ( _ ) | 6.55 |
| pH 8.5 | 0.05 | 2.31 |

Note: * Oscillatory rheology data was measured for 5% (w/w) TBSP and beta-lactoglobulin.

** Storage modulus (G') at 90°C

( _ ) Indicates no gelation and G' values not above instrument noise.

Several environmental factors, such as pH, salts, and other additives, also impact on the ability of the progel to set into a gel. The type of gel formation is affected by the pH. At highly acidic and alkaline pH, proteins assume a molecule charge. The strong electrostatic repulsion inhibits gel network formation. At the isoelectric pH, proteins have zero net charge and tend to aggregate via hydrophobic interactions. This leads to formation of a coagulum-type gel with a coarser network and loser gel strength. Only at the optimum pH, which permits an optimum balance of protein-protein and protein-solvent interactions, can a uniform gel matrix with high gel strength and water-binding capacity be formed. Generally, the optimum pH is about 7-8 for most proteins (Damodaran, 1996b). The gelation of TBSP at pH 7 is the same as many studies, such as, Hultin and Kelleher (2000) indicated that protein gels prepared from alkali solubilization of cod muscle exhibited good gel quality. However, there are exceptions. For example whey protein gels formed at pH 6-7 were stronger than those formed at pH 4.0 (De Wit, Hontelez-Backz, and Adamse, 1988). In contrast, the gels of ovalbumin formed at acidic pH (< 4.0) were stronger than those that were formed at pH 6.0 (Damodaran, 1996b), also beta-lactoglobulin is the same as this study.

Recently, Hultin and Kellerher (1999) patented the acid solubilization process based on solubilization of both myofibrillar and sarcoplasmic proteins at acidic conditions (pH 2.5) and subsequent precipitation of all soluble proteins at their isoelectric point. At a pH above the pI, proteins become negatively charged, resulting

in increased solubilization and solubility. Lin and Park (1998) showed that solubility of salmon myosin was highest at pH 2 and 8 to 10. Therefore, a process in which a higher yield of surimi can be produced might not be restricted to only acid solubilization but also alkaline treatment. Therefore, both acid and alkali solubilization processes could be alternative (Yongsawatdigul and Park, 2004). However, Choi and Park (2002) found that the gel produced by the acid solubilization process of Pacific whiting mince yielded a lower breaking force, which resulted from activity of cathepsin L retained in the recovered proteins.

#### 4.3.5.3 Effect of enzyme inhibitors on TBSP gelation

Many researchers reported that fish sarcoplasmic protein inhibit the gel formation of myofibrillar proteins due to the presence of proteases. Therefore, proteases present in TBSP might affect gelation. Investigations were performed to examine the effect of protease inhibitors on TBSP gelation.

The gelation of TBSP (5% w/w) was examined by oscillatory rheology temperatures of 20-90°C with a rate of 1°C/min (Ramp rate) using distilled water as solvent which pH was adjusted to 7, after inhibitors were added as described in section 3.3.5.4. Values for the storage modulus (G') showed that TBSP started to denature at temperatures $\geq$ 50°C (Figure D4 in Appendix D).

The gel strength of TBSP with inhibitors was the same as data from the section 4.3.5 without the effect of inhibitor. The results showed that protease inhibitors (iodoacetate, pepstatin A, 1,10-phenanthroline) did not affect TBSP gelation as shown in Table 4.13.

The inhibition studies reported in Table 4.5 showed that TBSP contained protease activity sensitive to PMSF, iodoacetate and 1,10-phenanthroline. However, perhaps the levels of enzyme activity are too low to affect gelation. In the research reported in this section, there was no conclusive evidence for the effect of protease inhibitors on TBSP gelation.

Table 4.13 Effect of inhibitors on G' (Pa) of TBSP

| Inhibitors / Solvent | G' (Pa)* |
|---|---|
| Control-water | 2.44 |
| Iodoacetic acid (1 mM, water) | 1.71 |
| Control-methanol | 4.13 |
| PMSF (1 mM, methanol) | 5.26 |
| 1,10-Phenanthroline (1 mM, methanol) | 3.16 |
| Pepstatin A (1 $\mu$M, methanol) | 4.15 |

Note: - Oscillatory rheology data was measured for 5% (w/w) TBSP

* Storage modulus (G') at 90°C

#### 4.3.5.4 Effect of transglutaminase addition on TBSP gelation

The effect of tranglutaminase on the gelation of TBSP (5% w/w) was examined by oscillatory rheology with at temperatures of 20-90°C at a rate of 1°C/min (Ramp rate) using distilled water as solvent which pH was adjusted to 7. Afterwards 0.2% (w/w) transglutaminase (Ajinomoto, Activa®-TGase) was added as described in section 3.3.5.5. Values for the storage modulus (G') showed TBSP at temperatures $\geq$ 50°C (Figure D5 in Appendix D).

In section 4.2.7 Determination of enzyme transglutaminase in TBSP, shown that TGase activity was absent in TBSP, so there was no TGase activity in the protein samples. From Table 4.14 in the treatment of TBSP plus TGase (0.2%) with NaCl (100 mM) and $CaCl_2$ (5 mM), has G' lower than others. This TGase is Ajinomoto, Activa®-TGase. This commercial TGase from microbial culture (MTGase) offers a means of upgrading the gelling quality of surimi. This MTGase is not calcium sensitive; therefore, neither chelating agents nor calcium salts have any marked effect on its activity. Although, some reports have indicated that benefits from MTGase addition are mainly noticeable in products from lower quality surimi, there is also a substantial increase in the strength of gels made with high-quality pollock surimi. MTGase is widely used in Japan to strengthen surimi gels and many other protein foods (Lanier, 2000). However, the data of TGase with effect of NaCl and $CaCl_2$ on

gelation of TBSP is not clear because their G' values were very close while G' values beta-lactoglobulin (pure protein) were much higher. TBSP is a mixture of proteins, so many components of TBSP can affect gelation.

Table 4.14 Effect of transglutaminase on G' (Pa)** of TBSP

| Additive | beta-lactoglobulin* | TBSP* |
|---|---|---|
| No additive | ( _ ) | 4.32 |
| TGase (0.2%) | ( _ ) | 5.94 |
| NaCl (100 mM) & $CaCl_2$ (5 mM) | 68.73 | 10.58 |
| TGase (0.2%) with NaCl (100 mM) & $CaCl_2$ (5 mM) | 63.59 | 2.96 |

Note: * Oscillatory rheology data was measured for 5% (w/w) TBSP and beta-lactoglobulin

** Storage modulus (G') at 90°C

( _ ) Indicates no gelation and G' values not above instrument noise.

Nowsad, Katoh, Kanoh, and Niwa (1995) described the effect of sarcoplasmic proteins from various fish fleshes on the setting of TGase-free paste. It has long been believed that sarcoplasmic proteins in fish flesh reduced the elasticity of resultant kamaboko. It was observed, however, that the suwari gel from Alaska pollack surimi was weakened by washing it repeatedly but was strengthened again by returning wash-water to it. This dichotomy was resolved by finding the existence of TGase in sarcoplasmic proteins, for the effect of various sarcoplasmic proteins on the breaking force of the suwari gel. The force was increased at the initial stage of setting, and somewhat decreased thereafter. This increment was enlarged by the addition of sarcoplasmic proteins in the order of Alaska pollack, Pacific mackerel, sardine, horse mackerel, carp and Spanish mackerel, nearly in the same order as that of suwari gel formability of fish paste depending on species. The extent of enzymic action of TGase was found to be influenced also by the conformational factor of substrate proteins, depending on fish species.

## 4.4 Modification of TBSP

The functional properties of a protein determine its behavior during food processing, storage and preparation, and ultimately govern its suitability as a food ingredient. The critical functional attributes required by a protein include solubility, heat coagulation, water and fat absorption, gelation, emulsifying properties, whippability and good sensory properties (Kinsella, 1979). Many native proteins possess limited functionality. Therefore, chemical modification, such as acylation (succinylation, acetylation, etc.) and enzymatic modification (trypsin, chymotrypsin, papain, etc.) are often performed to expand the range of functional properties available. There are no published data on functional properties of freeze-dried fish sarcoplasmic proteins and or the modified protein. The aims of the study presented in this chapter to understand the functional properties of modified freeze-dried fish sarcoplasmic proteins.

### 4.4.1 Chemical and enzymatic modification of TBSP

#### 4.4.1.1 Chemical modification of TBSP by acylation

The effect of chemical modification (succinylation and acetylation, section 3.4.1.1) on free amino content of TBSP is shown in Table 4.15. From this data is possible to estimate the percentage of free amino group modified. The free amino content (lysine $\varepsilon$-$NH_2$) was determined by methods described in section 3.4.1.1.3. In these experiments, original TBSP had amino group content 3.18 ($\pm$ 0.23) x $10^{-4}$ moles/g protein. During modification of the TBSP with succinic acid and acetic acid anhydride, free amino groups in a protein molecule, mainly the lysine $\varepsilon$-$NH_2$ are masked by acylation. Acylation is useful for structure and composition analysis or for blocking $\varepsilon$-$NH_2$ to protect them form other reactions, so the amino group content of succinylated TBSP and acetylated TBSP was expected to be zero when excess succinic anhydride and acetic acid anhydride was used. The % degree of modification (% MD) of succinylated TBSP and acetylated TBSP were 98.83 and 100%, respectively (Table 4.15). Incubation of TBSP at 55°C, under conditions used to assay endogenous protease (section 4.4.1.2), led to a net loss of 55.72% free amino

groups. The amino group content in TBSP that was modified by trypsin was increased 86.54% because peptides were hydrolyze and gave free the amino group.

The amount of free amino group in TBSP samples is calculated from the equation below (Adler-Nissen, 1979):

$$\text{Amino group (moles/g protein)} = \frac{A_{420} \times 0.001025\ (L)}{20300(L\ mol^{-1}\ cm^{-1}) \times 1\ cm \times W(g)}$$

where W (g) is the amount of protein present in 0.025 mL of the TBSP (1% w/w) solution added to each cuvette for the TNBS assay. It can be shown that;

W (g) = 0.025 mL x 0.01 g/mL x Kjeldahl protein (%)

Table 4.15 Degree of chemical modification

| Type of proteins | Amino group content (moles/g protein) | % MD amino group |
|---|---|---|
| TBSP | $3.18 \times 10^{-4} \pm 0.23 \times 10^{-4}$ | - |
| Succinylated TBSP | $3.74 \times 10^{-6} \pm 0.72 \times 10^{-6}$ | 98.83 |
| Acetylated TBSP | $0.00 \pm 0.00$ | 100.00 |
| Trypsin-1h TBSP | $5.94 \times 10^{-4} \pm 0.08 \times 10^{-4}$ | a* |
| Endogenous protease TBSP | $1.41 \times 10^{-4} \pm 1.95 \times 10^{-4}$ | 55.72 |

Note: - Protein contents in modified TBSP were analyzed by Kjeldahl method.

- Test of free amino content with TNBS method, read at 30 min, A420, 1% (w/w) TBSP in deionized water pH 7.
- % MD of amino group = $100 \times \frac{\text{amino group (initial) - amino group (final)}}{\text{amino group (initial)}}$
- a = 86.54%
- * Final amino group content is more than initial amino group content due to hydrolysis by trypsin.

After chemical modification, the free lysine content decreased when compared to unmodified protein as expected. The succinylating agent reacts with lysine. Each free amino group was succinylated transforms. The net charge of the protein changed from +1 to –1. Lysine was modified and the ($-CH_2CH_2CH_2COOH$) succinyl group

was added. At pH 7, $-CH_2CH_2CH_2COOH$ group ionized and lost $H^+$ to become $-CH_2CH_2CH_2COO^-$. In summary, every lysine which is succinylated, the charge on the modified lysine residue changes from a value of +1 (for amino group $NH_2$) to a charge value of -1 for $COO^-$ group. Overall effect is that protein becomes more negatively charged. The pI for modified and unmodified protein are therefore different. When a protein is acetylated, the lysine-ε-$NH_2$ group is replaced by lysine-ε-$NH.CO.CH_3$ group which has a charge of zero at pH 7.0.

Protein acylation concerns the reaction of a nucleophile such as the amino or hydroxyl group of the protein with the carbonyl group of an acylating agent, and results in the addition of a new functional group to the protein. The most common acylating agents are acetic anhydride and succinic anhydride. Reaction of protein with acetic anhydride results in elimination of the positive charges of lysyl residues and a corresponding increase in electronegativity. Acylation with succinic or other dicarboxylic anhydrides result in replacement of positive charge with negative charge at lysyl residues as described above. This causes an enormous increase in the electronegativity of proteins, and causes unfolding of the protein if extensive reaction is allowed to occur.

Acylated proteins are generally more soluble than native proteins at neutral pH. In fact, the solubility of caseins and other less soluble proteins can be increased by acylation with succinic anhydride. However, succinylation, depending on the extent of modification, usually impairs other functional properties. For example, succinylated proteins exhibit poor heat-gelling properties because of the strong electrostatic repulsive forces. The high affinity of succinylated proteins for water also lessens their adsorption at oil-water and air-water interfaces, thus impairing their foaming and emulsifying properties. Also, because several carboxyl groups are introduced, succinylated proteins are more sensitive to calcium induced precipitation than is the parent protein.

Much attention has been given to the effects of acylation on protein digestibility and nutritional value. Siu and Thompson (1982) reported that both in-vivo and in-vitro digestibility of lysine, cysteine, methionine, and threonine were greatly reduced at high levels of succinylation.

### 4.4.1.2 Enzymatic modification of TBSP

Table 4.16 shows the effect of incubation time on the degree of hydrolysis (% DH) of TBSP by 0.2% trypsin solution. DH was determined by the slightly modified methods of Adler-Nissen (1979) as described in section 3.4.1.2.2. DH of the proteins was calculated from a numerical comparison of free amino group between the modified (section 3.4.1.2.1) and unmodified original protein. From Table 4.16, a steady increase in % DH was observed with increased hydrolysis time. TBSP with 0.2% trypsin (w/w) had the highest % DH at 48 h of hydrolysis time.

Proteases are the most widely used enzymes for modification of food protein. They hydrolyze selected peptide bonds to promote reduction of molecular weight, possible conformation changes, and enhanced hydrophilicity due to newly exposed amino and carboxyl groups. Effective hydrophobicity of certain globular proteins conceivably could be increased as well through exposure of apolar amino acid residues upon limited hydrolysis and subsequent unfolding of the polypeptide chain (Kester and Richardson, 1984).

General effects of proteolysis on size, structure, and polarity could result in dramatic changes of protein functional behavior. Specific properties of the hydrolysate are dependent upon DH, which is influenced and chemical character of the protein substrate, and reaction conditions.

The enzymatic modification proteins by trypsin have been studied on many occasions. Kristinsson and Rasco (2000a, 2000b) studied biochemical, functional properties and kinetics of the hydrolysis of Atlantic salmon muscle proteins by alkaline proteases. Protein hydolysates (5, 10 and 15% DH) were made from minced salmon muscle treated with alkaline proteases (Alcalase 2.4L, Flavourzyme® 1000L, Corolase® PN-L, and Corolase® 7089) or endogenous digestive proteases. Reaction conditions were controlled at pH 7.5, 40°C, and 7.5% protein content, and enzymes were added on the basis of standardized activity units (Azocoll units). When the enzymes were added at the same activity levels to compare hydrolytic efficiencies, Corolase® 7089 and the endogenous extract (pyloric caeca extract, PCE) were the most efficient and Alcalase was the least efficient. PCE was very efficient on the salmon muscle substrate. The extract contained a complex mixture of several enzymes, mainly chymotrypsin, trypsin and elastase with chymotrypsin as the major

active component, and the protein breakdown is largely attributed to it. In Table 4.16, data for TBSP modified by 0.2% trypsin shows the shape of the hydrolysis curve is typical of published for fish protein hydrolysis (Kristinsson and Rasco, 2000a, 2000b), crayfish protein hydrolysis (Baek and Cadwallader, 1995), and whey protein hydrolysis (Mutilangi, Panyam, and Kilara, 1995, 1996).

Table 4.16 Effect of incubation time on degree of hydrolysis (% DH) of TBSP modified by 0.2% trypsin

| Time (h) | free $-NH_2$ content (mmole/g) | Changed free $-NH_2$ content (mmole/g) | % DH* |
|---|---|---|---|
| 0 | 0.96 | 0.000 | 0.0 |
| 1 | 1.12 | 0.160 | 2.0 |
| 2 | 1.19 | 0.234 | 2.9 |
| 4 | 1.15 | 0.192 | 2.4 |
| 6 | 1.22 | 0.264 | 3.3 |
| 12 | 1.30 | 0.344 | 4.3 |
| 24 | 1.33 | 0.377 | 4.7 |
| 36 | 1.40 | 0.444 | 5.5 |
| 48 | 1.59 | 0.633 | 7.9 |
| 60 | 1.54 | 0.581 | 7.3 |
| 72 | 1.43 | 0.475 | 5.9 |

* calculated as shown by Adler-Nissen (1979)

There seems to be a difference in the hydrolysis of myofibrillar and sarcoplasmic proteins by serine protease, as suggested by Hara et al. (1985). The authors found that myofibrillar proteins were degraded to a greater extent than the sarcoplasmic proteins, which were highly resistance to degradation. When sarcoplasmic and myofibrillar proteins were mixed together and treated with trypsin, the rates of degradation decreased. This protective action of the sarcoplasmic fraction may explain the relatively slow degradation of minced muscle by tryptic enzymes at

alkaline pH. The results of this work are of particular interest, when it is considered that kamaboko production relies on the use of water-washed proteins with the myofibrillar proteins as the principal protein involved in the gel setting process. The hydrolysis involves the action of selected proteolytic enzymes to split specific peptide bonds in proteins, resulting in a decrease in the size of protein, with consequent changes in solubility and functional properties (Beirao et al., 2001).

### 4.4.2 Properties of modified TBSP

#### 4.4.2.1 Surface hydrophobicity of modified TBSP

The surface hydrophobicity of BSA, TBSP and modified TBSP are shown in Table 4.17. The method to determine surface hydrophobicity was described in section 3.1.4. Briefly in Table 4.17 the surface hydrophobicity was measured as the initial slopes ($S_o$) of the fluorescence intensity versus protein concentration plot, calculated by linear regression analysis, (Nakai, Li-Chan, and Arteaga, 1996). Alizadeh-Pasdar and Li-Chan (2000) also calculated surface hydrophobicity by adjusting $S_o$ values to values for BSA (last column in Table 4.17).

The data shows that most of chemically modified TBSP had increased surface hydrophobicity compared to control. In this study, the order of surface hydrophobicity values was acetylated TBSP < native TBSP < succinylated TBSP. Yamauchi et al. (1979) and Seifert and Schwenke (1995) reported succinylation and acetylation may result in aggregation of the modified proteins. Acetylation of ε-amino groups of lysine residues renders them electrically natural. At the same time, acetylation also decreases the number of positive charges by substituting hydrophobic acetyl groups for the positively charged ε-amino groups. By comparison, succinylation introduces anionic succinate residues covalently linked to the ε-amino groups of lysine residues. The resulting change from positive to negative charge leads to greater changes in electrostatic relationships and frequently brings about the dissociation of aggregated or subunit proteins and/or rather major conformation changes. Therefore, succinylation is sometimes preferable to acetylation for the modification of amino groups because, in some cases, products of the former are likely to be more soluble (El-Adawy, 2000). Succinylation and acetylation do not

have the same effect on modified protein because different groups are introduced. Succinylation introduces the group -C(=O)$CH_2CH_2COO$, but acetylation give only –$COCH_3$ to the modified protein. Therefore, succinylation may cause more disruption to protein structure than the introduction of smaller group by acetylation leading to difference in surface hydrophobicity of the modified protein.

Table 4.17 Hydrophobicity of BSA, TBSP and modified TBSP

| Modified TBSP | Surface hydrophobicity | Surface hydrophobicity ($S_{TBSP}/S_{BSA}$) x 1000** |
|---|---|---|
| BSA | $42.40^{g} \pm 0.14$ | 1,000 |
| TBSP | $69.36^{d} \pm 0.31$ | 1,636 |
| Succinylated TBSP | $75.27^{c} \pm 0.22$ | 1,775 |
| Acetylated TBSP | $67.27^{e} \pm 0.02$ | 1,587 |
| Trypsin-1h TBSP* | $105.46^{a} \pm 0.25$ | 2,487 |
| Trypsin-24h TBSP* | $76.44^{b} \pm 0.23$ | 1,803 |
| Endogenous protease TBSP* | $53.40^{f} \pm 0.26$ | 1,259 |

Note: (1) a-g; means with different letters represent significantly different value at $p<0.05$ using SAS program

(2) * samples heated to 55°C for 1 h or 24 h

(3) ** calculated as shown by Alizadeh-Pasdar and Li-Chan (2000)

For TBSP incubated at 55°C with trypsin for 1 h, there was higher surface hydrophobicity compared to TBSP incubated at 55°C for 1 h with no enzyme added (Table 4.17). This suggests that hydrophobic binding sites for the ANS were exposed by trypsin digestion during incubation (Wu, Hettiarachchy, and Qi, 1998). In contrast, with incubation at temperature 55°C for 1 h with no trypsin [cf. endogenous protease modification], there was a decrease surface hydrophobicity compared to control perhaps due to heat aggregation. Heat aggregation could also decrease solubility after 24 h compared to 1 h trypsin sample (Figure 4.15).

#### 4.4.2.2 Solubility of modified TBSP

The pH-solubility profiles for TBSP and modified TBSP were determined using protein analysis methods as described in section 3.3.1. The results in Table 4.18 show that succinylated TBSP has the highest solubility at pH 5-9 but the lowest solubility at pH 3-4. Acetylation did not increase protein solubility compared to unmodified TBSP.

It can be seen that trypsin modification-1h increased protein solubility compared to unmodified TBSP (Figure 4.15 and Table C2 in Appendix C). However, trypsin treatment for 24 h (55°C) decreased protein solubility compared to untreated TBSP, probably because of the protein aggregation due to the prolong treatment at 55°C.

In this study it is reported that the solubility of TBSP was high in both acid and alkaline pH regions. It had lowest solubility in pH 5, while for all of modified TBSP the solubility minimum was at pH 3-4. Succinylated proteins exhibited a shift in their isoelectric pH, thus resulting in an enhanced solubility at neutral to alkaline pH (Bora, 2002). The shift reflects an increase in the negative charge, as a result of replacing the α-amino groups of lysine with negatively charged carboxyl groups. In comparison with native TBSP, succinylated TBSP showed better solubility in the pH range of 5-9 but the solubility was greatly reduced below pH 4. The greater the extent of succinylation, the higher was the effect of pH on their solubility. Similar observation was also made by Bora (2002) for the succinylated lentil globulins. From the minimum solubility at pH 2-4, it may be become acetylation decreased the isoelectric point of the TBSP from about pH 5 to pH 2-4. The solubility of the acetylated protein was higher between pH 5-9 than enzyme modification, but lower than native and succinylated TBSP. Trypsin modification decreased the isoelectric point of the TBSP and the solubility below pH 5 to pH 3-4. The solubility of the trypsin modified TBSP did not differ between pH 2, 5, 6, 7, 8 and 9.

Table 4.18 Solubility of TBSP and modified TBSP at pH 2-9

| pH | TBSP | Succinylated-TBSP | Acetylated-TBSP |
|---|---|---|---|
| 2 | 45.86 ± 0.92 | 4.41 ± 1.43 | 0.01 ± 0.03 |
| 3 | 42.88 ± 1.30 | 0.00 ± 0.14 | 0.00 ± 0.03 |
| 4 | 41.92 ± 0.60 | 0.00 ± 0.19 | 0.00 ± 0.13 |
| 5 | 16.09 ± 0.85 | 52.35 ± 1.32 | 6.92 ± 0.81 |
| 6 | 27.28 ± 2.94 | 62.27 ± 0.69 | 17.09 ± 0.35 |
| 7 | 44.68 ± 0.60 | 60.29 ± 0.22 | 19.25 ± 0.37 |
| 8 | 43.65 ± 0.83 | 57.48 ± 2.95 | 24.03 ± 0.93 |
| 9 | 43.67 ± 4.24 | 61.13 ± 1.69 | 40.07 ± 1.14 |

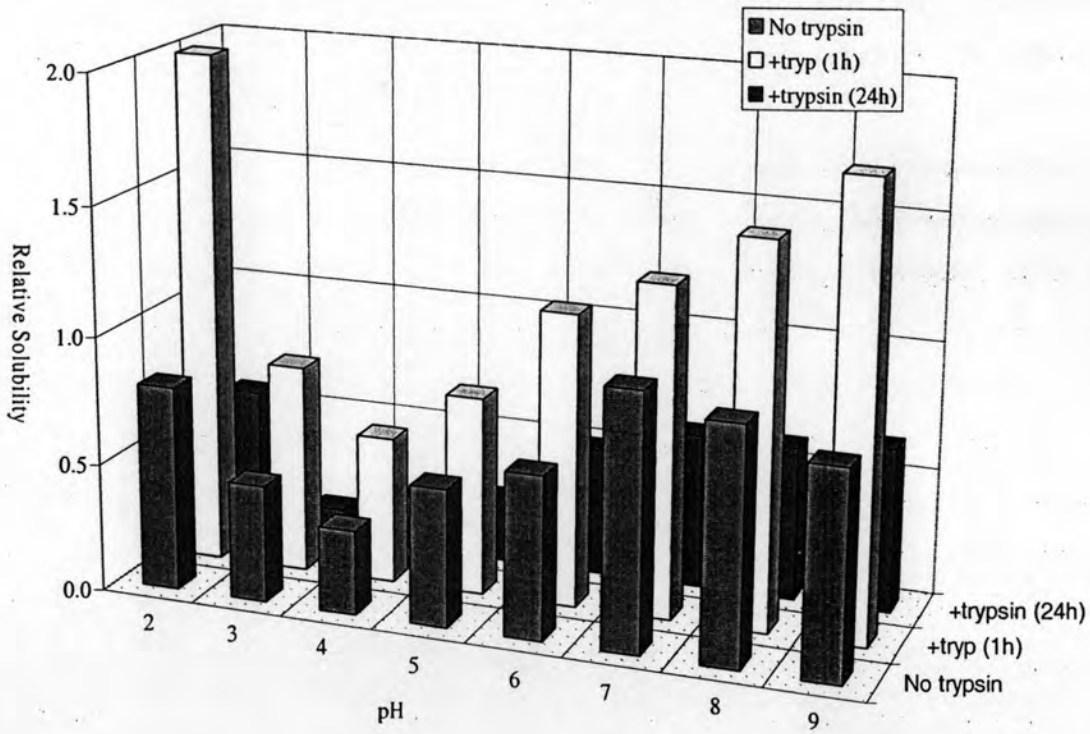


Figure 4.15 pH profiles of solubility of TBSP and trypsin treated TBSP. Reference sample relative solubility value = 1.0 (pH 7). All samples were incubated at 55°C

From changes in the pH-solubility profile, it seems that succinylation shifted the pH that has lowest solubility of TBSP approximately 1.0 pH unit from pH 5 to 4. By appropriate chemical derivatization the pI may be moved up or down so that it is out of the pH range where solubility is required (Franzen and Kinsella, 1976a, 1976b). Nevertheless, the slight shift in the pH that has lowest solubility, upon succinylation or acetylation may have implications in the successful development of food products such as high protein acidic beverages. However, acetylation and succinylation may reduce protein digestibility (Siu and Thompson, 1982).

Effect of trypsin hydrolysis on TBSP (Figure 4.15) agrees with similar studies reported in the literature. Many studies on protein hydrolysates reported an increasing solubility with increasing DH (Chobert, Bertrand-Harb, and Nicolas, 1988; Chan and Ma, 1999). This connection between solubility and DH is believed to be primarily due to the decrease in peptide size, because smaller and more soluble peptides are produced at higher DH (Kristinsson and Rasco, 2000). In this study, protein solubility increased when TBSP was hydrolyzed with trypsin for 1 h (DH = 2.0%) but declined when TBSP was enzymically hydrolyzed for 24 h at 55°C (DH = 4.7%) probably because high temperature denatures the fish protein (Figure 4.15). A high temperature was used in this study, because previous tests showed that endogenous fish proteases were active at 55°C (section 4.2) and produced maximum hydrolysis. From DSC data, TBSP began to denature at 55°C and would then be expected to aggregate after 24 h at 55°C.

Intact fish proteins are commonly believed to lack solubility in water (Venugopal and Shahidi, 1994). Although, some studies have shown that fish protein can be quite soluble at very low ionic strength (Stefansson and Hultin, 1994; Feng and Hultin, 1997). Enzymatic breakdown of protein involves a major structural change in that the protein is gradually cleaved into smaller peptide units, having increasingly higher solubility than the intact protein. This increased solubility is partly due to the smaller peptide size but, most importantly, to the delicate balance of hydrophilic and hydrophobic forces of the peptides. The smaller peptides from myofibrillar proteins are expected to have proportionally more polar residues, with increased ability to form hydrogen bonds with water and increasing solubility compared to that of the intact protein (Kristinsson and Rasco, 2000c).

Solution pH is also important because pH influences the charge on the weakly acidic and basic side-chain groups; thus, proteins and protein hydrolysates generally exhibit lowest solubility at their isoelectric point and highest solubility when maximally charged at low and high pH. Table 4.18 shows the effect of pH on the solubility of TBSP and modified TBSP, which succinylated TBSP was the most soluble. The lowest solubility of modified TBSP was shift pH 5 to pH 3-4. However, the solubility of trypsin modifications, endogenous protease modification and acetylated TBSP are lower than TBSP. Based on the results from the current study, it is recommended that trypsin modification should be done at 25-37°C. The use of 55°C (optimum temperature for fish protease) was found to be unsuitable when exogenous trypsin was used for fish protease hydrolysis.

#### 4.4.2.3 Foaming properties of modified TBSP

The foaming capacity and foam stability of TBSP and modified TBSP were determined as described in detail in section 3.3.3.1. In this experiment, protein dispersions were whipped for 1 min. The results are shown in Figure 4.16 - 4.17. The concentration of TBSP and modified TBSP used was about 2.2% protein (5% sample, 44.20% total protein content). The results for foaming capacity and foam stability of TBSP and modified TBSP are in Figure 4.16 and 4.17, respectively.

To determine foam capacity, 5% TBSP or modified TBSP solution (5% w/w; pH 7) was blended under standard conditions and the volume increased due to air incorporation was measured (section 3.3.3.1). The degree of air incorporation of TBSP was 160%. The % overrun values for succinylated and acetylated TBSP were approximate 170%. The highest overrun was obtained for TBSP treated with trypsin-1h and trypsin-24h TBSP with values of 250% and 230% respectively. This study shows increases of TBSP foaming due to enzymatic modification.

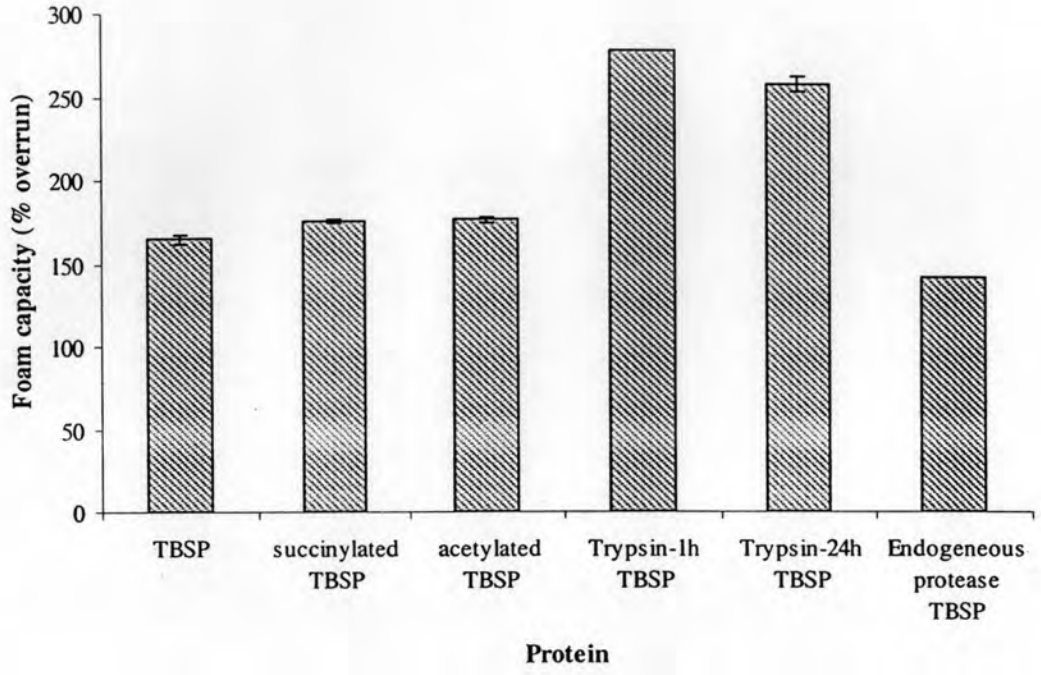


Figure 4.16 Effect of modification of TBSP on foam capacity.

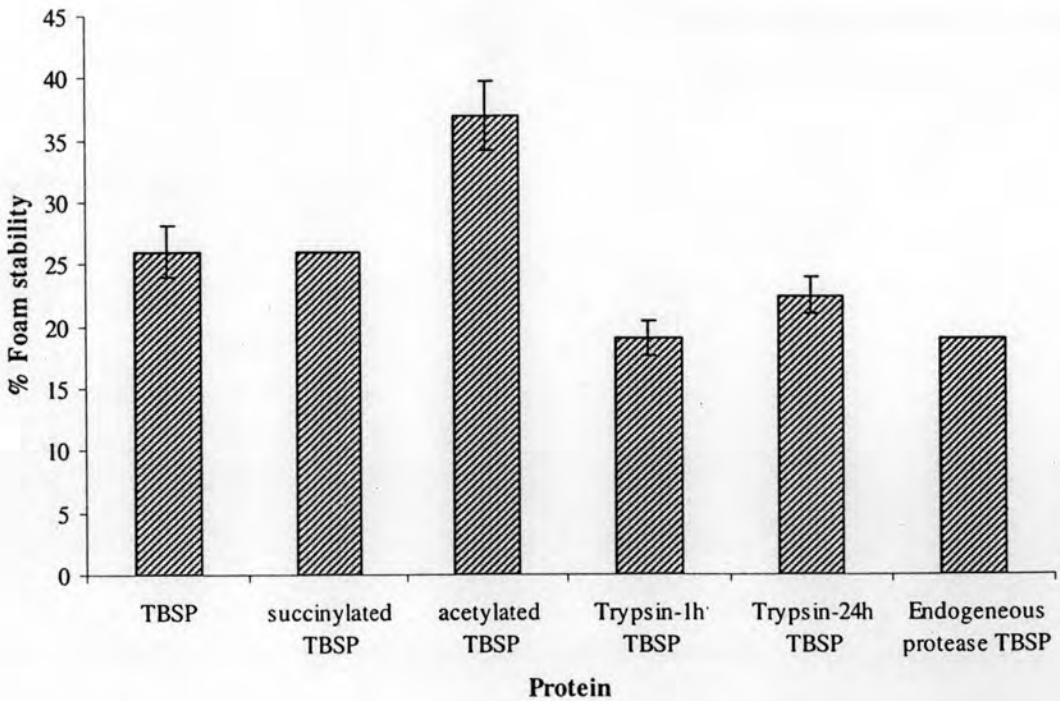


Figure 4.17 Effect of modification of TBSP on foam stability.

In this study, foam stability, acetylated TBSP was the most stable. The enhanced foaming properties of modified TBSP as compared to unmodified TBSP could be related to higher solubility and surface hydrophobicity at pH 7 of the modified TBSP described in the previous sections (section 4.4.2.1).

Heat denaturation (trypsin-1h, trypsin-24h were incubated in 55°C) results in increased foaming properties probably due to increased surface hydrophobicity (Table 4.17) and flexibility of denatured protein. This may explain why the unmodified had lower foaming properties than the modified samples (Damodaran, 1994). Although, there was an enhancement of foaming capacity after modification, the foam stability of trypsin modified TBSP was low compared to TBSP, succinylated and acetylated TBSP because foaming capacity and foam stability require different molecular characteristics. Foam capacity requires rapid adsorption of protein at an air-water interface during whipping or bubbling, ability to undergo rapid conformational change, and rearrangement at the interface. Conversely, foam stability requires a thick, elastic, cohesive, continuous, air-permeable protein film around each gas bubble (Zayas, 1997)

The high surface hydrophobicity may have been a result of increased flexibility of the modified molecule resulting in increased exposure of hydrophobic groups to the probe (Were, Hettiarachchy and Kalapathy, 1997). The high surface hydrophobicity of modified TBSP as shown in Table 4.17 may contribute to differences in foaming properties as the unfolding of protein molecule results in exposure of hydrophobic groups which can result in increased interactions at the air/water interface. The high surface hydrophobicity of chemically modified TBSP could be a contributing factor for the enhanced foaming capacity of modified TBSP compared to TBSP (section 4.4.2.1), as foam capacity is related to high surface hydrophobicity (Damodaran, 1994).

#### 4.4.2.4 Emulsifying properties of modified TBSP

The emulsifying activity index (EAI) and Emulsion stability index (ESI) of TBSP and modified TBSP were determined using a method described in details in section 3.3.4.1 and section 3.3.4.2. The concentration of TBSP used was 2.2%

protein (5% sample). The results for EAI and ESI of TBSP and modified TBSP are in Table 4.19.

The EAI of succinylated TBSP were higher than unmodified TBSP. The ESI of trypsin-1h and endogenous protease TBSP, that were incubated at 55°C 1 h, were lower than unmodified TBSP. The ESI of TBSP is 60.45 min. In this experiment, turbidity of modified TBSP did not decrease during 10 min, observation indicated that these proteins formed stable emulsion. Data suggest that modified TBSP formed good emulsions. More work is needed to determine better defined ESI values for modified TBSP.

From studies using whey protein, it was reported that lower EAI of the unmodified compare to modified protein could be related to their lower solubility of the later. A high solubility is suggested to aid in film formation due to rapid migration and adsorption at the interface (Chobert, Sitohy, and Whitaker, 1988). Film formation is related also to the ability of proteins to form cross-links at the oil-water interface. The high surface hydrophobicity (Table 4.17) and high emulsifying properties of the modified TBSP were the same from reported results, where a high surface hydrophobicity and high emulsification were demonstrated (Damodaran, 1994). Succinylation increased the EAI and ESI of TBSP perhaps because of its enhancement of solubility (Table 4.18). Acetylation which also increases emulsification etc. did not increase TBSP solubility compared with unmodified protein.

Table 4.19 EAI and ESI of TBSP and modified TBSP

| Proteins | EAI ($m^2$/g sample) | ESI (min) |
|---|---|---|
| TBSP | 46 ± 3.13 | 60.45 ± 2.05 |
| Succinylated TBSP | 145 ± 2.87 | * |
| Acetylated TBSP | 35 ± 0.78 | * |
| Trypsin-1h TBSP | 14 ± 6.93 | * |
| Trypsin-24h TBSP | 6 ± 1.80 | * |
| Endogenous protease TBSP | 14 ± 1.02 | * |

Note: * Indicates stable emulsion within study time (10 min)

After trypsin modification leading to partial hydrolysis, the protein structure and function were altered. The hydrolysate with a higher solubility and smaller molecular size might facilitate the diffusion and spread at oil-water interfaces. Exposed hydrophobic (Table 4.17) groups may enhance the interaction between proteins and lipids. High EAI for the hydrolysates were obtained. High correlation between surface hydrophobicity and emulsifying activity has been reported (Wu, Hettiarachchy, and Qi, 1998). However, Shimizu, Saito, and Yamauchi (1986) reported that there was no correlation between these parameters. Hence, solubility and molecular size, rather than surface hydrophobicity, might be the major factors for the high emulsion properties of the small peptides.

#### 4.4.2.5 Gelation property of modified TBSP

The gelation of TBSP and modified TBSP (5% w/w) was examined by oscillatory rheology using 100 mM NaCl with 5 mM $CaCl_2$ solution at pH 7 as solvent (section 3.3.5.1). Values for the storage modulus (G') (Table 4.20) showed that TBSP and modified TBSP started to denature at temperature $\geq$ 50°C (Figure D6 in Appendix D).

The data in Table 4.20 shows the lower G' of TGase modified of beta-lactoglobulin and TBSP than the native one. Truong et al. (2004) reported gelation changes for whey protein treated with microbial TGase. With high TGase/substrate ratios (1.2-10 units/g WPI), extensive cross-linking of the major components of WPI, alpha-lactalbumin and beta-lactoglobulin, was evident. The extensive intra- and interchain cross-linking probably caused formation of polymers that were too large and unfavorable for thermally induced unfolding for effective network development, resulting in and increase in gel point temperature and a severe decrease in gel strength.

The increase in G' signifies an enhance in the protein-protein interactions and in gelation (Joseph, Lanier, and Hamann, 1994). However, the significantly higher G' values for TBSP protein resulting from acetylation as compared to succinylation would indicate that the protein-protein interactions contributing to G' were greater in the acetylated TBSP. This data is the same as reported by Gruener and Ismond (1997a, 1997b) who studied the effects of acetylation and succinylation on the

physicochemical and functional of the canola 12S globulin. Paulson and Tung (1988) tentatively identified the bonds involved in gel formation and stability of canola proteins to be hydrophobic interactions and hydrogen bonding. Therefore, the higher G' of the acetylated concentrates may be attributed to their higher surface hydrophobicity (Gruener and Ismond, 1997b). The effect of modification on protein hydrophobicity cannot be whole explanation for gelation effect because succinylation increases surface hydrophobicity more than acetylation (Table 4.17) but produces less increase in gelation.

Table 4.20 Gelation of TBSP and modified TBSP

| Proteins* | G' (Pa)** |
|---|---|
| TBSP | 10.58 |
| TGase TBSP*** | 2.96 |
| Succinylated TBSP | 0.20 |
| Acetylated TBSP | 4.70 |
| Trypsin-1h TBSP | 0.52 |
| Endogenous protease TBSP | 1.72 |

Note: * Oscillatory rheology data was measured for 5% (w/w) proteins in NaCl (100 mM) with $CaCl_2$ (5 mM) (pH 7.0)

** Storage modulus (G') at 90°C

*** Transglutaminase modification was added 0.2% w/w TGase (Ajinomoto) in 5% (w/w) protein in NaCl (100 mM) with $CaCl_2$ (5 mM) (pH 7.0)

In this study, it can be revealed that chemical modification reduce gelation. The extent of hydrolysis brought about by enzymes also seems to prevent good gel formation because of the reduction in molecular weight of the proteins. However, these modifications reduce gelation are good for some products that do not want gelation, such as drinking food. The loss of the gelation ability of soy protein isolate is used to advantage in the manufacture of soy protein hydrolysates that can be heat processed without changing their flow properties (Panyam and Kilara, 1996). So we can apply modified technique for suitable food. This behavior of modification that

reduced gelation is in direct contrast to whole myofibrillar proteins in surimi-based products. This is supported by the work of Miller and Groninger (1976) where acylated myofibrillar proteins showed gelling properties in a model system at a minimum concentration of 3% when heated at 70 to 75°C for 20 min. When hydrolyzed by bromelain, the acylated proteins only formed gels at a minimum of 5 to 7% solutions. Hall and Ahmad (1992), working with 10% solutions of fish protein hydrolysate heated for 30 min at 70°C or 80°C at pH from 2 to 8, could not obtain gels.

Kristinsson and Rasco (2000c) observed that enzymatic breakdown of protein involves a major structural change in the protein is gradually cleaved into smaller peptide units, having increasingly higher solubility than the intact protein. This increased solubility is partly due to the smaller peptide size but, most importantly, to the delicate balance of hydrophilic and hydrophobic forces of the peptide. The smaller peptides form myofibrillar proteins are expected to have proportionally more polar residues, with increased ability to form hydrogen bonds with water and increasing solubility compared to that of the intact protein.

Hydrolysis was presumed to be detrimental to the gelling properties of proteins because of the reduced hydrophobicity of hydrolysates (Mahmoud, 1994). From the data of trypsin treated TBSP (section 4.4.1.2), trypsin hydrolyze peptide bonds of TBSP to promote reduction of molecular weight, possible conformation changes, and enhance hydrophilicity due to newly exposed amino and carboxyl groups, there for the solubility of trypsin treated TBSP (1h) has solubility (protein-water interaction) higher than the native as shown in Figure 4.15.

Van Kleef (1986) studied soybean protein and ovalbumin gels at 10-35% concentrations and found the characteristics of the gels to be highly dependent on pH. The gels formed at a higher pH exhibited more protein-water interactions. At a higher pH, less intramolecular interactions occurred, due to electrostatic repulsion. This may lead to more flexible protein chains that could extend further. Because the protein molecules interacted less, the overall distribution of the protein chains in the gel was more uniform.

After chemical modification of TBSP by succinylation and acetylation, protein surface hydrophobicity was higher than the original TBSP (Table 4.17). In this study,

the functional properties improved by acylation include solubility, surface hydrophobicity, EAI and ESI. The improvement of a specific aspect of functionality depends on reaction conditions, particularly the type and extent of acylation. For instance, high levels of acetylation are effective in masking lysine residue, exposing hydrophobic interiors and causing subunit dissociation (Shih, 1992).

Pavlova, Damshkaln and Vainerman (1991) found a definite correlation exists between the acetylation degree and the dynamic viscosity of the modified pollack protein isolates. The viscosity falls dramatically with the acetylation degree increasing. One of the reasons of viscosity decreasing may be connected with the fact that acetylation promotes dissociation if actomyosin complex and myosin into subunits. The curve of thermally induced viscosity changes for myofibrillar proteins dispersion has a complicated character. It implies the existence of several macromolecular conformational transitions which influence the protein-water and protein-protein interactions.

Succinylation of a protein converts the cationic amino groups to anionic residues and the increase in net negative charge produced by succinate anions alters the physicochemical character of the protein resulting in an enhanced aqueous solubility and subsequent changes in emulsifying and foaming capacity. Franzen and Kinsella (1976b) explained the increase in aqueous solubility observed following succinylation and acetylation of soy protein. The structure of succinylated proteins results from their high net charge and the replacement of short range attractive forces in the native molecule with short range repulsive ones with subsequent unfolding of polypeptide chains. Thus, in soy isolate electrostatic attractions between neighboring ammonium and carboxyl groups enhance protein-protein interactions which lower solubility. Upon succinylation at neutral pH values, however, the ammonium cations of lysine are replaced by succinate anions. Electrostatic repulsions occur between the added carboxyl groups and the neighboring native carboxyl groups producing fewer protein-protein interactions and more protein-water interactions to enhance aqueous solubility. Since net negative charge is proportional to the extent of derivatization. Significantly, the aqueous solubility of succinylated soy isolate derivatized with 0.5 and 2 g quantities of succinic anhydride were similar, i.e., 1.65 and 1.70 mg/mL of

protein. Hence, exhaustive succinylation was not required to produce a substantial increase in solubility.

Upon acetylation ammonium cations were replaced by neutral acetyl groups ($-C(=O)CH_3$) producing fewer electrostatic repulsions, and the aqueous solubility of acetylated soy isolate was intermediate in magnitude between that of soy and succinylated soy isolate. Franzen and Kinsella (1976) noted that the electrophoretic motilities of acetylated bovine serum albumin and beta-lactoglobulin were intermediate between those of the corresponding native and succinylated proteins.

Therefore, the acylation modification of TBSP, gave lower solubility and hydrophobicity, that may result in aggregation of the modified proteins (Yamauchi et al., 1979; Seifert and Schwenke, 1995), so gelation of acylation modification is lower than original TBSP, but a little bit higher than succinylated-TBSP. Gelation mechanism and gel appearance are fundamentally controlled by the balance between attractive hydrophobic interactions and repulsive electrostatic interactions. These two forces in effect control the balance between protein-protein and protein-solvent interactions in a gelling system. Therefore, modification of TBSP alters the balance of forces needed for optimum gelation.