CHAPTER II

LITERATURE REVIEW

2.1 Sources of food proteins

The use of extracted protein as ingredient dates from the 1970's with concerns about world protein shortages. This area has developed over 30-years. Now many protein concentrates and isolates have been developed from microbial, plant and animal sources. Whole protein ingredients as well as hydrolysates have been used. Protein isolates, concentrates, as well as hydrolysates are used in the agro-food and biotechnology business, e.g. agricultural feed, microbiological protein broths, and as protein ingredients in food manufacturing.

The biggest users of protein ingredients is in the food manufacturing areas including dairy, confectionary and deserts, beverage and cold drinks, soups, cooked meat products. To manufacture processed foods, extracted protein (concentrated or isolated proteins) can be used as raw material or combining with more complex range of new products of food proteins.

There are many research studies on the characteristics, nutritional and functional properties of extracted proteins as shown in Table 2.1 and 2.2. Food proteins have been modified to improve functional properties for application in food products. These proteins can be potentially used as nutrition supplements, and as functional ingredients.

Table 2.1 The major food proteins groups: Plant proteins

Protein source	References
• Leaf proteins	- Franzen and Kinsella (1976a); Wanasundara and Shahidi (1997); Aletor, Oshodi, and Ipinmoroti (2002)
• Cereal proteins (wheat gluten, oat)	- Ma (1984); MacRitchie and Lafiandra (1997)
• Legumes (beans)	- Aluko and Yada (1995); El-Adawy (2000); Neto et al. (2001); Bora (2002); Adebowale and Lawal (2003); Bora and Neto (2005); Lawal (2005)
• Oils seeds (soy protein isolate)	- Franzen and Kinsella (1976b); Paulson and Tung (1988); Gruener and Ismond (1997a, 1997b); Qi, Hettiarachchy, and Kalapathy (1997); Were, Hettiarachchy, and Kalapathy (1997); Wu, Hettiarachchy, and Qi (1998); Achouri, Zhang, and Shiying (1999); Chan and Ma (1999); Achouri and Zhang (2001); Bandyopadhyay and Ghosh (2002); Gensheng et al. (2005); Jung, Murphy, and Johnson (2005);

Table 2.2 The major food proteins groups: Animal proteins

Protein source	References
• Milk (caseins, whey proteins isolate)	- Siu and Thompson (1982); Kester and Richardson (1984); Shimizu, Saito, and Yamauchi (1985, 1986); Modler and Jones (1987); Mulvihill and Kinsella (1987); Chobert, Bertrand-Harb, and Nicolas (1988); Chobert, Sitohy, and Whitaker (1988); Kinsella and Whitehead (1989); Nakagawa et al. (1989); Closs, Courthaudon, and Lorient (1990); Phillips and Kinsella (1990); Phillips, Schulman, and Kinsella (1990); Kneifel et al. (1991); Phillips, Yang, and Kinsella (1991); Phillips (1992); Dickinson et al. (1993); Chen, Swaisgood, and Foegeding (1994); Althouse, Dinakar, and Kilara (1995); Ju, Otte, and Madsen (1995); Mutilangi, Panyam, and Kilara (1995, 1996); Dickinson (1997); Guo et al. (1997); Foegeding et al. (2002); Giardina et al. (2004); Panyam and Kilara (2004); Severin and Xia (2006);
• Egg (dried or liquid egg white, yolk, ovalbumin)	- Van Kleef (1986); Zayas (1997)
• Meat proteins (extracts, hydrolysates, BSA)	- Li-Chan, Nakai, and Wood (1984); Ziegler and Acton (1984); Kim and Kinsella (1985); Stanley, Stone, and Hultin (1994); Miyaguchi, Nagayama, and Tsutsumi (2000); Feng and Hultin (2001)

Table 2.2 The major food proteins groups: Animal proteins (continued)

Protein source	References
• Fish and seafood	- Chen, Richardson, and Amundson (1975); Spinelli and Dassow (1982); Beas et al. (1990); Seki et al. (1990); Pavlova, Damshkaln, and Vainerman (1991); Hall and Ahmad (1992); Morioka, Kurashima, and Shimizu (1992); Morioka and Shimizu (1993); Stefansson and Hultin (1994); Haard (1995); Nowsad, Katoh, Kanoh, and Niwa (1995); Gomez-Guillen, Javier-Borderias, and Montero (1996); Diniz and Martin (1997); Morioka et al. (1997); Lin and Park (1998); Jeon, Byun, and Kim (1999); Liceaga-Gesualdo and Li-Chan (1999); Wahyuni, Ishizaki, and Tanaka (1999); Kim and Park (2000); Kristinsson and Rasco (2000a, 2000b, 2000c); Kristinsson and Hultin (2003); Karthikeyan et al. (2004); Normah et al. (2004); Petursson, Decker, and McClements (2004); Yongsawatdigul and Park (2004); Sathivel et al. (2005); Sathivel, Smiley, Prinyawiwatkul, and Bechtel (2005); Sathivel and Bechtel (2006)
• Blood	- Morrissey, Mulvihill, and O'Riordan (1991); (Zayas, 1997); Benjakul et al. (2004); Rawdkuen et al. (2004)
• Byproducts, processing waste	- Marti et al. (1994); Okazaki (1994); Baek and Cadwallader (1995); Benjakul et al. (1996); Sathivel et al. (2003), Sathivel et al. (2004); Slizyte et al. (2004); Lin, Park, and Morrissey (1995)

In summary, many food proteins have been produced and used as ingredients for food manufacturing, agricultural feedstock and biotechnology industry (e.g. microbiological media). The constituents of proteins or waste, such as casein and whey, blood, abattoir waste, etc., need to be refined and studied further before wider use.

Fish proteins are the good source of protein. There are a lot of proteins that can be recovered from waste or byproducts from fish industry. In this case, the understanding of fish proteins concentrates or isolates and their characteristics and functional properties is important for application in food products.

2.2 Fish

2.2.1 Production of fish

In the annual report of Fisheries Department of FAO (2004), global production from capture fisheries and aquaculture supplied about 101 million tons of food fish in 2002, providing an apparent per capita supply of 16.2 kg (live weight equivalent). Overall, fish provided more than 2.6 billion people with at least 20% of their average per capita animal protein intake. The share of fish proteins in the world animal protein supplies grew from 14.9% in 1992 to a peak of 16% in 1996 and remained close to this level (15.9%) in 2001. In 2002, countries in Asia accounted for 91.2% of the production quantity and 82% of the value. Of the world total, China is reported to produce 71.2% of the total quantity and 54.7% of the total value of aquaculture production. World fish trade has increased in terms of both value and quantity. In 2002, China overtook Thailand for the first time to become the world's main exporter of fish and fish products, with exports valued at an estimated US$ 4.5 billion. The largest importer in 2002 was Japan, with over one fifth of the world's imports. Thailand is one (the 6^{th}) of the top ten producers in aquaculture production with 644,900 tons (FAO, 2004).

In 2002, about 76% (100.7 million tons) of estimated world fisheries production was used for direct human consumption. The remaining 24% (32.2 million tons) was used for non-food products, mainly the manufacture of fishmeal and oil (FAO, 2004).

Fish paste products, such as fish balls and fish cakes, are believed to have originated form China with fresh fish used as raw material. Since then, the Japanese and Chinese immigrants to Southeast Asia have made these traditional products, and fish paste products are now widely consumed in the Asian region. In the 1960s, the traditional methods of making surimi-based products were changed by arrival of frozen surimi as a raw material. In 1980, the Marine Fisheries Research Department of the Southeast Asian Fisheries Development Centre introduced the concept of surimi to fish-processing industry in Southeast Asia. After that, there has been huge development in the processing of surimi for export and in the upgrading of the local fish jelly product industries in Southeast Asia, especial in Thailand (Morrissey and Tan, 2000).

The fish species used in Thailand for production of surimi are mainly threadfin bream. Threadfin bream (*Nemipterus* spp.) is in family Nemipteridae, and about 10 species are commonly found in the Indo-West Pacific region in tropical and subtropical waters. Catches of threadfin bream are usually not identified by species, but the most commonly found in Thailand's surimi production industry, are *N. hexodon*, *N. japonicus* and *N. fergosus*.

2.2.2 Proteins in fish

Proteins, the most important functional components in muscle, give many of the physicochemical and sensory attributes of muscle foods. Muscle proteins comprise 15-22% of total muscle wet weight (Xiong, 1997). As with all contractile muscle, fish flesh can be divided into three major groups which are separated by extraction techniques of water and salt solutions (solubility characteristics): connective tissue or stromal proteins (insoluble), myofibrillar proteins (salt-soluble) and sarcoplasmic proteins (water-soluble). These proteins play different in each species as show in Table 2.3. Of the three groups, myofibrillar proteins are the most critical role during processing treatments. Sarcoplasmic and connective tissue proteins also possess desirable functionality, which are important for producing acceptable muscle foods. Recent advances in muscle protein research have

contributed to the discovery that many of the functional properties of muscle proteins are related to their structures and physicochemical changes.

Table 2.3 Protein (%) composition of fish muscle

Species	Connective tissue proteins	Myofibrillar proteins	Sarcoplasmic proteins	Ref.
Sardine	1.1-1.6	58.0-62.9	32.8-39.4	(1)
Mackerel	1.1	59.9	37.8	(1)
Albacore	2.4-2.9	45.9-50.4	46.0-51.4	(2)
Yellowfin tuna	1.3-1.5	55.6-56.0	40.9-42.6	(2)
Skipjack	1.2-2.4	51.0-53.4	44.2-45.7	(2)
Sea bass	3.4-4.2	55.0-66.9	24.8-28.0	(3)
Shark	3.8-11.3	56.7-66.8	20.4-26.1	(3)

References: (1) Hashimoto et al. (1979), (2) Kanoh et al. (1986), (3) Watabe et al. (1983)

2.2.2.1 Connective tissue proteins

The interstitial space of muscle cells contains the extracellular proteins such as collagen, reticulin and elastin and the supporting ground substance. These and related proteins substances are called connective tissue proteins or stromal proteins since they make up tissue connecting the muscle cells. Connective tissue proteins contain 3-10% of total protein in muscle (Mackie, 1996).

Connective tissue proteins of fish skeletal muscle consist mostly of collagen. The collagen molecule is a long cylindrical protein comprising three polypeptide chains wound around each other in a supra helical coil and with inter- and intra-molecular cross-linking generally increasing with the age of the animal. In its native form, collagen is insoluble in low and high ionic strength salt solutions but on heating it breaks down to its denatured form, gelatin, which is soluble in water. Compared with those of mammals, fish connective tissue proteins are present in relatively low concentration reflecting the different structural arrangement of contractible muscle,

and in consequence they do not contribute much to the textural properties of fish muscle (Mackie, 1997).

2.2.2.2 Myofibrillar proteins

The myofibrillar proteins are those which compose the contractile apparatus within the muscle cell. Myofibrillar proteins comprise 65-75% of total protein in muscle (Mackie, 1996). Based on their physiological and structural, myofibrillar proteins can be further divided into three groups:

1. Major contractile proteins, including myosin and actin, which are directly responsible for muscle contraction and are the backbone of the myofibril. Myosin and actin are the myofibrillar proteins that directly function in the contraction-relaxation cycle. The myosin molecule consists of two subunits called heavy chains (200 kDa), and is a long structure with two globular heads, and up to four subunits called light chains (16-30 kDa).

2. Regulatory proteins, including tropomyosin, the troponin complex, and several other minor proteins, which are involved in the regulation of filamentous structure of myofibrils. These proteins are distributed in different parts of the myofilaments, e.g., A-bands, I-bands, and Z-disks.

3. Cytoskeletal or scaffold proteins, including titin or connectin, nebulin, desmin.

2.2.2.3 Sarcoplasmic proteins

The term "sarcoplasmic proteins" usually refers to the proteins of the sarcoplasm, the components of the extracellular fluid, and the proteins contained in the small particles of the sarcoplasm. They can be extracted by homogenizing the muscle with water or dilute salt solutions (< 0.15 M KCl). The sarcoplasmic proteins contain generally low molecular weight compounds consisting of albumins, myoglobin, hemoglobin and enzymes of the glycolytic pathway, lysosomes, and various proteases as well as peptides (Venugopal and Shahidi, 1996; Haard, 1995). This protein group may include some proteins that are not strictly soluble in the sarcoplasm, notably membrane proteins (Haard, 1995). These proteins make up 20-35% of the total protein content of muscle, depending upon the species (Mackie,

1996). The contents of sarcoplasmic proteins are generally higher in pelagic fish such as sardine and mackerel and lower in demersal fish like plaice and snapper (Suzuki, 1981).

The unique nature of the sarcoplasmic proteins of each species can be obtained from the separation patterns or profiles of electrophoresis. This pattern can be used for the accurate identification of the species. Many researches were done on the methods of identifying species of raw and processed fish using differentiable sarcoplasmic proteins (Hashimoto et al., 1984; Nakagawa, Watabe, and Hashimoto, 1988a, 1988b; Scobbie and Mackie, 1988; An et al., 1989; Sotelo et al., 1993; Huang, Marshall, and Wei, 1995; Rehbein et al., 1995; Chen et al., 1996; Chen and Hwang, 2002). This situation arises when the identifying features of different fish have been removed on processing, as into fish fillets or fish products which are completely lacking in any features that would enable the identity of the fish to be established. It may be then necessary to establish the identity of the species, for example, that it is as declared on the label of a product or specified in a commercial contract (Mackie, 1997).

Beside this, sarcoplasmic proteins especially those that function as glycolytic enzymes can also be used to categorize fish. Nakagawa, Watabe, and Hashimoto (1988a, 1988b) used SDS-PAGE patterns of glyceraldehyde-3-phosphate dehydrogenase, creatine kinase and aldolase, to differentiate 16 fish species into three groups: bottom marine, pelagic and freshwater fish.

The muscles of fish and lower vertebrates differ from those of land animals in that they contain large quantities of Ca^{2+}-binding proteins called parvalbumins (Haard, 1995). Parvalbumin (molecular weight about 12 kDa) has been investigated as a biomarker in fish because it is found at the millimolar level in the white muscle of most species, it withstands high temperatures without denaturing, and it is resistant to protease degradation (Ross et al., 1997).

Recent research has resulted in properties of fish sarcoplasmic proteins being better defined. The characteristics, nutritional and functional properties of fish proteins are listed in Table 2.2. These indicate that fish sarcoplasmic proteins have potential applications as nutritional, food, and protein functional ingredients.

Most of enzymes are component in sarcoplasmic proteins. There are many researches about enzymes in fish that affect the quality of fish and fish products. Enzymes in fish are good reviewed by Haard (1995), Kolodziejska and Sikorski (1996) and Park (2000)

Fish muscle enzymes are involved with physiological events of cell activities, and they are the biological catalysts of the chemical reactions which muscle cells carry out in life. On this basis, all six classes of enzymes (oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases) are present in fish muscle. The main enzyme groups known to affect the edible qualities of fish are hydrolases and transferases, especially in surimi gelation as described by An, Peters, and Seymour (1996).

2.2.2.3.1 Proteolytic enzymes

Hydrolytic enzymes, hydrolases, of importance in post harvest fish include proteases and peptidases, lipases and phospholipases, and glycogen hydrolases (Haard, 1995). Most of the research has been undertaken on proteases (proteolytic enzymes). Protease refers to the enzymes that hydrolyze peptide bond to shorter chain peptide or amino acids.

Proteases are classified according to active sites, mechanism of action, and three-dimensional structure. Four mechanistic classes are recognized by the International Union of Biochemistry. These classes have a characteristic set of functional amino acid residues arranged in a particular configuration to form the active site. They are (Neurath, 1989):

1. Serine proteases (EC 3.4.21); serine proteases are endopeptidases which contain a hydroxyl group (-OH) of serine at the active site. Examples of these enzymes are trypsin, chymotrypsin, elastase and subtilisin.

2. Cysteine proteases (EC 3.4.22); they contain sulfhydryl (thiol group, -SH) of cysteine at the active sites. They are also called sulfhydryl or thiol protease. Examples of these enzyme are papain, calpains and cathepsins.

3. Aspartic acid proteases (EC 3.4.23); these enzymes contain a carboxyl group (-COOH) of aspartic acid. They are also called carboxyl proteases or acid protease. Examples of these enzymes are pepsin, rennin and chymosin.

4. Metallo-proteases (EC 3.4.24); these enzymes have metal ions at their active sites, such as Zn^{2+} and Mn^{2+}. Examples are thermolysin, carboxypeptidases A and B.

Only the serine and cysteine (thiol or sulfhydryl) proteases appear to be involved in surimi gel degradation.

From a practical standpoint, fish muscle proteases may also be grouped according to the optimum pH of their activity on muscle proteins, such as acid (cathepsins B, H, and L), neutral (calpain D), and alkaline protease (trypsin, chymotrypsin) (Kolodziejska and Sikorski, 1996; Kang and Lanier, 2000).

The proteases are responsible for many of the deteriorative reactions in seafood, such as texture softening in fillets, gaping between myotomes, and the modori phenomenon in surimi gels. The localization of the enzyme in fish muscle and its solubility in water can affect its role in surimi gelation. Proteases in the sarcoplasm are easily extractable, whereas those in myofibrils are tightly associated and not easily leached during surimi manufacture. For this reason, the gelling properties of the minced muscle of certain species can be similar to that of surimi made from it, whereas for others it is not. Under some circumstances, proteases can also be used for seafood processes, such as production of fish protein hydrolysates, fish sauce, or to facilitate roe processing and flavor development (Kang and Lanier, 2000). Fish proteases have been compiled (Haard, 1994, 1995; Kokodziejska and Sikorski, 1996; Kang and Lanier, 2000). The source, type and content of proteases can vary greatly for each species.

Muscle proteases have been largely studied for their "modori" producing effect. These proteases are classified into several groups including alkaline proteases and acid proteases.

The amount of alkaline proteases activity found in muscle varies considerably within families of freshwater and marine fish. The pH and temperature optima with casein substrate are normally 8.0 and 60-65 °C, respectively. Most alkaline proteases are classified as cysteine and serine enzyme but metallo-proteases have also been reported (Kolodziejska and Sikorski, 1996).

The acid proteases are the major proteases that can exist in muscle cells. Cathepsins are responsible for the intracellular protein degradation that is important in

living tissue physiologically and pathologically. The cathepsins of muscles from various fishes have been reviewed (Haard, 1994; Kolodziejska and Sikorski, 1996; Kang and Lanier, 2000). Aoki, Yamashita and Ueno (2000) examined the distribution of several cathepsins (cathepsins B, B-like, L and D, and latent cathepsin L) in red and white muscles among 24 species of marine and freshwater fish. All activities of cathepsins except cathepsin D of marine red-flesh fish were relatively higher than that of marine white-flesh fish and freshwater fish. The activity of cathepsin D was detected in red or white muscle of all fish, and no difference can be seen between fish species. Cathepsin L (EC 3.4.22.15) from both fillets and surimi of Pacific whiting has the highest activity at 55°C, and thus can degrade gel texture during conventional cooking of surimi seafood. Among the highly active lysosomal cathepsins (B, H, and L) in whiting fillets, a large portion of cathepsin B and almost all of cathepsin H is removed during the washing process in surimi manufacture, whereas cathepsin L is not washed out (An et al., 1994). Cathepsin L purified from Pacific whiting consists of a single peptide with a molecular weight of 28.8 kDa and a pH optimum near 5.5, although it readily degrades the myofibrillar proteins at near neutral pH (Seymour et al., 1994).

Many protease assay methods are based on the measurement of TCA-soluble peptide products in the solution. The activity of enzymes depend on many factors, such as substrates, pH of the reaction medium (usually and aqueous solution), temperature at which the reaction occurs, inhibitor and activator or frozen storage and freeze-drying.

Substrates: The two mains groups of protease substrates are, natural and synthetic. The natural substrates are cheaper and easier to handle. Benjakul et al. (1998) characterized protease from Pacific whiting surimi wash-water by using various natural substrates. They concluded that casein, acid-denatured hemoglobin and myofibrils were hydrolyzed more readily than native hemoglobin or BSA.

Casein, azocasein and endogenous substrate are in this natural substrate group. Many proteases were studied using casein or sodium caseinate for more soluble substrate, as substrates (An et al., 1994; Choi, Cho, and Lanier, 1999; Choi et al., 1999; Hurtado et al., 1999; Visessanguan et al., 2001; Benjakul, Visessanguan, and Leelapongwattana, 2003).

Azocasein is also used as substrate for protease activity test (An et al., 1994; Choi, Cho, and Lanier, 1999; Hurtado et al., 1999; Visessanguan et al., 2001). This substrate consists of casein conjugated with azo-dye. Degradation of the casein liberates free dye into the supernatant that can be quantitatively analyzed at A450 nm. Azocasein provided additional advantages for the assay requiring shorter time and easier handling (An et al., 1994).

Endogenous substrate is the protein of the fish itself that can be used as substrate. Choi et al. (1999) used actomyosin that was prepared from Atlantic menhaden muscle for characterization protease in its muscle. Stoknes, Rustad, and Mohr (1993) used myofibrillar proteins extracted from herring muscle as substrates in experiment; they reported that the proteolytic activity on myofibrils follows the same temperature profile as the activity on hemoglobin.

pH: The forces that hold a protein chain in its particular shape result from a number of interactions, involving both the peptide backbone and amino acid side chains. The side chains of the amino acid can be charged, either positively or negatively depending on the pH of the solution. The ability of the amino acids at the active site of an enzyme to interact with the substrate depends on their electrostatic state, i.e., whether they are properly charged or uncharged, as well as their spatial orientation. If the pH is not correct (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). Therefore, 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. Therefore, the pH-activity profile for protease enzymes can provide information about the active site groups and help to identify the proteases.

Temperature: An increase in temperature accelerates the rate of enzyme catalyzed reactions and affects the thermal stability of an enzyme (Mathewson, 1998). High temperatures increase the exposure of hydrophobic amino acid residues and unfolding of the enzyme molecule. At low temperatures, enzyme inactivation is

essentially negligible compared to the rate of reaction catalysis, but becomes more significant at higher temperatures.

The optimum temperature of purified protease from threadfin bream muscle showed a temperature optimum of 60°C (Kinoshita, Toyohara, and Shimizu, 1990). A novel myofibril-bound serine protease from carp muscle had an optimum temperature of 55°C (Osatomi et al., 1997). Kinoshita et al. (1991) studied modori-phenomenon (thermal gel degradation) in threadfin bream muscle. They suggested that a latent serine proteases was responsible for the modori-phenomenon occurring at 60°C. In another work from the same group Kinoshita et al. (1992), found a novel substrate specificity for trypsin-like protease and inhibitor patterns characteristics of serine proteases. They concluded that trypsin-like serine protease that is active at 50°C play important roles in developing modori-phenomenon in commercial fish jelly production.

Inhibitors: It is possible to recommend a limited set of inhibitors to utilize for initial classification of a newly discovered protease. The most commonly available commercial protease inhibitors were reviewed by Beynon and Salvesen (1989). These are the serine protease inhibitor: phenylmethylsulfonylfluoride (PMSF), the cysteine protease inhibitor: iodoacetic acid, aspartic protease inhibitor: pepstatin A; metallo-protease inhibitor: 1,10-phenanthroline. Each inhibitor should be incubated with the enzymatic activity for up to 1 h at suitable temperature, and the free enzyme should be incubated under the same conditions without the inhibitor to serve as the control.

Garcia-Carreno (1996) gave a nice review protease inhibitors and discussed how protease inhibition is involved in some processes of laboratory and food products. Most of the researches to characterize proteases always use standard inhibitors (Kinoshita, Toyohara, and Shimizu, 1990; Benjakul et al., 1996; Osatomi, 1997; Cao et al., 2000; Visessanguan et al., 2001; Benjakul, Visessanguan, and Leelapongwattana, 2003)

Activators: Many enzymes incorporate divalent cations (Mg^{2+}, Ca^{2+}, Zn^{2+}) and transition metal ions (Fe, Cu, Ni, Co, etc.) within their structures to stabilize the folded conformation of the protein or to make possible direct participation in the chemical reactions catalyzed by the enzyme. Metals can provide a template for protein folding. Metal ions can also serve as redox centers for catalysis; examples

include heam-iron centers, copper ions and nonheam ions. Other metal ions can serve as electrophilic reactants in catalysis, as in the case of the active site zinc ions of the metallo-proteases. Most metal ions are bound to the protein portion of the enzyme by formation of coordinate bonds with certain amino acid side chains; histidine, tyrosine, cysteine, and methionine, and aspartic and glutaminc acids (Copeland, 2000).

The calcium activated calpain families of enzymes are found in fish (Jiang, Lee, and Chen, 1994). Enzymes with more than on subunit, such as transglutaminase and m-calpain, also need calcium to activate. Past research shows that calpain, which is an intracellular cysteine protease is controlled by calcium ions and calpastatin (Wang, Su and Jiang, 1992). Wang et al. (1993) reported the calcium requirement for half-maximum activation of the calpain from tilapia and grass shrimp muscles, with casein as a substrate, while the optimum concentration of the ions is 1-2.5 mM and 5 mM, respectively. Other metal ion in 5 mM concentration either activate the enzyme (Sr^{2+}, Ba^{2+}, Mn^{2+}) or have a significant inhibitory effect (Fe^{2+}, Fe^{3+}, Ni^{2+}, Cu^{2+}, Zn^{2+}, Cd^{2+}, Hg^{2+}). Jiang, Lee, and Chen (1994) found cysteine protease from mackerel was inhibited by Zn^{2+}, Cd^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Fe^{2+} and Fe^{3+}.

2.2.2.3.2 Transglutaminase

Transglutaminase (TGase, EC 2.3.2.13) is an enzyme that catalyzes the polymerization and cross-linking of proteins through the formation of covalent bonds between protein molecules. Non covalent bonds are formed between glutamic acid and lysine residues in proteins. This link enhances the physical strength of surimi gels (An, Peters, and Seymour, 1996; Lanier, 2000).

Kishi, Nozawa, and Seki (1991) reported reactivity of muscle TGase on carp myofibrils and myosin B. Carp ordinary muscle contained TGase activity, easily extractable with a solution at low ionic strength, of 1.5 unit/g of wet weight. The TGase, partially purified by DEAE-cellulose and gel filtration chromatography (Sephacryl S-300), showed molecular weight of about 80 kDa and Ca^{2+}requirement for full activation.

Nowsad, Kanoh and Niwa (1995) studied the contribution of TGase to the setting of fish flesh paste by using TGase-free actomyosin (AM) from five fishes. Sarcoplasmic proteins were extracted from each fish and one part of them was treated

with N-ethylmaleimide in order to inactivate TGase contained in them (NEM-SP). The addition of sarcoplasmic proteins to the AM paste promoted the cross-linking of myosin heavy chain during its setting and increased the breaking force of resulting suwari gel. In the breaking force, the gel added with sarcoplasmic proteins was higher than the one added with NEM-SP. They concluded that TGase increases the breaking force of the suwari gel from the fish flesh paste nearly by a few ten percent.

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 enzymatic action of TGase was found to be influenced also by the conformational factor of substrate proteins, depending on fish species.

2.2.3 Technological aspects of fish proteins

2.2.3.1 Preparation and extraction of fish proteins

Fish protein can be processed by various methods to separate protein components from muscle cell, also separate particular protein from other proteins and non-protein components present in the cell. Before the protein extraction step, raw fish is normally prepared by heading, gutting and deboning. The fresh fish fillet is then chopped into small pieces and homogenized or blended. The next step of protein extraction is to extract the fish with a suitable solvent for the aim of separating fish muscle proteins, connective tissue, myofibrillar and sarcoplasmic proteins. The different proteins have different solubility in different solvents. Connective tissue

proteins are almost totally insoluble in water or salt solution. Myofibrillar proteins are soluble in mild salt (NaCl) solution (0.5 M), but are largely insoluble in water of lower ionic strength. Sarcoplasmic proteins are soluble in water or salt solutions of low ionic strength (< 0.15 M) (Lanier, 2000). Morioka et al. (1997) extracted three different sarcoplasmic protein solutions from Pacific mackerel. These proteins had different effect on the strength of myofibrillar proteins gel.

2.2.3.2 Proximate analysis

The main chemical components of fish flesh are water, protein and lipids which make up about 98% of the total mass of the flesh, as show in Table 2.4. However, carbohydrate content in fish muscle is very low, usually below 0.5% (Huss, 1995). Fish proteins powders usually have a high protein content, as show in Table 2.5.

Table 2.4 Chemical compositions of the fillets of various fish species

Species	Scientific name	Water (%)	Lipid (%)	Protein (%)
Blue whiting	*Micromesistius poutassou*	79-80	1.9-3.0	13.8-15.9
Cod	*Gadus morhua*	78-83	0.1-0.9	15.0-19.0
Eel	*Anguilla anguilla*	60-71	8.0-31.0	14.4
Herring	*Clupea harengus*	60-80	0.4-22.0	16.0-19.0
Plaice	*Pleuronectes platessa*	81	1.1-3.6	15.7-17.8
Salmon	*Salmo salar*	67-77	0.3-14.0	21.5
Trout	*Salmo trutta*	70-79	1.2-10.8	18.8-19.1
Tuna	*Thunnus spp.*	71	4.1	25.2
Carp	*Cyprinus carpio*	81.6	2.1	16.0

Source: Huss (1995)

Table 2.5 Chemical compositions of fish protein powders

Fish protein powder	Protein (%)	Fat (%)	Moisture (%)	Ash (%)	Ref.
Herring soluble fraction powder (HSFP)	62.0±0.3	20.5±0.1	3.5±0.1	13.7±0.1	(1)
Herring body protein powder (HBP)	73.4±0.8	3.6±0.3	5.3±0.3	17.7±0.3	(2)
Whole herring protein powder (WHP)	76.2±0.4	3.9±0.4	5.3±0.0	14.8±0.3	(2)
Arrow tooth flounder protein powder (AFP)	81.4±0.4	3.0±0.0	10.7±0.4	4.8±0.2	(2)
Arrowtooth flounder					
- soluble protein powder (AFSP)	79.1±2.6	14.9±1.0	2.4±1.0	3.6±1.2	(3)
- insoluble protein powder(AFISP)	79.9±0.7	5.9±0.3	4.9±1.5	9.3±0.7	(3)

References: (1) Liceaga-Cesualda and Li-Chen, 1999, (2) Sathivel et al., 2004, (3) Sathivel et al., 2005

2.2.3.3 Amino acid profiles

Fish proteins contain all the essential amino-acids found in protein isolated from milk, eggs, and meat and have a very high biological value (Huss, 1995). Fish are considered as an excellent source of high-quality protein, particularly of the essential amino acids lysine and methionine (Sathivel et al., 2004). Cereal grains are usually low in lysine and/or the sulfur containing amino-acids (methionine and cysteine), whereas fish protein is an excellent source of these amino acids (Huss, 1995). Many researchers reported amino acid profiles of fish protein powder as shown in Table 2.6.

Table 2.6 Amino acid profiles of fish proteins powders

Amino acid	mg/g protein							
	HSFP[1]	HBP[2]	WHP[2]	AFP[2]	AFP[2]	AFISP[3]	EAA[a]	EAA[b]
Hydroxyproline	-	20.4	28.5	5.9	5.5	1.1		
Aspatic acid	118.0	78.5	75.0	99.2	106.8	104.7		
Threonine[c]	42.0	34.8	29.3	38.2	40.7	50.7	9	43
Serine	42.2	41.5	40.3	41.6	42.2	41.8		
Glutamic acid	133.6	169.7	130.1	204.3	209.4	144.9		
Proline	21.4	50.8	56.0	26.4	26.6	34.8		
Glycine	81.7	105.5	144.1	50.9	46.7	39.1		
Alanine	84.7	81.9	94.6	63.0	62.1	51.3		
Valine[c]	39.2	37.4	29.3	39.6	43.1	56.9	13	55
Methionine[c]	27.2	29.2	28.8	30.0	24.3	34.1	17[d]	42[d]
Isoleucine[c]	32.0	26.0	22.0	33.0	34.9	55.3	13	46
Leucine[c]	69.3	72.0	57.8	90.4	88.6	86.9	19	93
Tyrosine	18.7	16.4	14.3	19.5	23.2	48.6		
Phenylalanine[c]	46.4	30.8	37.7	28.2	28.0	50.2		
Histidine[c]	13.4	17.0	28.0	20.8	18.0	25.6		
Lysine[c]	116.3	94.6	91.7	117.4	115.5	98.6	16	26
Arginine	111.8	82.7	73.3	76.0	72.4	70.9	16	66
Cysteine	2.2	8.4	6.4	12.2	-	-		
Tryptophane	-	-	-	-	-	-		

Note: General abbreviations are as shown in Table 2.6

[1]Liceaga-Cesualda and Li-Chen, 1999

[2]Sathivel et al., 2004

[3]Sathivel et al., 2005

[a]Suggested profile of essential amino acid requirements for adult humans by FAO/WHO

[b]Suggested profile of essential amino acid requirements for infants by FAO/WHO/UNU

[c]Essential amino acids, [d]Methionine + cysteine

2.2.3.4 Fish proteins analysis by electrophoresis and gel filtration

SDS-PAGE is the most widely used method for analyzing protein samples in a qualitative manner and the method is based on the separation of proteins according to size. The component subunits of a protein are held together by disulfinde bonds, these linkages is broken prior to electophoresis. In addition, gel filtration chromatography is also known as molecular sieving, gel permeation and size exclusion chromatography. The primary objective of gel filtration is to achieve rapid separation of molecules base on size. Gel filtration separates proteins as native components (Roe, 2001). Therefore, the studies of molecular weight of proteins by SDS-PAGE and gel filtration are different.

2.2.3.4.1 Electrophoresis

Sodium dodecyl sulfate polyacrylaminde gel electrophoresis (SDS-PAGE), is widely use in determination of protein molecular weight. The basis of the method is a migration of proteins under electric field in a gel matrix depends on their net charge, shape and size. Proteins are denatured by heat, denaturing detergent or by treatment with a disulfide reducing agent (mecaptoethanol or dithreitol). Treatment of proteins with electrophoresis sample buffer leads to their being coated with the anionic detergent SDS. In the denatured state, most proteins bind SDS in a constant weight ratio, so that they end up possessing similar charge densities. Under these conditions, the migration rate of the proteins in the electric field is determined only on the basis of molecular size, but is not dependent on the charge of molecules. The larger proteins will be harder to migrate in the polymeric gel than smaller proteins (Copeland, 1994). To estimate the molecular weight of a protein, SDS-PAGE is run using standard marker proteins. A graph of the relative migration distance for marker protein against logarithmic molecular weight is plotted. The unknown protein molecular weight will be determined on the same electrophoresis gel slab as the molecular weight markers and can be calculated from the standard curve.

The patterns of proteins on SDS-PAGE also can identify fish proteins and species of fish. Muscle aqueous extracts consist mainly of sarcoplasmic proteins which account for 20-35% of total muscle proteins (Mackie, 1996). Most of these proteins are enzymes involved in metabolic processes. Differences appear especially

for enzyme proteins performing specific functions, which allow the particular exploitation of resources, by different organisms. Electrophoresis techniques were employed for detecting these differences. SDS-PAGE, which separates proteins on the basis of differences in molecular weights, were used by Mackie (1990, 1996, 1997), Rehbein et al., (1995) and also Sotelo et al. (1993). The unique nature of the sarcoplasmic proteins of each species is the separation patterns or profiles obtained on electrophoresis can be used for identification of the species (Mackie, 1996). When the fish sarcoplasmic proteins are separated by electrophoresis, they give profiles that are unique to the species which, can be compared with the reference profiles for authenticity (Mackie, 1997).

Nakagawa, Watabe, and Hashimoto (1988a) used PAGE technique to determine quantitatively the sarcoplasmic protein patterns of various fish species. Sarcoplasmic proteins were extracted from the ordinary muscle of twelve species of marine and four species of freshwater fish. Patterns of marine fishes widely differed from those of freshwater fishes. Polyacrylamide slab gel electrophoresis (10% gel) in the presence of SDS made it possible to categorize those fishes into three groups by patterns: marine white-fleshed, bottom fish (rich in 43 kDa and 35 kDa components), marine red-fleshed, pelagic fish (rich in 43 kDa, 40 kDa and 35 kDa components), and freshwater fish (rich in 43 kDa component). Red sea bream, Pacific mackerel and carp, as representatives of the three groups, were analyzed for relative amounts of 43 kDa, 40 kDa and 35 kDa components in the total sarcoplasmic proteins: 12.6, 13.7 and 13.5% in red sea bream, 13.7, 18.7 and 11.6% in Pacific mackerel, and 19.5, 12.3 and 10.7% in carp, respectively.

SDS-PAGE (10.4% gel) was used to identify species-specific protein bands of raw and cooked fish and surimi sample from Alaska pollack and red hake. In raw samples, species-specific bands were found in the water extracts. While in cooked samples, it was found that 1% SDS extract was more effective for species identification in both fish and surimi (An et al., 1989).

Huang, Marshall and Wei (1995) investigated the feasibility of using SDS-PAGE, IEF, and two-dimensional (2-D) gel electrophoresis to prepare protein profiles for red snapper identification compared with other eleven closely related fish species that may be substituted illegally for red snapper. Comparing the distinctive patterns

of water-soluble sarcoplasmic proteins for each species on IEF and SDS gels, red snapper could be clearly identified. Thus, they suggested, these techniques could be applied to identify fish species.

Chen and Hwang (2002) studied species identification of puffer species SDS-PAGE. A comparison of identifying was also made among extracts of sarcoplasmic, myofibrillar, SDS-soluble, and urea-soluble proteins, protein bands of lower molecular weight showed the species-specific characteristics. Species identification of all tested puffers could be achieved by judging from the SDS-PAGE patterns (15% gel).

2.2.3.4.2 Gel filtration

Gel filtration is one of the chromatography methods that separates proteins on the basis of their size. Proteins partition between a mobile (solvent) and a stationary phase (porous matrix). Large proteins are excluded from the matrix pores of stationary phase, therefore move faster than smaller proteins. The gel filtration method can be used as a tool to estimate molecular weight of proteins as well as for purification. Molecular weight estimation is carried out by comparing sample and standard proteins eluted under the same conditions. A linear plot of relative elution volume of each standard component (V_e/V_o) against log of MW is used for MW estimation; where V_e is elution volume and V_o is void volume of the column (Copeland, 1994).

Kinoshita et al. (1991, 1992) characterized trypsin-like serine protease and found its molecular weight was estimated as 77 kDa by SDS-PAGE analysis and 70 kDa by gel filtration using TSK-gel G3000SWxl. Mati et al. (1994) used Sephadex G-200 column-gel filtration to distributed molecular weight to two main peaks of recovered proteins from fish meal factory wastewater.

2.2.3.5 Differential scanning calorimetry

Differential scanning calorimetry (DSC) is a thermoanalytical technique for monitoring changes in physical or chemical properties of materials as a function of temperature by detecting the heat change associated with such processes. In DSC, the measuring principle is to compare the rate of heat flow to the sample and to an inert

material which are heated or cooled at the same rate. Changes in the sample that are associated with absorption or evolution of heat produce a change in the differential heat flow which is then recorded as a peak. The area under the peak is directly proportional to the enthalpy change and its direction indicates whether the thermal event is endothermic or exothermic (Biliaderis, 1983)

From the DSC experiment it is possible to measure the sample "melting" peak temperature (Tm or T_{max}) and the peak area which is the heat absorbed for protein denaturation (watts/g protein). This is also called enthalpy of denaturation. The DSC thermogram of whole mammalian muscle showed three transitions with T_{max} values of 57-60, 62-67, and 74-80°C (Wright, Leach, and Wilding, 1977; Wagner and Anon, 1986; Xiong, Brekke and Leung, 1987)

2.3 Functional properties of proteins

Functional properties are most important in determining the usefulness of proteins in food systems. Kinsella (1976) gave the definition of "protein functional properties" as, those physicochemical properties of proteins which affect their behavior in food systems during preparation, processing, storage and consumption, and contribute to the quality and sensory attributes of food systems. The most important functional properties of proteins in food application are: solubility, water and oil holding capacity, foaming properties, emulsifying properties and gelation. As the number of processed and fabricated foods increases, greater reliance will be placed on the consistent behavior of ingredients in specific formulations.

Studying the functionality of food proteins is essential in that their role may be understood and they may be used effectively in food products. The overviews of testing methods for proteins functionality were provided (Hall, 1996; Zayas, 1997; Owusu-Apenten, 2004). Testing of proteins functionality should be performed with protein concentrates or isolates, to help developing new sources of protein and incorporation of these proteins in food systems. This testing is necessary to demonstrate the suitability of new protein ingredients as replacements for conventional proteins.

In developing new sources of food proteins, the functional property is a main criterion in assessing their potential utilization in foods. The knowledge of the functional property of food proteins provides information about performance of these additives in foods. Currently a variety of proteins with functional properties acceptable for many foods are commercially produced (Hettiarachchy and Ziegler, 1994; Zayas, 1997; Linden and Lorient, 1999; Park, 2000).

2.3.1 Solubility

The solubility of proteins is considered as that proportion of nitrogen in a protein product which is in the soluble state under specific conditions. Solubility is the amount of protein in a sample that dissolves into solution. Proteins recommended as food additives can be partly or completely soluble or completely insoluble in water.

Protein solubility is the first functional property usually determined during development and testing of new protein ingredients. Protein solubility is a physicochemical property that is related to other functional properties. Many functional properties of food proteins have been related to solubility. Solubility is considered one of the main characteristic of proteins selected for use in liquid foods and beverages. High soluble proteins possess good dispersibility of protein molecules or particles, and lead to the formation of finely dispersed colloidal systems. Potential applications of proteins can be dramatically expanded if they possess high solubility.

Protein solubility is influenced by amino acid composition and sequence, molecular weight, and conformation and content of polar and nonpolar groups in amino acids. Protein solubility is also affected by environmental factors: ionic strength, type of solvent, pH, temperature, and processing conditions.

The pH of the medium is very important factor affecting solubility of proteins. The degree of protein solubility in an aqueous medium is the result of electrostatic and hydrophobic interactions between the protein molecules. Solubility is increased if electrostatic repulsion between the molecules is higher than hydrophobic interactions. Protein solubility is affected by sensitive balance between repulsive and attractive intermolecular forces and proteins are soluble when electrostatic repulsion between proteins is greater than hydrophobic interactions (Kinsella, 1982).

As solvent pH reaches the isoelectric point (pI), a protein has a net zero charge, attractive forces predominate, and molecules tend to associate, resulting in insolubility. At solvent pH values above the pI, the net protein charge is negative and solubility is enhanced. Protein-water interactions increase at pH values higher or lower than the pI because protein carry a positive or negative charge. Minimal interactions with water were observed for protein molecules at pH values not far from pI. If the protein solubility is plotted as a function of the pH, a U-shaped curve is obtained and the minimum solubility corresponds with the pI (Vojdani, 1996). Consequently, solubility and yield of extraction is greater at alkaline than acid pH and can be enhanced by increasing the net electrical charge of the proteins. Alkali treatment increases the solubility of soy protein and other proteins by causing dissociation and disaggregation of the proteins.

Caseins and caseinates are least soluble at their pI of 4.6, which limits their uses in high-acid foods. Limited proteolysis of casein with trypsin decreased the average molecular weight to < 15,000 Da and improved solubility remarkably in the pH range 4.0-5.0 (Chobert, Bertrand-Harb, and Nicolas, 1988). Whey proteins have very good solubility over a wide pH range but denaturation tends to decrease their solubility at low pH as a result of increased aggregation. Hydrolysis of heat-denatured whey-protein isolate, especially by trypsin, partially restored the loss of solubility (Mutilangi, Panyam, and Kilara, 1996).

The pH-solubility profile for proteins can be determined using a variety of protein analysis methods. The concentration of dissolved protein can be determined using the modified Lowry's method. The total protein used in the solubility test is usually determined as crude protein as determined by Kjeldahl analysis.

The solubility property is usually calculated using equation (Vojdani, 1996):

$$\% \text{ solubility} = \frac{\text{amount of soluble protein} \times 100}{\text{total amount of protein}}$$

2.3.2 Water and oil holding capacity

2.3.2.1 Water holding capacity

The interaction of proteins with water is usually described in terms of: water holding capacity, water hydration and holding, water retention, water binding, water imbibing, water adsorption, and others. The water holding capacity (WHC) of foods can be defined as the ability to hold its own and added water during the application of forces, pressing, centrifugation, or heating (Zayas, 1997). Hermansson (1986) defined WHC as the ability of a food structure to prevent water from being released from the three-dimensional structure of the protein. Protein-water interactions influence other functional properties of proteins in foods such as: solubility, emulsifying properties, and gelation. WHC plays a major role in the formation of food texture, especially in comminuted meat products and baked dough. In processed food formulations, WHC of various protein ingredients must be determined. Protein ingredients with excessively high WHC may dehydrate other formula components.

A standard test for WHC of proteins has been approved. According to this procedure, distilled water is added incrementally to a weighed amount of sample, and a homogeneous paste is produced by vigorous stirring. The paste is then centrifuged at 2000 x *g* for 10 min before the supernatant liquid is discarded and the slurry weighed (Zayas, 1997).

The physical chemistry of water retention by food proteins has been studied. Water interacts with proteins in a number of ways. Interactions between molecules of water and hydrophilic groups of the protein side chains occur via hydrogen bonding. The polar hydrophilic groups, such as imino, amino, carboxyl, hydroxyl, carbonyl, and sulfhydryl groups are related to binding of water in protein. The capacity of proteins to retain moisture is affected by the type and number of these polar groups in the protein polypeptide chain. The binding of water is due to the dipolar character of water. Proteins that contain numerous charged amino acids will tend to bind large amounts of water. Water binding of proteins can be predicted from their amino acid composition. The absorbed water is tightly bound to the protein molecules.

Amino acids are classified according to their ability to bind water (Zayas, 1997) into: (1) polar amino acids have highest water binding. Polar side chains bind 2 or 3 water molecules, and ionic side chains (aspartic and glutamic acids, and lysine)

bind 4 to 7 water molecules/molecule of amino acid, (2) non-ionized amino acids, bind intermediate amounts of water, and (3) hydrophobic groups which bind little or no water.

Gensheng et al. (2005) studied on the properties of soy protein hydrolysate. They found that water holding capacity was high (66.2%) for peptides of molecular weight < 1000 Da. Slizyte et al. (2004) determined hydrolysis of cod byproducts. They found that sludge yield was high and sludge powders had markedly higher water holding capacity than fish protein hydrolysate, suggesting that utilization of all fractions from fish byproduct hydrolysis may be improved by giving more attention to the non-soluble parts.

2.3.2.2 Oil holding capacity

Oil holding capacity (OHC) is important in many types of foods including, emulsions, powders, dairy foods, and sausage products. The ability of proteins to absorb and retain fat and to interact with lipids influences food texture. 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. 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. Kinsella (1976) described mechanism of oil absorption, is attributed mostly to physical entrapment of the oil and thus the higher bulk density of the protein the more fat absorption. Barbut (1996) explained methods used to measure OHC expressed as the volume of oil/g protein. Commonly, protein powders are mixed with a specified amount of excess for a particular time and then centrifuged at a low centrifugal force. OHC can be expressed as milliliter or gram of oil bound by 1 g protein.

The OHC for proteins has been correlated with protein surface hydrophobicity (S_o) and with S_o* S where S = protein solubility (Voutsinas and Nakai, 1983).

Sathivel et al. (2004) investigated properties of protein powders from arrow tooth flounder and herring byproduct. The OHC of all freeze-dried fish protein powders were higher than those of soy protein concentrate.

2.3.3 Foaming properties

The chemistry underlying foaming properties of protein and protein hydrolysates have many things in common with emulsifying properties. Both rely on the surface properties of protein. Food foams consist of air droplets dispersed in a liquid containing a soluble surfactant. The role of the surfactant is to lower the surface and interfacial tension of the liquid. The amphiphilic nature of proteins makes this possible; the hydrophobic portion of the protein extends into the air and the hydrophilic protein into the aqueous phase (Kristinsson and Rasco, 2000c).

2.3.3.1 Foaming capacity

Whipping of protein solutions is most widely utilized method of foam testing because it most closely resembles the commercial process of foaming. Phillips et al. (1990) described a standard method assessing foaming properties of food proteins.

The most stable foams are formed with soluble proteins which can interact and form thick viscous films. In some studies (German and Phillips, 1994; Guo et al., 1997; Were, Hettiarachchy, and Kalapathy, 1997) a correlation was found between the solubility of proteins and their foaming capacity (FC) and foam stability (FS). Protein solubility makes an important contribution to the foaming behavior of proteins. According to Zayas (1997), proteins in the liquid film surrounding a foam should (1) be in the soluble state in aqueous media, (2) be concentrated at the liquid-air interface, and (3) be in the denatured state to possess high viscosity and strength, foam was not formed with suspensions of the insoluble components.

The properties of proteins which enable them to form stable films in foams are affected by the molecular configuration of proteins, their intermolecular bonds, and the content and disposition of hydrophobic residues. Conformational changes of proteins at the air/water interface with unfolding expose hydrophobic regions and this accelerates the association of the polypeptides at the interface. Exposure of the hydrophobic groups at the interface facilitates the association of the polypeptides. As a result, a continuous cohesive film is formed around the air bubbles. The foams with high stability are produced when the hydrophobic region of proteins becomes situated at the interface, and molecules resist migration to the aqueous phase.

Surface hydrophobicity measured by fluorometry and the FC and FS are related (Kato and Nakai, 1980; Townsend and Nakai, 1983; Damodaran, 1994; Nakai, Li-Chan, and Arteaga, 1996). Surface hydrophobicity is generally increased by denaturation as the result of exposing more non-polar groups. The foaming properties of proteins are enhanced by moderate heating and heat denaturation because of partial unfolding and exposure of hydrophobic groups and increased surface hydrophobicity and decreased surface tension. However, excessive heating and denaturation cause coagulation and aggregation and foam destabilization. An increase in FC was obtained with hydrophobic derivatives of proteins as a result of better orientation at the air/water interface (Were, Hettiarachchy, and Kalapathy, 1997). Proteins with low hydrophobicity showed poor FC, and some proteins with low solubility showed a good FC.

Were, Hettiarachchy, and Kalapathy (1997) determined effect of alkaline treatment followed by papain hydrolysis on functional properties of soy protein isolates. They found modified soy protein was improved foaming and water holding capacity. Liceaga-Gesualdo and Li-Chan (1999) found that 36% degree of hydrolysis (DH) of herring protein hydrolysate presented adequate foam expansion as compared to the soluble fraction from the unhydrolyzed control. Reductions in protein molecular weight allow greater flexibility which leads to a more stable interfacial layer. Rate of diffusion to the interface is also increased improving the foamability properties.

The FC of proteins is influenced by environmental conditions such as pH. Relationships between surface properties and FS of BSA as a function of pH have been reported (Kim and Kinsella, 1985). The surface pressures were measured at different pH values to determine their effect on foam stability. The maximum surface pressure was found at the near pI of BSA and decreased rapidly below pH 5 and above pH 6. At pI electrostatic attractions between proteins are at a maximum at the pI and more proteins adsorb at the interface reducing interfacial tension. Interfacial films are thickest, with high viscosity and elasticity because of extensive electrostatic bonding between molecules.

A significant negative relationship was found between FS and charge density (Townsend and Nakai, 1983). The FS increased considerably at low charge density.

These data showed that foams can be destabilized as a result of intermolecular repulsion between protein molecules.

The foam volume and stability is influenced by protein concentration. Foams obtained with a higher concentration of proteins are more dense and more stable because of an increase in the thickness of interfacial films. Film formation on foam bubbles is influenced by protein concentration, and surface pressure is a function of protein conformation and concentration at the air/water interface. The maximum overrun of foam was obtained at the optimum protein concentration (2-8%) as a result of an appropriate viscosity of the liquid phase and thickness of the adsorbed film (German and Phillips, 1994).

The effect of different salt ions on foaming properties depends on their influence on structure and protein conformation. Addition of NaCl influences the FC of proteins because salt affects the solubility, viscosity, unfolding and aggregation of proteins. NaCl increases foam overrun and reduces foam stability. Multivalent metal ions improve the FC of proteins. Kinsella (1976) reported that NaCl added to soy protein suspensions increased FC, however decreased FS. 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.

2.3.3.2 Foam stability

Foam stability (FS) requires the formation of cohesive, viscous, elastic, continuous, air-impermeable film around each gas bubble. FS is affected by the "self-healing" ability of proteins, i.e. their ability to move from a region of low interfacial tension to a region of high interfacial tension (Marangoni effect). The FS is influenced by film thickness, mechanical strength, protein-protein interactions and environmental factors such as pH and temperature (Kinsella and Whitehead, 1989; Phillips, Schulman, and Kinsella, 1990). The resistance of protein foam to coalescence and to the collapse of air bubbles is determined by ability of the protein to form a multimolecular matrix.

Lamellar liquids in the foam tend to drain and when the space between films decreases (Kinsella, 1981). The viscosity of the surface film and bulk liquid is a major factor affecting foam stability. Strong foams are formed by high surface viscosity of film as a result of significant cohesive forces between protein molecules. The stability of the foam is also influenced by the properties of the protein, i.e. concentration, solubility, and ionic strength. Generally, FS is directly affected by protein concentration in solution by influencing the thickness, mechanical, strength and cohesiveness of the film.

During foam ageing gravitational forces will cause water to drain, and air cells will come closer together. Separation of liquid from foam is either by leakage from the lamellae or by rupture of the bubbles. The reduction of water drainage in foam is obtained if proteins at the air-water interface bind water tightly and cause an increase in surface viscosity. Proteins with high rigidity and surface viscosity have low foaming power, however, they form stable foams (Phillips, 1981). The stability of the foam is at a maximum at the isoelectric point, when the surface elasticity is also at a maximum.

The FS of protein should be at a maximum near the isoelectric point of proteins because of a reduction in the surface tension and an increase in film elasticity. The stability of foams of BSA to drainage was maximal in the isoelectric pH range because of the maximum surface pressure, surface viscosity, yield stress and elasticity. Electrostatic repulsions between the interfacial films of foam bubbles influence foam stability. The coalescence will be minimal if neighboring bubbles are repelled at a critical distance by electrostatic repulsions between proteins in the interfacial film (Kinsella, 1981).

2.3.4 Emulsifying properties

Based on the size of the droplets in the dispersed phase, food emulsions are classified as macroemulsions with droplet size 0.2 to 50 μm. Food emulsions can be of the oil in water (O/W) or water in oil (W/O) type. The difference between O/W and W/O emulsions is that an O/W emulsion commonly exhibits a creamy texture, while a W/O system has greasy textural properties (Zayas, 1997).

Emulsions are thermodynamically unstable and therefore the oil and water phase separate with time. The stability of emulsions can be improved by adding amphiphilic surface active molecules that adsorb at the oil-water interface and reduce the interfacial tension. Proteins, being amphiphilic, are well suited to act macromolecular surfactants. In addition to lowering interfacial tension, proteins can form continuous cohesive films at the oil-water interface and thus impart mechanical stability to emulsions (Damodaran, 1996a).

The factors that affect the emulsifying properties of proteins are similar to those that affect the foaming properties of proteins. These include the rate of adsorption at the oil-water interface, the amount of protein adsorbed, conformational rearrangement at the interface, the extent of reduction in interfacial tension, and formation of a cohesive film. The emulsifying properties of proteins are also affected by the hydrophobicity and solubility of proteins. Highly insoluble proteins display very poor emulsifying properties (Damodaran, 1994).

Sathivel et al. (2005) studied the functional, nutritional and rheological properties of protein powders from arrowthooth flounder (AFSP), and developed mayonnaise products (emulsion properties) with AFSP. They reported AFSP had desirable essential amino acid and mineral content, and also concluded the soluble protein powder from arrow tooth flounder can be use as a potential emulsifier. These researches indicate the sarcoplasmic proteins powder can be potentially used as functional ingredients and nutrition supplements.

The characteristics used to describe emulsifying properties of proteins are emulsifying activity index (EAI), and emulsion stability index (ESI).

2.3.4.1 Emulsifying capacity

The emulsifying capacity is the ability of the protein solution or suspension to emulsify oil. Emulsifying properties are useful functional characteristics which play an important role in the development of new sources of plant protein products for uses as foods (Hettiarachchy and Ziegler, 1994; MacRitchie and Lafiandra, 1997; Utsumi, Matsumura and Mori, 1997; Linden and Lorient, 1999). Proteins are the components that dominate in most food emulsions.

Emulsifying capacity (EC) is the amount of oil (mL) that is emulsified under specific conditions by 1 g protein. EAI is maximal emulsion interfacial area (m^2) stabilized per 1 g of protein of a stabilized emulsion (Zayas, 1997). The EAI and EC for a protein emulsifier depends on its ability: (l) to form the adsorption film around the oil globule, and (2) to lower the interfacial tension at the oil-water interface. ES is the capacity of emulsion droplets to remain dispersed without separation by creaming, coalescing, and flocculation.

EAI is usually determined by a turbidimetric method (Pearce and Kinsella, 1978) using the relation:

$$\text{EAI } (m^2/\text{g sample}) = \frac{2T}{c\Phi}$$

$$T = \frac{2.303(A \times \text{dilution factor})}{l}$$

where c is the protein concentration (g/m^3) in the aqueous phase, Φ is the volume fraction of the oil phase, where A is the observed absorbance, l is the path length of the cuvette and T is the turbidity of the emulsion measured at 500 nm. Since, according to Mie theory of light scattering, the turbidity is half the interfacial area of emulsion droplets, EAI is essentially a measure of the interfacial area of emulsion generated per gram of protein.

The factors that affect the emulsifying properties of proteins are similar to those that affect the foaming properties of proteins (Damodaran, 1997; Xiong, 1997; Zayas, 1997); they include the rate of adsorption at the oil-water interface, the amount of protein adsorbed, the extent of conformational rearrangement at the interface, the extent of reduction in interfacial tension, and the ability to form a continuous, cohesive, and viscous film via both covalent (disulfide bonds) and noncovalent interactions.

Emulsifying properties show strong correlation with surface hydrophobicity (Zayas, 1997). Kato and Nakai (1980) have reported a strong correlation between the interfacial tension, EAI, and surface hydrophobicity of various native and heat-denatured globular proteins. The poor correlation between the emulsifying properties and Bigelow's average hydrophobicity indirectly suggests that the adsorbed proteins

at the oil-water interface do not undergo extensive denaturation as they do at the air-water interface (Kato and Nakai, 1980). This is thought to be because the interfacial free energy at the oil-water interface is considerably lower than that at the air-water interface; this lower interfacial energy probably is insufficient to overcome the activation energy barrier for extensive unfolding of proteins at the oil-water interface (Damodaran, 1997).

According to the hydrophobicity mechanism of protein emulsifying capacity, proteins having high surface hydrophobicity are adsorbed at the oil/water interface. Adsorbed proteins reduce the interfacial or surface tension facilitating the formation of emulsions. The concentration of protein at the interface and decrease of the interfacial tension is affected by protein hydrophobicity. The surface hydrophobicity of proteins correlated significantly with an increasing index of emulsifying activity and decreasing interfacial tension (Kato and Nakai, 1980).

2.3.4.2 Emulsion stability

Emulsion stability (ES) is the ability of emulsion droplets to remain dispersed without coalescing, flocculation, or creaming. ES is not a characteristic of maximum oil addition, but rather the ability of the emulsion to remain stable and unchanged. Emulsions with low stability will appear visually as fat separation or creaming, which is caused by flocculation and coalescence. Protein emulsifiers possess the ability to reduce the tendency of the droplets in an emulsion system to coalesce and tendency of the emulsion to break down.

Different procedures have been used to measure ES, but it is commonly measured in terms of the oil and/or cream separating from and emulsion during a certain period of time at a stated temperature and gravitational field (Hill, 1996). The stability of emulsion is usually expressed as emulsion stability index (ESI), which is defined as the time at which the turbidity of the emulsion is half its original value (Voutsinas, Cheung and Nakai, 1983). A common relation for estimating ESI is,

$$\text{ESI (min)} = \frac{T \times \Delta t}{\Delta T}$$

Where ΔT is the change (increase or decrease) in turbidity, T, occurring during the time interval Δt (10 min).

Emulsion stability is affected by the nature, and properties of the interfacial film. Protein stabilized emulsions have thick, hydrated interfacial films and possess a net charge. Thickness, viscosity, cohesiveness, and charge of the layer formed is determined by the nature of the protein and the conditions of emulsification. Stability of emulsion particles is enhanced by hydration of the proteins adsorbed in surrounding film (Zayas, 1997).

2.3.5 Gelation

Protein gel formation is a result of intermolecular interactions resulting in the three dimensional networks of protein structures which develop high structural rigidity. Damodaran (1996b) defined gels as consisting of polymeric molecules cross-linked either covalently or noncovalently to form three-dimensional net works capable of entrapping water and other small molecular weight substances. Zayas (1997) defined a gel as a well hydrated insoluble three-dimensional network in which the protein-protein and protein-solvent interactions occur in an ordered manner. Gelation in food systems has been extensively reviewed by Ziegler and Acton (1984), Clark and Lee-Tuffnell (1986), Hermansson (1994), Matsumura and Mori (1996), Zayas (1997), Oakenfull, Pearce, and Burley (1997), Kim and Park (2000) and Lanier (2000).

Gels are formed when partially unfolded proteins develop uncoiled polypeptide segments that interact at specific points to form a three dimensional cross-linked network. Partial unfolding of proteins with slight changes in secondary structure is required for gelation (Clark and Lee-Tuffnell, 1986). Most globular food proteins form gels upon heating. Because of their chemistry, proteins have many advantages for gelation. Comparing with carbohydrate, proteins are able to form a wider range of cross-links and have a higher nutritional value.

Many different instruments are available for measuring gel strength. A recent advance has been the development of instruments for dynamic testing that rely on computer for recording and analyzing the data. Typically the sample is subjected to

rotational strain between parallel plates or between a cone and plate. The moving cone or plate has an air bearing with very low friction and is rotated by a computer-controlled induction motor; movement is measured optically. Such instruments can measure viscosity versus rate of shear, creep, stress relaxation, and, using oscillation, the bulk modulus and storage modulus, etc. The complex calculations required to produce these values are done by the computer (Oakenfull, Pearce and Burley, 1997).

The parameters of protein network formation which are most often examined by dynamic rheology are the storage modulus (G'), loss modulus (G") and loss tangent or tan-delta (G"/G'). The G' relates to the elastic nature of the material, the G" relates to the viscous nature of the material, and the tan delta denotes the relative energy from the viscous and elastic components (Zayas, 1997).

The storage modulus, G' expresses the magnitude of the energy that is stored in the material or recoverable per cycle of deformation. G" is a measure of the energy that is lost as viscous dissipation per cycle of deformation. Therefore, for a perfectly elastic solid, all the energy is stored; that is, G" is zero and the stress and the strain will be in phase. In contrast, for a liquid with no elastic properties, all the energy is dissipated as heat; that is G' as zero and the stress and the stain will be out of phase by 90°. If G' is much greater than G", the material will behave more like a solid; that is, the deformations will be essentially elastic or recoverable. However, if G" is much greater than G', the energy used to deform the material is dissipated viscously and the material's behavior is liquid-like (Rao, 1999). Joseph, Lanier, and Hamann (1994) studied dynamic rheological properties of actomyosin from Alaska Pollock and Atlantic croaker with the addition of TGase. They reported that TGase enhanced gelation, as indicated by increase in storage modulus (G').

Gelation property of various proteins has been studied, such as milk, whey protein, plant proteins and meat proteins. The gelling capacity of fish proteins in comminuted fish products is one of their most important functional properties.

Sarcoplasmic proteins after heat coagulation contribute to gel formation and the binding structural elements of meat. Considerably more gelation has been obtained with myofibrillar proteins than with sarcoplasmic proteins. Gel formation is influenced by sarcoplasmic proteins to a small extent because these proteins coagulate and precipitate when heated above 40°C (Zayas, 1997). Many researchers reported

heat-stable proteolytic enzymes (An et al., 1994; Lanier, 2000) that are in sarcoplamic proteins, and decrease the gelation of fish myofibrillar proteins. However, Nowsad, Katoh, Kanoh, and Niwa (1995) showed that the sarcoplasmic fraction of fish can actually enhance the gelling ability when added back to surimi because of its higher TGase activity. Karthekeyan et al. (2004) found gelation profile of washed sardine meat in combination with different sarcoplasmic proteins fraction revealed fraction V can enhance the storage modulus values during heating.

Morioka, Kurashima, and Shimizu (1992) studied heat-gelling properties of fish sarcoplasmic proteins (9 fish species) under different conditions. They found gelation was observed at least 10% fresh sarcoplasmic proteins (puncture test), at 60°C or more, pH 7.5-9.0, NaCl concentration up to 2.5% and fish species were different in gelling property. Morioka and Shimizu (1993), described the difference in jelly strength among sarcoplasmic proteins gels is related to a difference in the composition of sarcoplasmic proteins mainly on a high amount of heat-coagulable proteins, especially those of 94, 40 and 26 kDa components of sarcoplasmic proteins.

2.4 Modification of proteins

The effective utilization of proteins in food systems is dependent on tailoring the proteins functional characteristics to meet the complex needs of the manufactured food products. The particular functionality required varies with the food system and application. No single protein can meet all the functional properties required in various foods (Kinsella, 1982). The individual functionality of proteins in food is a result of the physicochemical properties of the proteins and their changes during the processing of foods.

Protein modification is important because it allows an extension of functionality. Protein structure is modified to improve functional properties such as solubility, foaming properties and emulsifying properties and other functional properties of proteins thereby enhancing their effective use as foods and food ingredients. The purpose of modifying the structure of proteins is to create new and

unique products that would possess better functional properties in food systems than the unmodified protein.

Since 1970's, protein modification has been the subject of many reviews; most of the works deal with milk protein the principles described are the same as for fish proteins (Hamada, 1992). Modified whey proteins display a highly variable range of functional properties (Kinsella and Whitehead, 1989; Gauthier et al., 1993; Foegeding et al., 2002) and the modified fish proteins have been studied (Sikorski, 1981; Gomez-Guillen, Javier-Borderias, and Montero, 1996; Gomez-Guillen, De Castro, and Montero, 1997; Huidobro, Alvarez, and Tejeda, 1998; Normah et al., 2004).

Current knowledge related to modification of protein functional properties in food systems has been presented in books by Zayas (1997) and also Yada (2004). In section 2.4, the literature review will be confined only to succinylation and acetylation (chemical) and hydrolysis of protease (enzymatic) modification in proteins.

The objective of chemical derivatization is to alter the non-covalent forces determining protein conformation in a manner that results in desired structural and functional changes. Noncovalent forces of importance in terms of influencing protein conformation include van der Waals forces, electrostatic interactions, hydrophobic interactions, and hydrogen bonds. Proteins that have been modified (acylated) to alter functionality include whey protein (Siu and Thompson, 1982; Kester and Richardson, 1984; Ponnampalam et al., 1988; Phillips and Kinsella, 1990), leaf protein (Franzen and Kinsella, 1976a), soy protein (Franzen and Kinsella, 1976b; Allaoua, Wang, and Xu, 1999), Canola protein (Gruener and Ismond, 1997a, 1997b), mung bean (El-Adawy, 2000), lentil protein (Bora, 2002), oat protein (Ma, 1984) and fish protein (Chen, Richardson, and Amundson, 1975; Pavlova, Damshkaln, and Vainerman, 1991). Mostly the ε amino group of lysine is acylated most readily.

Proteolysis of food proteins yields a mixture of peptides. Functional behavior usually is altered depending on extent of hydrolysis. Enzymatic modification has been studied to improve functional properties of casein (Giardina et al., 2004; Panyam and Kilara, 2004), whey protein (Kester and Richardson, 1984; Althouse, Dinakar and Kinsella, 1995; Mutilangi, Panyam, and Kilara, 1995; Severin and Xia, 2006), soy protein (Qi, Hettiarachchy, and Kalapathy, 1997; Were, Hettiarachchy, and Kalapathy, 1997; Wu, Hettiarachchy, and Qi, 1998; Chan and Ma, 1999), sesame

protein (Bandyopadhyay and Ghosh, 2002), crayfish processing byproduct (Baek and Cadwallader, 1995), as well as fish protein (Diniz and Martin, 1997; Liceaga-Gesualdo and Li-Chan, 1999; Kristinsson and Rasco, 2000a, 2000b).

2.4.1 Chemical and enzymatic modifications of proteins

2.4.1.1 Chemical modification of proteins by acylation

Functional properties of protein are closely related to size of the protein, structural conformation and level and distribution of ionic charges. Chemical treatments that could cause alteration of these properties include reactions which introduce a new functional group to the protein or remove a component part from the protein. Therefore, reactions such as succinylation, acetylation, phosphorylation, limited hydrolysis and specific amide bound hydrolysis (deamidation) have been used to improved functional properties to the protein.

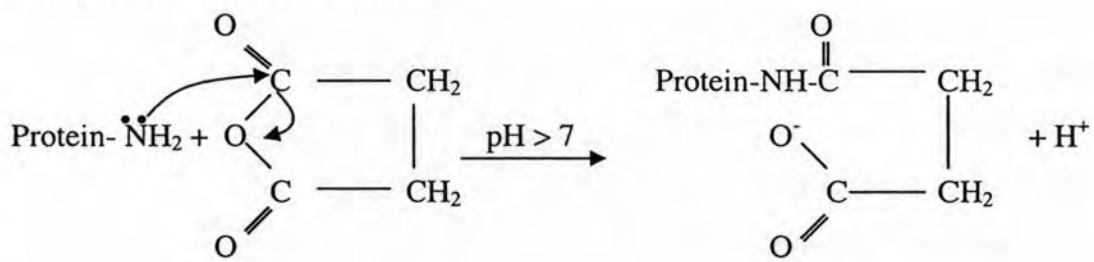
Protein acylation involves the reaction of nucleophilic groups such as the amino or hydroxyl group of the protein with the carbonyl group of an acylating agent. Compared to ε-amino group of lysine, the phenolic group of tyrosine and hydroxyl groups of serine and threonine are weak nucleophiles and they are not easily acylated in aqueous solution. Acylation has been useful in protein characterization and modification. Free amino groups in a protein molecule, especially the ε-amino group of lysine, are often marked by acylation for structure and composition analysis or are blocked to protect them from other reactions. In food processing, protein acylation produced derivatives with new functional groups and, depending on the acylating agent used, alters the physical and chemical properties of the protein. Succinic anhydride and acetic anhydride are the most often used acylating agents.

Succinylation introduces anionic succinate residues to be covalently linked to the ε-amino groups of lysine residues (Figure 2.1). 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 conformational changes. However, acetylation of ε-amino groups of lysine residues renders them electrically natural, whereas, acetylation decreased the number of positive charges by substituting hydrophobic acetyl groups for the

positively charged ε-amino groups (El-Adawy, 2000). The increase in negative charge of proteins caused by neutralizing amino groups via acetylation or by introducing additional carboxyl groups via succinylation may result in a drastic change in the native protein conformation. Such changes are more pronounced for succinylated proteins than for acetylated proteins (Schwenke, 1997).

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 gives 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.

Succinylation with succinic acid



Acetylation with acetic acid

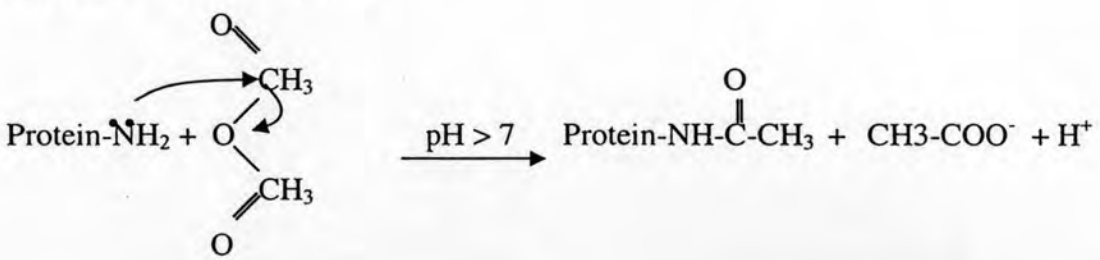


Figure 2.1 Reaction of succinic and acetic anhydrides with the ε-amino group of lysine (modified from El-Adawy, 2000).

Acylated proteins are generally more soluble than native proteins. 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 decreases 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 can sometimes reduces their adsorptivity 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 the parent protein (Damodaran, 1996a)

Much attention has been given to the effects of acylation on protein digestibility and nutritional value. Acetylation and succinylation reactions are irreversible. The succinyl-lysine isopeptide bond is resistant to cleavage catalyzed by pancreatic digestive enzymes. Furthermore, succinyl-lysine is poorly absorbed by the intestinal mucosa cells. Thus, succinylation and acetylation greatly reduce the nutritional value of proteins (Damodaran, 1996a). 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.

2.4.1.2 Enzymatic modification of proteins

Many enzymic methods for the modification of food proteins have been reported including treatment with transglutaminases, deaminases, and proteases (Hamada and Marshall, 1992; Nowsad, Kanoh, and Niwa, 1995; Kristinsson and Rasco, 2000a, 2000c; Truong et al., 2004; Severin and Xia, 2006). Enzymatic hydrolysis of proteins using proteases accompanies by three distinct effects: (1) a decrease in molecular weight, (2) an increase in the number of ionizable groups, and (3) the exposure of hydrophobic groups (Panyam and Kilara, 1996). The functional properties of proteins are changed by direct result of these effects. Food-grade proteolytic enzymes, which can be of animal, bacterial, fungal or plant origin, and may have different pH and temperature optima, hydrolyze a variety of peptide bonds. Depending on the specificity of the enzyme, environmental conditions and extent of hydrolysis, a wide variety of peptides are generated. The resultant protein hydrolysate contains low molecular weight peptides as well as higher molecular weight peptides and unhydrolyzed proteins.

Hydrolysis of food proteins using proteases, such as pepsin, trypsin, chymotrypsin, papain and thermolysin, alters their functional properties (Chobert,

Sitohy, and Whitaker, 1988; Mutilangi, Panyam and Kilara, 1996; Chen and Ma, 1999; Liceaga-Gesualdo and Li-Chan, 1999; Kristinsson and Rasco, 2000c; Bandyopadhyay and Ghosh, 2002; Severin and Xia, 2006). Extensive hydrolysis by nonspecific proteases, such as papain, causes solubilization of even poorly soluble proteins (Were, Hettiarachchy, and Kalapathy, 1997; Bandyopadhyay and Ghosh, 2002; Wu, Hettiarachchy, and Qi, 1998). Such hydrolysates usually contain low molecular weight peptides of the order of two to four amino acid residues. Extensive hydrolysis damages several functional properties, such as gelation, foaming, and emulsifying properties. These modified proteins are useful in liquid-type products, such as soups and sauces, digest solid foods. Partial hydrolysis of proteins either by using site-specific enzyme (such as trypsin or chymotrypsin) or by control of hydrolysis time often improves foaming and emulsification properties, but not gelling properties. With some proteins, partial hydrolysis may cause a temporary decrease in solubility because of exposure of buried hydrophobic regions (Damodaran, 1996a).

Mutilangi, Panyam, and Kilara (1996) studied heat-denatured whey protein isolate hydrolyzed by trypsin, α-chymotrypsin, Alcalase® and Neutrase® with 2.8, 4.3, 6.0 and 8.0% DH. Hydrolysates were fractionated by ultrafiltration and freeze-dried. They reported that surface hydrophobicity increased with hydrolysis. Specificity of the enzyme, and degree of hydrolysis influenced the functional properties of the peptides.

Functionality changes are best observed following a controlled or limited hydrolysis (Panyam and Kilara, 1996). Protein hydrolysates that tend to be used in nutritional formulations are hydrolyzed to a greater extent, and are classified as slightly, moderately or extensively hydrolyzed, depending on the molecular weight distribution of the resultant hydrolysate. Extensive hydrolysis is normally used to produce hypoallergenic hydrolysates, with no peptides > 5000Da and almost 90% of them < 500Da. Hydrolysates used as protein supplements may undergo less-extensive hydrolysis (Mahmoud, 1994). Whey protein hydrolysate (WPH) produced by enzymatic hydrolysis has been on the market for many years. They are thought to possess excellent nutritional value and functional properties. WHP are used for, improved heat stability, reduced allergenicity, production of bioactive peptides,

tailoring amounts and size of peptides for special diets and altering the functional properties of gelation, foaming and emulsification (Panyam and Kilara, 1996).

2.4.2 Properties of modified proteins

Fundamental principles of protein hydrophobicity and surface hydrophobicity are presented in section 2.3.3.1 of this literature review. This section reviews past work on the effect of chemical modification and protease digestion on food protein functionality.

2.4.2.1 Hydrophobicity of modified proteins

Yamauchi et al. (1979) and Seifert and Schwenke (1995) reported that hydrophobicity and acylation may result in aggregation of the modified proteins. Wu, Hettiarachchy, and Qi (1998) reported that partial hydrolysis by papain of soy protein isolate, significantly increased surface hydrophobicity, solubility, emulsion properties (EAI and ESI). In a native protein molecule, hydrophobic groups are buried inside the core of the folded structure. After partial hydrolysis, some of these groups are exposed, resulting in increased surface hydrophobicity. Many food proteins have been subjected to limited proteolysis, including whey protein (Althouse, Dinakar, and Kilara, 1995; Mutilangi, Panyam, and Kilara, 1995, 1996; Foegeding et al., 2002), crayfish processing byproduct (Baek and Cadwallader, 1995) and fish protein (Kristinsson and Rasco, 2000b). Maybe moderate hydrolysis increases surface hydrophobicity by exposing internal groups, but excessive hydrolysis to form small peptides leads to net decrease.

2.4.2.2 Solubility of modified proteins

The solubility of protein depends on many factors as described earlier in section 2.3.1. Bora (2002) reported that succinylated proteins exhibit a shift in their isoelectric pH, thus resulting in an enhanced solubility at neutral to alkaline pH. Similar observation was also made by Franzen and Kinsella (1976a, 1976b). They studied functional properties of succinylated and acetylated leaf protein and soy protein. The solubility of proteins was markedly improved by succinylation. They

also found that the effects of pH on emulsifying and foaming properties of succinylated proteins paralleled theirs effects on protein solubility. However, acetylation of proteins caused negligible changes in the functional properties studied.

Gruener and Ismond (1997b) examined effects of acetylation and succinylation on the functional properties of canola 12S globulin. They reported that protein solubility below the isoelectric point was decreased, but solubility at neutral to alkaline pH values was greatly enhanced. Bora (2002) evaluated the functional properties of native and succinylated lentil globulins. He found that succinylation caused a shift on the pI of native globulins from 4.5 to 3.5 and improved the solubility above pH 4.0. However, below this pH the solubility of succinylated globulins was reduced.

Effect of enzymatic hydrolysis on solubility properties of proteins was observed (Chobert, Bertrand-Harb, and Nicolas, 1988; Mutilangi, Panyam, and Kilara, 1996; Chan and Ma, 1999; Kristinsson and Rasco, 2000c). Many studies on protein hydrolysates reported an increasing solubility with increasing degree of hydrolysis (DH). 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, 2000b).

Intact fish proteins are commonly believed to lack solubility in water (Venugopal and Shahidi, 1994). Kristinsson and Rasco (2000c) revealed that 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.

2.4.2.3 Foaming properties of modified proteins

Phillips and Kinsella (1990) examined effect of succinylation on food protein foaming properties using beta-lactoglobulin as model. The foaming properties of moderately succinylated beta-lactoglobulin were higher than native beta-lactoglobulin. Gruener and Ismond (1997b) examined effects of acetylation and succinylation on the functional properties of canola 12S globulin. They found that

foam capacity was significantly increased by acylation, but foam stability decreased significantly upon acylation. El-Adawy (2000) studied functional properties of acetylated and succinylated mung bean protein isolate. He reported that foam capacity and foam stability were increased due to acylation. Bora (2002) evaluated the functional properties of native and succinylated lentil globulins. He found that foaming capacity of the succinylated globulins was decreased slightly, while foam stability was extensively reduced. Succinylated globulins showed maximum foam stability at pH 2.5, although native was pH 3.5.

Were, Hettiarachchy, and Kalapathy (1997) examined effect of protein digestion on foaming. Digestion of soybean protein by papain enhanced foaming stability. Liceaga-Gesualdo and Li-Chan (1999) tested foamability and foam stability of endopeptidase modified herring hydrolysates. They found at 36% DH, the herring hydrolysate presented an adequate foam expansion as compared to the soluble fraction from the unhydrolyzed control herring. Althouse, Dinakar, and Kilara (1995) examined modification of whey protein isolate by proteolytic enzyme. They found that permeate from Alcalase exhibited the best foaming characteristics, comparable to egg white. However, Chan and Ma (1999) determined trypsin modification soymilk residue (okara) protein isolate (OPI). They reported that trypsin hydrolysis of OPI led to decreases in foamability and foam stability.

2.4.2.4 Emulsifying properties of modified proteins

Gruener and Ismond (1997b) examined effects of acetylation and succinylation on the functional properties of canola 12S globulin. They reported that emulsion stability significantly increased initially, and decreased at the highest levels of acylation. For oat proteins, emulsion activity was increased by succinylation. Similarly, the emulsion stability was also improved.

Effect of protease hydrolysis on emulsification has been examined for different proteins. Wu, Hettiarachchy, and Qi, (1998) studied papain modification of SPI and found that significantly improved the EAI. The correlation between surface hydrophobicity and EAI for the unmodified SPI, hydrolysates, and retentate was high, but for the unmodified SPI, hydrolysates retentate, plus permeates it was low. They concluded the low surface hydrophobicity of permeates cannot be credited for their

high emulsifying activity. Therefore, solubility and molecular size, rather than surface hydrophobicity, might be the major factors for the high emulsifying activity of the small peptides. Qi, Hettiarachchy, and Kalapathy (1997) examined SPI with varying DH produced using pancreatin. EAI of pancreatin modified SPI increased with increasing DH. These results showed that 15% DH had highest EAI, but ESI decreased with increasing DH.

Liceaga-Gesualdo and Li-Chan (1999) investigated emulsion properties of endopeptidase modified Herring hydrolysates. They reported at 36% DH, the herring hydrolysate presented good emulsifying stability.

2.4.2.5 Gelation of modified proteins

Gruener and Ismond (1997b) studied the effects of acetylation and succinylation on the gelation of the canola 12S globulin by measuring G' and G" values using small amplitude oscillatory rheology. They found an increase in G' signify an increase in gel strength, and in the protein-protein interactions. 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).

Miller and Groninger (1976) and Ahmad (1991), found that enzymatic hydrolysis does not increase 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. Hydrolysis was assumed to be destructive to the gelling properties of proteins because of the reduced hydrophobicity of hydrolysates (Mahmoud, 1994). The increased net charge on the proteins results in increased charge repulsion between peptides, decreasing gelling ability. 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).