CHAPTER 2

LITERATURE REVIEW

Sources of proteases

Proteases are found in a diversity sources such as plants, animals, and microorganisms. The disadvantage of the use the plant as source of protease was the availability of land for cultivation and the suitability of climatic conditions for growth. Moreover, production of proteases from plants is a time-consuming process. Papain, bromelain, keratinases, and ficin represent some of the well-known proteases of plant origin. The most familiar proteases of animal origin are pancreatic trypsin, chymotrypsin, pepsin, and rennins. Proteases from microbial sources are preferred to the enzymes from plant and animal sources since they possess almost all the characteristics desired for their biotechnological applications. Microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Organisms belonging to the genus *Bacillus* produce most commercial proteases, mainly neutral and alkaline. Bacterial neutral proteases are active in a narrow pH range (pH 5.0 to 8.0) and have relatively low thermotolerance (Rao, *et al*., 1998). The bacterial neutral proteases are characterized by their high affinity for hydrophobic amino acid pairs. Their low thermotolerance is advantageous for controlling their reactivity during the production of food hydrolyzates with a low degree of hydrolysis. Some of the neutral proteases belong to the metalloprotease type and require divalent metal ions for their activity, while others are serine proteinases, which are not affected by chelating agents. Bacterial alkaline proteases are characterized by their high activity at alkaline pH, e.g., pH 10.0, and their broad substrate specificity. Their optimal temperature is around 60 °C. These properties of bacterial alkaline proteases make them suitable for use in the detergent industry. The protease produced by fungi, such as *Aspergillus oryzae* produces acid, neutral, and alkaline proteases. The fungal proteases are active over a wide pH range (pH 4.0 to 11.0) and exhibit broad

substrate specificity. However, they have a lower reaction rate and worse heat tolerance than do the bacterial enzymes. Fungal enzymes can be conveniently produced in a solid-state fermentation process. Fungal acid proteases have an optimal pH between 4 and 4.5 and are stable between pH 2.5 and 6.0. They are particularly useful in the cheese making industry due to their narrow pH and temperature specificities. Fungal neutral proteases are metalloproteases that are active at pH 7.0 and are inhibited by chelating agents (Rao, *et al.*, 1998). In view of the accompanying peptidase activity and their specific function in hydrolyzing hydrophobic amino acid bonds, fungal neutral proteases supplement the action of plant, animal, and bacterial proteases in reducing the bitterness of food protein hydrolyzates. Fungal alkaline proteases are also used in food protein modification.

Classification of protease

Proteases are classified on the basis of three major criteria: (i) type of reaction catalyzed, (ii) chemical nature of the catalytic site, and (iii) evolutionary relationship with reference to structure (Barett, 1994). Proteases are subdivided into two major groups, i.e., exopeptidases and endopeptidases, depending on their site of action. Exopeptidases cleave the peptide bond proximal to the amino or carboxy termini of the substrate, whereas endopeptidases cleave peptide bonds distant from the termini of the substrate. Based on the functional group present at the active site, proteases are further classified into four prominent groups, i.e., serine proteases, aspartic proteases, cysteine proteases, and metalloproteases (Hartley, 1960).

1. Exopeptidases

The exopeptidases act only near the ends of polypeptide chains. Based on their site of action at the N or C terminus, they are classified as amino- and carboxypeptidases, respectively. Aminopeptidases act at a free N terminus of the polypeptide chain and liberate a single amino acid residue, a dipeptide, or a tripeptide.

Aminopeptidases occur in a wide variety of microbial species including bacteria and fungi. In general, aminopeptidases are intracellular enzymes, but there has been a single report on an extracellular aminopeptidase produced by *A. oryzae* (Labbe *et al.,* 1974). The substrate specificities of the enzymes from bacteria and fungi are distinctly different in that the organisms can be differentiated on the basis of the profiles of the products of hydrolysis (Cerny, 1978). Aminopeptidase I from *Escherichia coli* is a large protease (400,000 Da). It has a broad pH optimum of 7.5 to 10.5 and requires Mg^{2+} or Mn^{2+} for optimal activity (De Marco and Dick, 1978). The *Bacillus licheniformis* aminopeptidase has a molecular weight of 34,000. It contains 1 g-atom of Zn^{2+} per mol, and its activity is enhanced by Co^{2+} ions. On the other hand, aminopeptidase II from *B. stearothermophilus* is a dimmer with a molecular weight of 80,000 to 100,000 (Stoll *et al.*, 1976) and is activated by Zn^{2+}, Mn^{2+}, or Co^{2+} ions. The activity of carboxy and aminopeptidases of enzymic preparation obtained from bacteria *Bacillus subtilis, Bacillus mesenteroides, Pseudomonas aeruginosa, Bacillus thermophilus,* mould *Aspergillus oryzae, A. flavus* and actinomycetes *Streptomyces griseus* were studied. The bacterial preparations the proteolytic activity was 1.5-2.5 times than in the fungal and actinomycetes preparations. The carboxypeptidase activity of bacterial preparations was comparable with that of the mould lower than that actinomycete aminopeptidase activity of the bacterial preparations was different. It was comparable with that of the moulds and was higher than that case. In the *Streptomyces* preparation the aminopeptidase activity significantly higher than in the bacterial and fungal preparations. The carboxypeptidases act at C terminals of the polypeptide chain and liberate a single amino acid or a dipeptide. Carboxypeptidases can be divided into three major groups, serine carboxypeptidases, metallocarboxypeptidases, and cysteine carboxypeptidases, based on the nature of the amino acid residues at the active site of the enzymes. The serine carboxypeptidases isolated from *Penicillium* sp., *Saccharomyces* sp., and *Aspergillus* sp. are similar in their substrate specificities but differ slightly in other properties such as pH optimum, stability, molecular weight, and effect of inhibitors. Metallocarboxypeptidases from *Saccharomyces* sp. (Felix and Brouillet, 1966) and *Pseudomonas* sp. (Lu, *et al.,* 1969) require Zn^{2+} or Co^{2+} for protease activity. The enzymes can also hydrolyze the peptides in which the peptidyl group is replaced by acyl groups.

2. Endopeptidases

Endopeptidases are characterized by their preferential action at the peptide bonds in the inner regions of the polypeptide chain away from the N and C termini. The presence of the free amino or carboxyl group has a negative influence on enzyme activity. The endopeptidases are divided into four subgroups based on their catalytic mechanism, (i) serine proteases, (ii) aspartic proteases, (iii) cysteine proteases, and (iv) metalloproteases. Serine proteases are characterized by the presence of a serine group in their active site. Serine proteases are found in the exopeptidase, endopeptidase, oligopeptidase, and omega peptidase groups. Aspartic acid proteases, commonly known as acidic proteases, are the endopeptidases that depend on aspartic acid residues for their catalytic activity. Cysteine proteases occur in both prokaryotes and eukaryotes. About 20 families of cysteine proteases have been recognized. The activity of all cysteine proteases depends on a catalytic site consisting of cysteine and histidine. The order of Cys and His (Cys-His or His-Cys) residues differ among the families (Barett, 1994). Metalloproteases are the most diverse of the catalytic types of proteases (Barette, 1995). They are characterized by the requirement for a divalent metal ion for their activity. They include enzymes from a variety of origins such as collagenases from higher organisms, hemorrhagic toxins from snake venoms, and thermolysin from bacteria (Hibbs *et al.*, 1985, Okada *et al.*,1986). Based on the specificity of their action, metalloproteases can be divided into four groups, (i) neutral, (ii) alkaline, (iii) *Myxobacter* I, and (iv) *Myxobacter* II. The neutral proteases show specificity for hydrophobic amino acids, while the alkaline proteases possess a very broad specificity. *Myxobacter* protease I is specific for small amino acid residues on either side of the cleavage bond, whereas protease II is specific for lysine residue on the amino side of the peptide bond. All of them are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP. Elastase produced by *Pseudomonas aeruginosa* is another important member of the neutral metalloprotease family. The alkaline metalloproteases produced by *Pseudomonas aeruginosa* and *Serratia* sp. are active in the pH range from 7.0 to 9.0 and have molecular masses in the region of 48 to 60 kDa. *Myxobacter* protease I has a pH optimum of 9.0 and a molecular mass of 14 kDa (Rao, *et al*., 1998).

Proteases are broadly classified as endo- or exoenzymes on the basis of their site of action on protein substrates. They are further categorized as serine proteases, aspartic proteases, cysteine proteases, or metalloproteases depending on their catalytic mechanism. They are also classified into different families and depending on their amino acid sequences and evolutionary relationships. Based on the pH of their optimal activity, they are also referred to as acidic, neutral, or alkaline proteases.

Mechanism of action of proteases

Purification of proteases to homogeneity is a prerequisite for studying their mechanism of action. serine proteases usually follow a two-step reaction for hydrolysis in which a covalently linked enzyme-peptide intermediate is formed with the loss of the amino acid or peptide fragment (Fastrez and Brouilet, 1973). This acylation step is followed by a deacylation process, which occurs by a nucleophilic attack on the intermediate by water, resulting in hydrolysis of the peptide. Aspartic endopeptidases depend on the aspartic acid residues for their catalytic activity. A general base catalytic mechanism has been proposed for the hydrolysis of proteins by aspartic proteases such as penicillopepsin (James *et al*., 1992) and endothiapepsin (Pearl, 1987). The mechanism of action of metalloproteases is depend on the presence of bound divalent cations and can be inactivated by dialysis or by the addition of chelating agents. For thermolysin, based on the X-ray studies of the complex with a hydroxamic acid inhibitor, it has been proposed that Glu 143 assists the nucleophilic attack of a water molecule on the carbonyl carbon of the scissile peptide bond, which is polarized by the Zn^{2+} ion (Holmes and Matthews, 1981). Most of the metalloproteases are enzymes containing the His-Glu-Xaa-Xaa-His (HEXXH) motif, which has been shown by X-ray crystallography to form a part of the site for binding of the metal, usually zinc. Cysteine proteases catalyze the hydrolysis of carboxylic acid derivatives through a double-displacement pathway involving general acid-base formation and hydrolysis of an acyl-thiol intermediate. The mechanism of action of cysteine proteases is thus very similar to that of serine proteases. Studies of the mechanism of action of proteases have revealed that they exhibit different types of mechanism based on their active-site configuration. The

serine proteases contain a Ser-His-Asp catalytic triad, and the hydrolysis of the peptide bond involves an acylation step followed by a deacylation step. Aspartic proteases are characterized by an Asp-Thr-Gly motif in their active site and by an acid-base catalysis as their mechanisms of action. The activity of metalloproteases depends on the binding of a divalent metal ion to a His-Glu-Xaa-Xaa-His motif. Cysteine proteases adopt a hydrolysis mechanism involving a general acid-base formation followed by hydrolysis of an acyl-thiol intermediate (Rao *et al*., 1998).

Isolation of salt tolerant bacteria

Salt tolerant (halotolerant) microorganisms are found in highly saline environments and considered to be extremophiles. An extremophile can live in extreme habitats, which are characterized by high or low temperature, high or low pH, and high salinity. Several extremophilic microorganisms are used in biotechnological processes, for example halobacteria are used for the production of bacteriorhodopsin, the alga *Dunaliella* is used in commercial production of β-carotene, including the production of polymers (poly hydroxyalcanoates and polysaccharides), enzymes and compatible (Ventosa and Nieto, 1995). Salt tolerant microorganisms can be isolated from various types of hyper saline environment, such as salt lakes and fermented food products (fermented oriental soy products, fermented fishery products and fermented sea food products). A halotolerant strain of *Bacillus licheniformis* was isolated from marine sediments (Manachini and Fortina, 1998). The isolation and screening of halophilic bacteria from different hypersaline environments in South Spain were obtained total 122 moderately halophilic bacteria able to produce different hydrolase (amylases, Dnases, lipases, proteases and pullanases). These bacteria are able to grow optimally in media with 5-15% salts and in most cases up to 20-25 % salts. These strains were identified as members of the genera: *Salinivibrio*, *Halomonas*, *Chromohalobacter*, *Bacillus*, *Salibacillus*, *Salinicoccus* and *Marinococcus* (Porro *et al.*, 2003).

Salt tolerant bacteria can grow in presence of high salt concentration (up to 2.5 M) because they accumulate intracellular concentration of organic compounds called “compatible solutes” that are responsible for the osmotic balance of the cell and also compatible with the cellular metabolism. These osmolytes are sugars

(trehalose and sucrose) amino acids (glycine, alanine, proline, etc.) polyols (glycerol, manitol, sorbitol) betaine and ectoines (ectoine and hydroyectoine) (Ventosa and Nieto, 1995). The change of lipid composition of *Zygosaccharomyces rouxii* after transfer from NaCl-free to 2 M NaCl medium the sterol and phospholipid contents increased, and phospholipid had a higher oleic acid content. These changes in membrane lipid composition may be required for the adaptation of *Z. rouxii* cells to a high NaCl environment (Watanabe and Takakuwa, 1987). Cummings and Russell (1996) found that when three halotolerant bacteria that isolated from raw, olive- mill waste-water were grown in tryptic soy broth (TSB), two responded to lowering of water activity (aw) by addition of NaCl or sucrose in the expected manner: by increasing the proportion of membrane anionic lipids diphosphatidylglycerol or phosphatidylglycerol. In contrast, the third isolate did not alter its membrane phospholipid composition significantly in response to growth in NaCl, whereas in sucrose there was an increase in phosphatidylethanolamine. Quantitative and qualitative differences in compatible solute composition were observed when the three isolates were grown in TSB with NaCl or sucrose added to lower water activity. The major compatible solutes in two of the isolates were proline and betaine, whereas in the third they were proline, betaine and ectoine; one isolate also contained some trehalose when grown in TSB with NaCl but not accumulated trehalose when grown in TSB with sucrose.

The isolation of Genus *Geobacillus* from various source were reported. Two proteolytic thermophilic aerobic bacterial strains, PA-9 and PA 5 that isolated from Buranga hot springs in Western Uganda was identified as genus *Geobacillus* sp. by 16S rRNA. Both isolates grew optimally at pH 7.5-8.5. The crude enzyme showed optimum temperature and pH for casein degradation was at 70 °C, pH 6.5., PA-9. The isolate PA-5 activity was observed over a temperature and pH range of 50-70 °C and pH 5-10, respectively (Hawumba *et al*, 2002). *Geobacillus tobii*, a thermophilic, spore-forming rod, aerobic, gram positive, motile isolated from hay post in Korea. Growth of the isolate was observed at 45-70 °C and pH 6.0-9.0 (optimum pH 7.5) (Sung *et al.*, 2002). The halotolerant and thermophilic *Geobacillus* strains isolated from marine hydrothermal vents of Eolian Islands (Italy). The strains were only moderately related to species of *Geobacillus* and their relative, members of *Saccharococcus*. The ability of these three novel strains were

produce exopolysacchride and also able to utilize hydrocarbons, oil, kerosene and mineral lubricating oil (Maugeri and Gugliandolo, 2002). The new species of *Geobacillus*, K2T, thermophilic spore-forming bacteria was isolated biofilm on the surface of a corroded pipeline in an extremely deep (4680 m, 40-72 °C) in Ural. They grow on a complex medium with tryptone and yeast extract and on a synthetic medium with glucose and mineral salt additional growth factors. The optimum growth at 65 °C and at pH 5-9 (Popova *et al.*, 2002). Thermophilic bacterium *Geobacillus uralicus*, isolated from ultradeep weel, was grown at temperature from 40 to 75 °C and pH from 5 to 9 (Panikov *et al.*, 2003). *Geobacillus thermocatenulatus*, thermophillic bacterium, degrading nylon having a growth optimum at 55 °C (Tomita *et al.*, 2003). A total of 53 thermophilic, aerobic sporulating and non-sporulating bacteria were isolated from subsurface sample of environment in Northern Ireland represent to be species of genus *Geobacillus* (Rahman, *et al*., 2004). Thermophilic bacteria, *Geobacillus stearothermophilus* that isolated from Irish soil and man made thermophilic biotopes (McMullan *et al*., 2004). Thermophilic stains of *Geobacillus kautophilus* and *G. stearother-mophilus* isolated from the deepest mud of the MaTrench were identified by using 16S rRNA sequences and DNA-DNA relatedness (Takami *et al.*, 2004). The thermophilic strain, T4, *Geobacillus thermoleovorans* was isolated sugar refinery wastewater in southern Taiwan (Tai *et al*., 2004).

The protease production from bacteria

Bacillus sp. could produce alkaline protease such as *Bacillus licheniformis* (Manachini and Fortina, 1998), *Bacillus sphaericus* (Singh, *et al*., 1999) *Bacillus stearothermophilus* (Fujio and Kume, 1991; Dhandapani and Vijayaragavan, 1994), *Bacillus subtilis* (Hameed, *et al.*, 1996) and neutral protease produced by *Bacillus licheniformis* (Tanskul and Trirattanakul, 1998). The optimization condition for high yield of protease production was reported. The productions of protease using synthetic media are available recently. The synthetic media provides better control and monitoring, improve product recovery and quality, and simplified purification systems than complex media. The complex nitrogenous substances support neutral protease production of *Pseudomonas aeruginosa*. However higher concentration of

casamino acids suppressed the synthesis (Nigam *et al.*, 1981). Oh, *et al*. (2000) studied the various ingredient of C-source (glucose, lactose, carboxy methylcellulose, D (-) arabinose, D (+) xylose, cellulose, rice bran) on protease production of *Pseudomonas aeroginosa* when grown in shrimp and crab shell powder (SCSP) medium (minimal synthetic medium (MSM) added 5 % (SCSP). The protease production was slightly enhanced by the addition of carboxymethyl cellulose, lactose or rice bran into medium. One percent of lactose was the most effective substrate for protease production. The effects of different nitrogen sources on the protease production were tested in a medium (MSM + 5 % SCSP + 1 % lactose) containing yeast extract, polypeptone, beef extract, sodium L-glutamate, sodium nitrate, or ammonium nitrate. The secretion of protease was slightly enhanced by most of nitrogen sources tested with the exception of polypeptone and beef extract, which repressed protease production. The concentration effects of nitrogen sources were studied, 0.5 % ammonium nitrate was most effective for protease production. The optimum protease production from *Pseudomonas aeroginosa* was observed when 0.5 % $FeSO_4.7H_2O$ was supplement but copper sulfate, magnesium sulfate and sodium chloride inhibited protease production. But the protease production of *Pseudomonas fluorescens* in the medium containing sodium caseinate and supported good growth and protease production. Asparagine was the most effective amino acid inducer for protease production (Fairbairn and Law, 1987). The highest protease activity of *Bacillus subtilis* strain 103 was obtained on the medium containing maltose or hydrolyzes starch as carbon source, monopotassium phosphate as a phosphorus source ammonium sulphate as a nitrogen source. The addition of albumin, peptone or casein, methionine, isoleucine and valine stimulated the enzyme synthesis (Kaluniants *et al*., 1979). And 10 g/l of casein was the best nitrogen sources for alkaline protease production by *Bacillus subtilis* (Massucco *et al*., 1980). The maximum synthesis of alkaline protease had obtained in the media containing starch or its hydrolyzates dextrin, maltose as the carbon source. Ammonium phosphate and casein were to be the optimal nitrogen source for *Bacillus mesentericus* and *Bacillus subtilis.* Complex B vitamins added to the nutrient medium could increased the enzyme synthesis 2.5-4 fold (Emtseva, 1975). Pornsettakul (1991) optimized the growth condition of *Bacillus sp.* for the highest protease activities. It was found that the addition of 0, 6 and 12 % NaCl to

the medium in shake flask fermentation could give the highest protease activity of 52 (69 h), 132 (63 h) and 88 (69 h) units/ml, respectively. The incubation temperature of 35 °C and agitation rate of 700 rpm in 2.5 liters jar fermenter gave the highest protease activity of 210 units/ml. The proteolytic activity produced by a *Bacillus subtilis* isolated from hot spring showed maximum protease production after 38 h of fermentation. Starch and peptone was the best inducers for maximum protease production (Kembhavi *et al.*, 1993). The optimum temperature for highest yield of protease production of *Bacillus licheniformis*, isolated from hot spring in Southern Thailand was 50 °C (Tanskul and Trirattananukul, 1998). High level of extracellular protease by *Bacillus* sp. production on soybean flour, casein and starch but low levels on carboxymethylcelluloase, pectin polypectate and polygalacturonate (Mahmood *et al.,* 2000). Phenylalanine was one of inducers for proteolytic enzymes, extracellular protease, an endopeptidase and aminoeptidase by marine bacterium *Vibrio* SA1 (Leeuwenhoek, 1978).

The purification and characterization of protease and aminopeptidase from bacteria

The purification procedures of proteases, involving affinity chromatography, ion-exchange chromatography, and gel filtration techniques, have been well documented. Khaziev *et al.* (2002) studied the effectiveness of methods for purification of metalloprotease produced by *Bacillus subtilis* strain 3H in different nutrient media. The use of the initial material obtained in nutrient medium made it possible to obtain proteolytic enzyme with highest specific activity by methods of salting out, gel and ion exchange chromatography, but the use of these methods on a production the deterioration of purify characteristics. Changes in the nutrient composition used for the cultivation of the production strain results greater effectiveness of the purified methods. Pornsettakul (1991) reported the optimal pH and temperature for protease activity of crude enzyme from *Bacillus* sp. were 7.0 and 40 °C, respectively. The protease was stable from pH 6 to 9 at room temperature for 24 hours, and stable at temperature from 30 to 40 °C for 60 min. Enzyme stability decreased with the increase in NaCl, temperature and time. Choorit and Prasertsan (1992) found that the optimal pH and temperature for highest protease

activity produced by *Bacillus* sp. were between 7.0-8.0 and 55 °C, respectively. The optimum condition for protease production in *Bacillus latersporus* was pH 8.0 and 60 °C, respectively (Sharma, *et al*., 1996). For metalloprotease of *Bacillus megaterium* was not inhibited by diisopropyl-fluorophosphate but inhibited by o-phenanthroline and EDTA, and is activated by Co^{2+} the maximum of enzyme activity at pH 6.5-7.0. The enzyme is stable at pH 7.0 retains its stability at 45-60 °C for several hours. The metllaloprotease hydrolyzes synthetic peptide substrates at the formed by the aminogroups of hydrophobic amino acids Phe, Leu, Val (Morozova *et al.*, 1993). Koka and Weimer (2000) purified acid protease from *Pseudomonas fluorescens* RO98 by anion exchange and gel filtration chromatography. The purified protease was active between 15 and 55 °C, and pH 9.0. Molecular weight of the enzyme was estimated to be 52 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and size exclusion chromatography. Values for Km of 144.28, 18.73 and 35.23 μmol were obtained for whole, α-,β and K-casein, with K-casein preferentially when incubated with artificial casein micelles. Oh, *et al*. (2000) purified protease from *Pseudomonas aeruginosa* K-187 by ammonium sulfate precipitation, DEAE-Sepharose CL-6B ion-exchange chromatography, and Sephacryl S-200 gel-permeation chromatography. The enzyme had a molecular weight estimated to be 58.8 kDa by using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The purified enzyme was active from pH 7.0 to 9.0 and its optimal pH was 8.0. They found that the highest protease activity of *P. aeruginosa* K-187 was as high as 21.2 U/ml, 10-fold that (2.2 U/ml) obtained prior to optimization. Kim *et al.* (2001) purified and characterized of protease from *Bacillus cereus* KCTC 3674. The 38 kDa and 36kDa that cultivated at 37 °C and 20 °C, respectively were purified. The 38 kDa and 36 kDa protease were identified as an extracellular metalloprotease. The 36 kDa shown to be the novel enzyme based on its N-terminal amino acid sequence, its identification as a metallo-enzyme that was strongly inhibited by EDTA and o-phenathroline, and its optimal pH and temperature for activity of 8.0 and 70 °C, respectively. Chantawannakul *et al.*, (2002) studied the protease of *Bacillus subtilis* strain 38 isolated from traditionally fermented soybean in Northern Thailand. The protease showed optimal pH and temperature at 6.5 and 47 °C, respectively. The protease was unstable at 60 °C. The enzyme activity was inhibited by 1, 10-phenanthroline indicating the presence

metlloprotease. The protease from moderated halophilic bacteria, *Pseudomonas* sp., was reported. The purified enzyme showed the molecular weight to be 120,000 Da. The optimum pH for acitivity at 8.0 and maximal activity at 18 % NaCl. The enzyme was activated by Mg^{2+}, Co^{2+} and Ca^{2+}, and heavy metal ion such as Fe^{2+}, Cu^{2+} and Hg^{2+} inactivated the enzyme. Thiol reagents and diisopropyl fluorophosphate did not affect the enzymatic activity of the protease. Metal reagent, ethylenediaminetetraacetic acid endo-phenanthroline, inhibited enzymatic activity, although citrate and oxalate did not affect it (Qua *et al.*, 1981). Oh *et al.* (2000) optimized condition for highest protease activity from *Pseudomonas aeruginosa* K-187. They found that the bacteria could produced higher protease activity when the initial pH and temperature were 8.0 and 25 °C, respectively. The protease activity from *Pseudoalteromonas* sp. strain CP76 showed maximal protease production at the end of exponential growth phase. The optimal protease activity at 55 °C, pH 8.5 and high tolerant a wide range of NaCl concentrations (0-4 M NaCl) and optimal activity at 7.5 % total salts. The purified protease has a molecular mass 38 kDa. The protease activity was showed metlloprotease, strongly inhibited by EDTA and PMSF. No significant inhibition was detected with E-64, bestatin, chymostatin, or leupeptin (Porro *et al.,* 2003). Kamekura and Seno (1990) reported halophilc extracellular protease from an unidentified halophilic archaebacterium strain 172 P1 in media containing 15-27 % salts. The purified enzyme is serine protease that can be inhibited by phenylmethylsulfonyl fluoride. The optimal concentration of NaCl was 10-14 % when assayed at 70 °C with azocasein as substrate. Hydrolyses of the synthetic substrates succinyl-alanyl, alanyl-prolyl-phenylalanyl-4-methylcoumaryl-7-amide or succinyl-alanyl-alanyl-p-nitroanilide at 26 °C were maximal at 25 and 30 % NaCl, respectively. The purified enzyme was most stable at pH 6.0-7.0, and optimal pH was 10.0. Its molecular weight was estimated as 44,000-46,000 Da.

The effects of inorganic salt on enzyme activity were studied. Sharma, *et al.* (1996) studied the effect of divalent cations (Ba^{2+}, Ca^{2+}, Fe^{2+}, Mg^{2+}, Mn^{2+}, Zn^{2+}) on the activities of protease from *Bacillus latersporus* and *Flavobacterium* sp. The enzyme activity of *B. laterosporus* was inhibited by Fe^{2+}, Mg^{2+}, Mn^{2+} and Zn^{2+} ions but enhanced by Ba^{2+}, Ca^{2+} and Fe^{2+}. The enzyme activity of *Flavobacaterium* sp. was inhibited by Mg^{2+} and Mn^{2+} ions but enhanced by Ba^{2+}, Ca^{2+} and Fe^{2+}. The

enzyme activity of the former was strongly inhibited by KCN, whereas that of the latter was only slightly inhibited by 8-hydoxyquinoline. Yang *et. al.* (2000) studied the effects of metal ions (Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Ca^{2+}, Hg^{2+} on protease activity by *Bacillus subtilis*. The protease activity was activated by Mn^{2+}, Fe^{2+}, Zn^{2+}, Mg^{2+}, Co^{2+}, but inhibited completely by Hg^{2+}. The protease was also inhibited by metal-chelating agent such as EDTA, sulhydryl reagents as, β-mercaptoethanol, and by cysteine hydrochloride, histidine, and glycerol. The EDTA was the most effective inhibitor. This enzyme is a metal-chelator sensitive neutral protease. Oh *et al.* (2000) studied the effect of inorganic salts (ferrous sulfate, copper sulfate, magnesium sulfate and sodium chloride) on protease activity by *Pseudomonas aeroginosa*. The alkaline serine protease from *Bacillus licheniformis* was activated by sodium chloride and stable in presence of 0.7 % $NaBO_3$, 0.5 % NaCl and 3 % H_2O_2 (Manachini and Fortina, 1998). The enzyme from *Bacillus pumilus*, isolated from soybean food was activated by the addition of 1 mM Mn^{2+} and Ca^{2+} ion in the reaction mixture (Yasuda and Aoyama, 2000).

Keungarp, *et al.* (1994) isolated and partially purified extracellular protease from the extreme halophile *Halobacterium halobium* (ATCC 43214). The major enzyme component has a Mr of 66,000 and is highly dependent upon salt concentrations near saturation for catalytic activity and stability. In aqueous solutions, a decrease in the NaCl concentration from 4 to 1 M results in an increase of nearly three orders of magnitude in the first order rate constant of inactivation at 30 °C. Salt effects the stability of the enzyme in cooperative manner, with a Hill coefficient of 4.1, which is similar to that of other enzymes from extreme halophiles. The enzyme activity is affected by the salt concentration, with a loss of 2.5 orders of magnitude in Kcat/Km in going from 4 to 0 M NaCl. This loss in catalytic efficiency is primarily due to increase in the Km for the substrate in low salt media.

The purification and characterization of aminopeptidase from bacteria were reported. Leucine aminopeptidase of *Aeromonas proteolytica* (EC 3.4.11.10) is a monomeric metlloenzyme having the capacity to bind two Zn^{2+} atoms in the active site. Comparison of deduced amino acid sequence of the *A. proteolytica* leucine aminopeptidase with other Zn^{2+} binding metallo enzymes failed to show homologies to the consensus binding sequence His-Glu-X-X-His for the metal ion (Guenet *et al.*,

1992). Lee *et al.*, 1998 purified and studied the properties of extracellular leucine aminopeptidase from halophilic bacterium, *Bacillus* sp. N2 that isolated from fermented anchovy sauce. The enzyme has molecular mass of 58000 Da using SDS-PAGE. This purified enzyme showed maximum activity at pH 9.5 and at 50 °C when L-leu-p-nitroanilide was substrate. The leucine aminopeptidase was inactivated by 1, 10-phenanthroline, dithiothreitol and sodium dodecyl sulphatd. Enzyme activity was increased by addition of $Co^{2+.}$ Sodium chloride (0-4.5 mol^{-1}) increases the hydrolytic activity towards L-Leu-p-nitroanilide. The N-terminal amino sequence was Glu-Arg-Glu-Leu-Pro-Phe-Lys-Ala-Lys-His-Ala-Tyr-Ser-Thr-Ile. The purified enzyme had a high specificity for L-Leu-p-nitroanilide. Kuo *et al.*, 2003 purified and characterized of the recombinant leucine aminopeptidase II of *Bacillus stearothermophilus*. The purified enzyme showed the molecular mass 44.5 kDa by SDS-PAGE. The temperature and pH optima for the purified protein were at 60°C and 8.0, respectively. Under the optimal condition, the purified enzyme showed a marked preference for Leu-p-nitroanilide, followed by Arg and Lys-derivatives. The His6-tagged enzyme was stimulated by Co^{2+} ions, but was strongly inhibited by Cu^{2+} and Hg^{2+} and by the chelating agents, DTT and EDTA. The EDTA-treated enzyme could be reactivated with Co^{2+} ions, indicating that it is a cobalt-dependent exopeptidase. Arora and Lee (1994) purified and characterized of aminopeptidase that extracted from cell lysis of *Lactobacillus casei* subsp. *rhamnosus* S93 during late exponential phase. The purified enzyme showed single protein band of 89 kDa. The maximum aminopeptidae activity was observed at pH 7.0 and 39 °C. The enzyme hydrolyzed a range of nitroanilide substituted amino acids, as well as dipeptides, and accounted for most of the aminopeptidase acitivity found in cell-free extracts. The enzyme activity was inhibited by metal chelators such as EDTA and 1,10-phenanthroline. Cobalt ions only stimulated aminopeptidae activity and were also able to re-activated the enzyme previously inhibit by metal chelators. The Km and Vmax. Values of the aminopeptidase for leucine p-nitroanilide were 0.06 mM and 12.6 mmol/min per mg of protein, respectively. This enzyme was stable over the pH range of 5-9 and below 45 ^{o}C. Macedo *et al.* (2003) purified and characterized of intracellular aminopeptidase from *Lactobacillus plantarum* isolated from traditional Serra da Estrela cheese. The enzyme molecular weight was 70 kDa and composed of two subunit, the molecular weight of which is

34 kDa. The purified enzyme hydrolyzed preferentially pNA adducts of hydrophobic and basic amino acid residues; no hydrolysis was observed of Glu-pNA, Gly-pNA or Pro-pNA. The enzyme activity was removed by the metal chelating agent EDTA, thus suggesting that it is a metallo enzyme; however, the EDTA-inhibited enzyme was reactivated in the presence of Co^{2+}. Optimal aminopeptidase activity was obtained at 28 °C (pH 7.0) and pH 6.5 (37 °C). The enzyme was inhibited by 10 mM $CaCl_2$ or $MgCl_2$. Chavagnat *et al.* (1999) purified and characterized of aminopeptidase N from *Streptococcus thermophilus* A. The purified enzyme has molecular mass 95 kDa, with maximal activity on N-Lys-7-amino-4-methycoumarin at pH 7.0 and 37 °C. It was strongly inhibited by metal chelating reagents, suggested that it is a metalloenzyme. The activity was greatly restored by the bivalent cations Co^{2+}, Zn^{2+} and Mn^{2+}. Except for proline, glycine, and acidic acid residues, PepN has a broad specificity on the N-terminal amino acid of small peptides, but no significant endopeptidase activity has been detected.

The characterization of thermostable protease and aminopeptidase from bacteria were reported. Thermostable enzymes are stable and active at temperatures, which are even higher than the optimum temperatures for the growth of the microorganisms. The need for thermostable catalysts, the optimum conditions for efficient catalytic activity of the enzymes and have considerable potential for many industrial applications. Because of that enzymes can be employed for use in harsh industrial conditions where their specific catalytic activity is retained. One extremely valuable advantage of conducting biotechnological process at elevated temperature is reducing the risk of contamination by common mesophiles. Allowing a higher operation temperature has also a significant influence on the bioavailability and solubility of organic compounds and provides efficient bioremediation (Becker, 1997). Thermostable enzymes, which have been isolated mainly from thermophilic organisms, have found a number of commercial applications because of their overall inherent stability. The isolation of Bacilli diverse sources has made these organisms the focus of attention in biotechnology (Johnevelsy and Naik, 2001). The thermophilic *Bacillus* sp. that produced protease have been isolated, *Bacillus stearothermophillus* (Salleh *et al.,* 1977) which is stable at 60 °C. Another *Bacillus* sp. has produced a thermostable protease that has an optimum activity at 60 °C

(Razak *et al*., 1993) while a different *Bacillus stearothermophilus* sp. produced an alkaline and thermostable protease, which is optimally active at 85 °C (Razak *et al*., 1995; Razak *et al*., 1997; Rahman *et al*., 1994). A species of *Bacillus stearothermophillus* TP26 produced extracellular protease and optimum temperature at 75 °C (Gey and Unger, 1995). Enhancement of protease activity excreted from *Bacillus stearothermophilus* had been possible using economical chemical additives in the proteolysis reactions involved in waste activated sludge (Kim *et al*., 2002). In a chemically defined medium, thermophilic and alkaliphilic *Bacillus* sp. Jb-99 was also reported to produce thermostable alkaline protease (Johnevelsy and Naik, 2001). Dominant producer of protease in fact, are the microorganisms of the genera *Pyrococcus, Thermococcus* and *Staphylothermus*. Extremely thermostable serine proteases are produced by the hyperthermophillic archaeum *Desulfurococcus* strain (Hanazawa *et al*., 1996), and the hyperthermophilic metalloproteases are reported from a gram negative thermophillic bacterium (Kanasawat and Wipapat, 1996). Cowan *et al.* (1987) studied the extremely thermostable extacellular proteinase from a strain of the archaebacterium *Desulfurococcus mucosus.* The purified enzyme was serine-type proteinase enzyme, had a molecular mass 52,000 Da by gel-permeation chromatography. Substrate-specific studies suggest a possible preference for hydrophobic residues C-terminal side of splitting point. The thermostability of this enzyme were (at 95 °C for 70-90 min; at 105 °C for 8-9 min. Ca^{2+} chelating does not appear to be implicated in stabilization of the protein structure. The stability of the *Desulfurococcus* proteinase was not greatly affected by the presence of reducing reagents (dithiothreitol) some chaotropic agents (NaSCN) and some detergents, but activity lost rapidly at 95 °C in the presence of the oxidizing agent $NaBO_3$. Proteolytic activity was readily detected including 125 °C, although denaturation was very rapidly above 115 °C. Minagawa *et al*.(1988) isolated and characterized of thermostable aminopeptidase T from *Thermus aquatus* YT-1. The enzyme had a meric structure, its relative molecular mass being 108,000 Da by gel filtration, and 48,000 Da by SDS-PAGE. The optimum pH of the enzyme activity was in the range of 8.5 to 9.0. The enzyme was significantly thermostable as it still retained 60% of its original activity even after heat treatment for 20 h at 80 °C. The enzyme activity was inhibited by metal chelating agents. The enzyme had low substrate specificity. Ando *et al.* (1999) reported thermostable aminopeptidase from *Pyrococcus*

horikoshii. The enzyme was stable at 90 °C, with the optimum temperature over 90 °C. The metal ion bound to this enzyme was calcium, but it was not essential for the aminopeptidase activity. This enzyme required the cobalt ion for activity.

Morikawa *et al*. (1994) purified and characterized of a thermostable thiol protease from hyperthermophilic archaeon strain, KOD1, *Pyrococcus* sp. isolated from a solfatara at a wharf on Kodaka Kagoshima, Japan. The growth temperature of the strain from 65 to 100 °C. The optimum growth pH and NaCl concentration were 7.0 and 3 % NaCl, respectively. The molecular mass was to be 44 kDa. The specific activity of the purified protease was 2,160 U/mg of protein. The enzyme showed maximum activity at pH 7.0 and 110 °C, with azocasein as a substrate. The enzyme activity was completely retained after heat treatment at 90 °C for 2 h, and the half–life of the activity at 100 °C was 60 min. The proteolytic activity was significantly inhibited by P-chloromercuric or E-64 but not by EDTA or phenylmethylsulfonyl fluoride. The partially purified enzyme from *Bacillus subtilis,* isolated from hot spring was stable at 60 °C for 30 min. Calcium ions were effective stabilizing the enzyme. The enzyme was extremely salt tolerant and retained 100 % activity in 5 $MnCl_2$ for 96 h. The molecular of the purified enzymes was 28,000 Da by SDS-PAGE. The enzyme was completely inactivated by PMSF, but little effected by urea, sodium dodecyl sulfate and sodium tripoly phosphate (Kembhavi *et al*., 1993). Sookkheo *et al*. (2000) purified and characterized of thermostable protease from *Bacillus stearothermophilus* TLS33. Three thermostable proteases were designated to be S, N and B. The molecular masses of the enzyme were estimated 36, 53 and 71 kDa, respectivley. The optimum pH of protease S, N and B were shown to be 8.5, 7.5 and 7.0, respectively. The maximum activities for the enzymes were at 70, 85 and 90 °C, respectively. All three thermostable proteases were strongly inhibited by the metal chelators EDTA and 1, 10-phenanthrolinem, and the proteolytic activities were restored by addition of $ZnCl_2$. The cleavage specificities of proteases S, N and B on a 30 residue synthetic peptide from pro-BPN'subtilisin were Tyr-Ile, Phe-Lys and Gly-Phe, respectively. Lin *et al*. (2004) reported thermostable leucine aminopeptidase (LAP) from *Bacillus kaustophilus* CCRC 11223. Phylogenic analysis of *B. kaustophilus* LAP showed closely related to the enzyme from *B. subtilis*. The pH and temperature optima for the purified enzyme

were 8 and 65 °C, respectively, and 50 % of its activity remained after incubation at 60 °C for 32 min. the enzyme preferentially hydrolyzed L-leucine-p-nitroanilide (L-Leu-p-NA) followed by Cys derivative. The report of thermostable proteolytic enzyme from various bacteria were showed in Table 2.1.

Resistance of thermophilics to high temperatures and denaturation

Microorganisms, like all living things, adapt to the condition in which they have to live and survive. Thermophiles are reported to contain proteins, which are thermostable and resist denaturation and proteolysis (Kumar and Nussinov, 2001). Specialized proteins known as “vhaperonins” are produced by these organisms, which help, after their denaturation to refold the proteins to their native form and restore their functions (Everly and Alberto, 2000). The cell membrane of thermophiles is made up of saturated fatty acids. The fatty acid provides a hydrophobic environment for the cell and keeps the cell rigid enough to live at elevated temperatures (Herbert and Sharp, 1992). The archae, which compose most of the hyperthermophiles, have lipids linked with ether on the cell wall. This layer is much more heat resistant than a membrane formed of fatty acid (De Rosa *et al*., 1994).

The application of proteases in foods

Proteases have a large variety of applications, mainly in the detergent and food industries. The use of proteases in the food industry such as cheese making, baking, preparation of soy hydrolyzates, and meat tenderization. The major application of proteases in the dairy industry is in the manufacture of cheese. In baking industry bacterial proteases are used to improve the extensibility and strength of the dough. Manufacture of soy products proteases have been used from ancient times to prepare soy sauce and other soy products. The alkaline and neutral proteases of fungal origin play an important role in the processing of soy sauce. Proteolytic modification of soy proteins helps to improve their functional properties. Treatment of soy proteins with alcalase at pH 8 results in soluble hydrolyzates with high solubility, good protein yield, and low bitterness. The hydrolyzate is used in protein-fortified soft drinks and in the formulation of dietetic feeds.

Table 2.1 Sources microorganisms and properties of thermostable proteolytic enzymes

Bacteria	Enzymes Properties		References
	Optimum Temperature (°C)	Optimum pH	
Bacillus brevis	60	10.5	Banerjee *et al*.,1999
Bacillus licheniformis	70	9.0	Manachini and Fortina, 1998
Bacillus stearothermophillus	60	-	Salleh *et al.*, 1977
Bacillus stearothermophillus	85	-	Rahman *et al.*, 1994
Bacillus sp. JB-99	80	6-12	Johnevelsy and Naik, 2001
Bacillus stearothermophilus TP26	75	-	Gey and Unger, 1995
Bacillus sp. No, AH-101	80	12.0-13.0	Takami *et al.*, 1990
Bacillus thermoruber	45	9	Manachini *et al.*, 1988
Pyrococcus sp. KODI	100	7	Fujiwara *et al.*, 1996
Staphylothermus marinus	-	9	Mayer *et al.*, 1996
Thermoacidophiles	60-70	7.0-8.5	Kocabiyik and Erdem, 2002.
Thermococcus aggreganes	90	7.0	Canganella *et al.*, 1998
Thermococcus celer	95	7.5	Zillag *et al.*, 1983
Thermococcus litoralis	85	8.5	Brown and Kelly, 1993
Thermotoga maritima	95	9.5	Huberg *et al.*, 1986

Modification of food protein can be carried out by chemical or enzymatic method. Enzymatic modification generally involves the use of proteolytic enzyme to hydrolyze specific peptide bonds. This has an advantage over chemical methods because it causes minimal undesirable side reaction. Enzymatic hydrolysis yields proteins that are smaller in molecular size, with secondary structure which may have

improved functional properties including solubility, emulsifying and foaming properties.

Rebeca, *et al.* (1991) used protease from *Bacillus subtilis*: Brew-N-zyme, Takabate 380 and HT proteolytic 200 (HT200), Maxatase LS, Pescalase 560, Protease N and from *Aspergillus oryzae*:Tendrin and Panzyma G for yield and nutritional value. The fish protein was solubilized with Pescalase 560 faster than with HT-200 or Protease N. The increase of protease concentration was associated with a quadratic increase in soluble nitrogen. Acalase hydrolyzed fish protein from Herring (*Clupea harengus*) was higher degree than papain. Papain hydrolyzates were more bitter than those made with alcalase (Hoyle and Merritt, 1994). In 1995, Stoknes and Rustad reported that the optimum condition for hydrolysis of protease in cray fish processing were pH 8-9, 65 °C, 2.5 h. reaction time, 75 % (w/v) substrate concentration and 0.3 % (w/v) enzyme. The maximum proteolytic activity in muscle of atlantic salmon (*Salmo salar*) were in the neutral to slightly alkaline pH range. Optimal activity of heat-stable alkaline protease was found at pH 8 and 65 °C. The activity dropped off markedly below 60 °C and above 70 °C. Preincubation of the muscle was especially susceptible to proteolytic degradation at elevated temperatures (Baek and Cadwallader, 1995). Gagne and Simpson (1993) used proteolytic enzymes, chymotrypsin and papain for hydrolyze protein associated with demineralized shrimp waste to recovery chitin. The Optimum conditions for deproteinization by chymotrypsin were 40 °C, pH 8.0 and Enzymes/Wastes (E/W) ratio of 7:1000 (w:w). With papain, a temperature of around 38 °C, pH of 8.7 and E/W ratio of 10:1000 (w:w) gave the optimum response. At these conditions, the yield of protein was maximum, and ranged from 22-48 % depending on the starting material. The used protease from *Bacillus subtilis* for deproteinize crustacean wastes. They found that the deproteinization test, liquid phase fermentation of untreated shrimp shell, crab shell, and lobster shell wastes with this microbe showed protein removal of 88, 67 and 83 %, respectively. In contrast, the protein removal of acid treated waste was 76, 62 and 56 %, respectively. The optimized conditions for protease production was found when the culture was shaken at 30 °C for 3 days in 100 ml of medium (phosphate buffer adjusted to pH 6.0) containing 7 % shrimp and crab shell powder (SCSP), 0.1 % K_2HPO_4, 0.05 % $MgSO_4$, 1.0 % arabinose, 1.5 %

$NaNO_3$ and 1.5 % $CaCl_2$. Under such conditions, the protease of *B. subtilis* Y-108 attained the highest activity (Yang *et al.*, 2000).

There are the reported about the use of enzyme for modified soybean protein. Ross and Bhatnagar (1989) modified proteins by enzyme protein kinase, isolated from cardiac muscle for improved the functional properties. Hamada and Mashall (1989) used enzyme for improve functional properties of soybean by deamidation with *Bacillus circulans* peptidoglutaminase. The deamidation increase protein solubility and substantially enhanced emulsifying activity under mildly acidic (pH 4.0-6.0) as well as alkaline conditions. Deamidation improved emulsion stability and foaming power of heat-denatured hydrolyzed soy protein. Zhang, *et. al.* (1996) hydrolyzed soybean proteins with protease from *Aspergillus*. They found that the degree of hydrolysis was 90 % at 35 °C and pH 7.0 and hydrolysis rate increase from 75 ml/min to 170 ml/min. Were *et al.* (1997) modified soy protein with alkali treatment at pH 10.0 followed by papain hydrolysis. Solubility, water hydration capacity (WHC), surface hydrophobicity, foaming and emulsifying properties of unmodified, alkali-treated, and papain-modified soy protein (PMSP) were compared. PMSP exhibited higher solubility (100 % at pH>7.0), WHC (3.13) and hydrophobicity (40.8) than unmodified soy protein which had solubility 68.5 %, WHC 0.21, and hydrophobicity 8.1. The PMSP had foaming capacity (22.0 ml) similar to egg albumin (21.2 ml) at pH 7.0 and enhanced foam stability (36.4) compared to the unmodified control (32.9). In general, alkali-treated soy had lower functional properties. Emulsifying properties of PMSP and alkali treated soy were unchanged by the modification. PMSP could be used as egg albumin substitute in foaming applications at neutral pH. Yasuda, *et al.* (2000) studied soybean milk coagulating enzyme (SMCE) from *Bacillus pumilus*, isolated from soybean food. They found that this enzyme was induced by the addition of soybean protein to the growth medium and the enzyme could improved smooth texture and mild taste of coagulated protein in soybean milk.

Madsen and Qvist (1997) hydrolyzed milk protein by *Bacillus licheniformis* protease. This protease was specific for acidic amino acid residues. A serine protease, which cleaved peptide bonds at the carboxylic site of Glu and Asp, was evaluated with milk proteins as substrate. The enzyme hydrolyzed casein almost 10

times more efficiently than whey protein. The enzyme may provide advantages in preparation of functional protein fractions and in cheese ripening.

Proteolytic enzymes are responsible for protein hydrolysis during traditional fish sauce fermentation. Aroonpiroj (1997) studied proteolytic enzymes in the traditional fish sauce fermentation. Total protease activities of fish visceral, muscular and indigenous bacterial origins were found to be at 48.23 %, 19.43 % and 32.35 %, respectively. Twelve fish sauce bacterial isolates giving high protease activities were identified to be *Bacillus firmus, Micrococcus varians, Corynebacterium* sp., *Micrococcus sedentarius* , *Bacillus pasteurii* and, *Halobacterium salinarium.*

The applications of aminopeptidase to reduce bitterness in foods were reported. Enzymatic hydrolysis of protein is an attractive of obtaining the change in the functional properties. Bitterness in foods may result from compounds naturally present. It may also due to the formation of bitter compounds by chemical reactions occurring during storage (Lemieux and Simard, 1991). Aminopeptidases from lactic acid bacteria are available under the trade name Debitrase. Carboxypeptidase A has a high specificity for hydrophobic amino acids and hence has a great potential for debittering. A careful combination of an endoprotease for the primary hydrolysis and an aminopeptidase for the secondary hydrolysis is required for the production of a functional hydrolyzate with reduced bitterness. Enzymatic synthesis of aspartame is therefore prefered. Although proteases are generally regarded as hydrolytic enzymes, they catalyze the reverse reaction under certain kinetically controlled conditions. An immobilized preparation of thermolysin from *Bacillus thermoprotyolyticus* is used for the enzymatic synthesis of aspartame. The wide diversity and specificity of proteases are used to great advantage in developing effective therapeutic agents.

Protease able to convert hydrophobic dipeptides into an insoluble precipitate and improve the flavor of bitter peptides extracted from protein hydrolyzates (Stevenson *et al.*, 1998). When exopeptidase catalyze hydrolysis of peptide bonds, the products may have a less bitter taste, and the free amino acids or small peptides formed may function in food as pleasant tasting flavor compounds or as flavor precursors. There are several classes of exopeptidase based on specificity for

hydrolysis of synthetic substrates. Exopeptidases in foodstuff may be of natural origin or may be extrinsic, that is, produced by microorganisms. Exopeptidases used to modify foods are also becoming increasingly available in the industrial enzyme market. Exopeptidases contribute a variety of quality changes in postharvest fruit, meat, and food fermentations. Foodstuff impacts by these enzymes during processing include coca, beer, age and cured meat products, koji, fish sauce, ripened cheeses, and protein hydrolyzates. An important role of exopeptidases in food is the hydrolysis of hydrophobic, bitter peptides (Rasakulthai and Haard, 2003). Numerous food and beverage products, bulking agents, and pharmaceuticals have pleasant as well as unpleasant bitter tasting components in their taste profile. In numerous cases, the bitter peptide taste modality is an undesirable trait of the product. Bitter characteristics found in some food systems have been removed or diminished by various processes (Roy, 1990).

Four new methods of debittering protein hydrolyzates and a fraction of hydrolyzates with high content of essential amino acids. Extraction of enzymatic protein hydrolyzates with azeotropic secondary butyl alcohol or aqueous ethanol or aqueous isopropanol, seems to be an efficient and generally applicable method for removal of bitter compounds. A reduction in bitterness of protein hydrolyzates could also be achieved by applying hydrophobic interaction chromatography. Of tested gels, hexyl-sepharose was found to be the most effective for debittering of protein hydrolyzates (Lalasidis, 1978).

Bitter peptide solutions, prepared by the enzymatic hydrolysis of soy protein and milk casein, were treated with an aminopeptidase from the edible basidiomycete *Grifola frondosa*. As the incubation time elapsed, the amount of free amino acids released increased and the bitterness of the enzyme reaction mixtures decreased. However, the debittering of the milk casein hydrolyzate by the aminopeptidase was less effective than that observed for the soy protein hydrolyzate. Hydrophobic amino acids such as valine, leucine, phenyllanine, tyrosine, and isoleucine were preferentially released from the bitter solution by the action of the aminopeptidase (Nishiwaki , 2002).

The intensity of the bitterness is proportional to the number of hydrophobic amino acids in the hydrolyzate. Some bitter peptides isolated from protein hydrolyzates showed in Table 2.2.

Table 2.2 Some bitter peptides isolated from protein hydrolyzates

Source	Peptides	Reference
Tryptic hydrolyzate of casein	Gly-Pro-Phe-Pro-Val-Ile Phe-Phe-Val-Ala-Pro-Phe-Pro-Glu-Val-Phe-Gly-Lys Phe-Ala-Leu-Pro-Gln-Tyr-Leu-Lys	Matoba *et al.*, 1970
Bacterial proteinase hydrolyzated of casein	Arg-Gly-Pro-Pro-Phe-Ile-Val	Minanimura *et al.*, 1972
Beer yeast residue	Trp-Phe, Trp-Pro, Leu-Pro-Trp	Matusita and Ozaki, 1993
Pepsin hydrolyzate of zein	Ala-Ile-Ala, Ala-Ala, Leu, Gly-Ala-Leu Leu-Gln-Leu, Leu-Glu-Leu, Leu-Val-Leu Leu-Pro-Phe-Asn-Gln-Leu, Leu-Pro-Phe-Ser-Gln-Leu	Wieser and Belitz, 1975
Pepsin hydrolyzate of hemoglobin	Val-Val-Tyr-Pro-Trp-Thr-Gln-Arg-Phe	Aubes-Dufau *et al.*, 1995
Alkalase hydrolyzate of hemoglobin	Val-Val-Tyr-Pro-Trp	Aubes-Dufau and Combes, 1997
Soy protein	Arg-Leu, Arg-Leu-Leu, Ser-Lys-Gly-Leu	Fujimaki *et al.*, 1971
Bovin β-CN (C-terminal)	Arg-Gly-Pro-Phe-Pro-Iie-Val	Takahashi *et al.*, 1995

The presence of a proline residue in the center of the peptide also contributes to the bitterness. The peptidases that can cleave hydrophobic amino acids and proline are valuable in debittering protein hydrolyzates. Proline peptides exhibited bitterness, this aspect being different from hydrophobic amino acids. The most significant role of proline residue in peptide bitterness was dependent on the conformational alternation of the peptide molecule by folding the peptide skeleton due to the amino ring of the proline molecule. It was demonstrated that two bitter taste determinant sites were essential for peptide bitterness and that they should be adjacent in the steric conformation of the peptides (Ishibashi *et al*., 1988). Kim *et al*. (1999) analyzed amino acid sequence of bitter peptide from soybean proglycinin. The most bitter tasting fractions contained peptides with average molecular weights lower than 1,700 Da. An analysis of the amino acid sequences indicated that many small bitter peptides (<1000 Da) are composed of uncharged polar amino acids as well as hydrophobic amino acids, with a charged residue often being present at either end. This suggests the involvement of a certain structural requirement in taste perception. Bouchier *et al.* (1999) found that the combinations of purified aminopeptidase, Aminopeptidase P (Pep P) (EC3.4.11.9 or post-proline dipeptidyl aminopeptidase (PPDA) EC 3.4.14.5) along with lysine paranitroanilide hydrlase (KpNA-H) (EC 3.4.11.1) and proline specific aminopeptidases from *Lactobacillus lactis* subsp. *cremoris* AM2 could hydrolyses peptides containing proline residues. Ishibashi *et al.* (1987) investigated the effect of leucine residues on the bitter taste. The hydrophobicity of leucine residues markedly caused the bitterness of peptides and stronger bitterness was always found when a leucine residue was located at the C-terminus of peptides.

Most bitter tasting peptides in cheese originate from the hydrolysis of caseins because contain many hydrophobic residues (Lowrie & Lawrence, 1972; Stadhouders & Hup, 1975). Bitter peptide from cheese has been found to be peptides with molecular weights ranging from 2000 to 3000 Da (Behnke and Schalinatus, 1975). Hydrolysis of casein in cheese during ripening is the main reason for development of bitter flavor as peptides known to give bitterer taste are produced (Lemieux and Simard, 1991).

Enzyme purification

Precipitation

Enzyme can be separated from other components by precipitation using certain salts. Two major methods used for protein precipitations are salting-out and solubility reduction. Salting-out of proteins is achieved by increasing the ionic strength of a protein-containing solution by adding inorganic salts such as $(NH_4)SO_4$ or Na_2SO_4. The added ions interact with water more strongly, causing protein molecules to precipitate. Organic reduction is functioned by adding organic solvents such as acetone and ethanol. They have effects similar to high level salts when added to protein solutions; that is, they lower the protein solubility (Deutcher, 1990)

Chromatography

Chromatography is of fundamental importance to enzyme purification (Table 2.3). Molecular are separated according to their physical properties (size, shape, charge, hydrophobic interactions), chemical properties (covalent binding), or biological properties (biospecific affinity).

Table 2.3 Chromatographic methods

Type of chromatography	Principle	Separation according to
Adsorption	Surface binding	Surface affinity
Distribution	Distribution equilibrium	Polarity
Ionexchange	Ionbinding	Charge
Gel filtration	Pore diffusion	Molecular size, molecular shape
Affinity	Specific absorption	Molecular structure
Hydrophobic	Hydrophobic chelation	Molecular structure
Covalent	Covalent binding	Polarity
Metal chelate	Complex formation	Molecular structure

Source : Gerhartz, 1990

Ion exchange chromatography

Ion-exchange chromatography (IEC) is a popular method for protein purification. The reasons for the popularity of IEC are its versatility, high resolving power, high capacity and straightforward basic principle. There are two types of exchanger: anion exchanger which the stationary phase carries a positive charge and cation exchanger in which the stationary phase carries a negative charge.

Each type of exchanger is also classified as strong or weak according to the ionizing strength of the fractional group. Strong anion exchangers contain quaternary amines and strong cation exchangers sulfonates, whereas the diethylaminoethyl (DEAE) group is usually used in weak anion exchange while the carboxymethyl (CM) groups is frequently used in weak cation exchange. Proteins carry both positive and negative charge groups on their surface, due largely to the side chains of acidic and basic amino acids. Positive charges are contributed by histidine, lysine, arginine and to a lesser extent, N-terminal amines. Negative groups are due to aspartic and glutamic acids, C-terminal carboxyl groups and to a lesser extent, cysteine residues. The net charge on a protein depends on the relative numbers of positive and negative charged groups; this varies with pH. The pH where a protein hs an equal number of positive and negative charged groups is termed its isoelectric point (pI). Above their pI proteins have a net negative charge while below it their overall charge is positive.

Ion-exchange chromatography is capable of separating molecules that have only slight differences in charge. The separation is based on the reversible interaction between charged molecules and oppositely charge chromatographic medium. Molecules bind as they are load onto the column. Conditions are then altered so that the bound substances are eluted differentially. Elution is usually performed by changes in salt concentration or pH. There is a variety of groups which have been closed for use in ion exchangers; some of these are shown in Table 2.4.

Table 2.4 Functional groups used ion exchangers

Functional groups	Name
Strong anion	
$-CH_2N^+(CH_3)_3$	Triaminoethyl (TAM-)
$-C_2H_4N^+(C_2H_5)_3$	Triethylaminoethyl (TEAE-)
$-C_2H_4N^+(C_2H_5)_2CH_2CH(OH)CH_3$	Diethyl-2-hydroxypropylaminoethyl (QAE-)
Weak anion	
$-C_2H_4N^+H_3$	Aminoethyl (AE-)
$-C_2H_4NH(C_2H_5)_2$	Diethylaminoethyl (DEAE-)
Strong cation	
$-SO_3^-$	Sulpho(S-)
$-CH_2SO_3^-$	Sulphomethyl (SM-)
$-C_3H_6SO_3^-$	Sulphopropyl (SP-)
Weak cation	
$-COO^-$	Carboxy (C-)
$-CH_2COO^-$	Carboxymethyl (CM-)

Source: Harris and Angal, 1989

Gel filtration

Gel filtration chromatography (GFC) is a form partition chromatography used for separating molecules of different sizes. The basic principle of GFC is that the stationary phase consists of porous beads with a well defined range of pore sizes. Four basic types of gel are available: dextran, polyacrylamide, agarose and combined polyacrylamide-dextran. The separation process is carried out using a

porous gel matrix (inbead form) packed in a column and surrounded by solvent. Consider a sample containing a mixture of molecules smaller and larger than the pores of the stationary phase matrix, as well as molecules intermediate in size. The smaller molecules can enter inside all the matrix pores and move more slowly through the column, appearing as that last component in the chromatogram. The larger molecules are excluded from the stationary phase and hence elute first from the column. Molecules intermediate in size can enter the stationary phase, but spend less time within it than smaller molecules do. Thus, these molecules are eluted between the large and small a molecules. A molecule of entering the pores of the matrix will have a partition coefficient Kav, is given by the equation;

$$Kav = \frac{(Ve-Vo)}{(Vt-Vo)}$$

Where Ve = volume of solvent required to elute the solute from gel column or bead

Vt = total volume of gel column or bed

Vo= elution volume is equal to the void/exclusion volume

It has been empirically the Kav is inversely proportional to the logarithm of the molecular weight of solute, at least for globular proteins. Each type of gel will be capable of excluding molecules larger than a particular size, and therefore of fractionating molecules within a particular range and of desalting solutions of proteins (Scopes, 1982; Harris and Angal, 1989, Janson and Ryden, 1989; Deutscher, 1990)

Electrophoresis

Electrophoresis is used to isolate pure enzymes on a laboratory scale. Depending on the conditions, the following procedures can be used: zone electrophoresis or porosity gradients. The heat generated in electrophoresis and the interference caused by convection is problems associated with a scale of this

method. And interesting contribution to the industrial application of electrophoresis is a continuous process in which the electrical field is stabilized by rotation.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is an excellent method with which to identify and monitor proteins during purification and to assess the homogeneity of purified fractions. SDS-PAGE is routinely used for determining the subunit compositions of purified proteins. SDS-PAGE can also be scaled up, for use in a preparative mode, to yield sufficient protein for further studies. In addition, two dimensional analysis, combining isoelectric focusing with SDS-PAGE, is very high resolution method for protein fraction, enabling thousands of polypeptides to be resolved in a single gel. When used in conjunction with blotting methods, SDS-PAGE provides one of the most powerful means available for protein analysis.

SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is an analytical method for identify and monitor protein during purification and assess the homogeneity of purified fractions. SDS-PAGE is routinely used for the estimation of protein submits molecular weights and for determining the subunit compositions of purified proteins. Proteins are charged at a pH other their isoelectric point (pI) and thus will migrate in an electric field in a manner dependent on their charge density (the ratio of charge to mass); the higher the ratio of charge to mass the faster the molecule will migrated. The movements of the proteins are retarded by interactions with the surrounding gel matrix, which acts as a molecular sieve. The opposing interactions of the electrical force and molecular sieving result in differential migration rates for the constituent proteins of a sample.

Polyacrylamide gels are formed by copolymerization of acrylamide and bisacrylamide (N,N'-methylene-bis-acrylamide). Gel polymerization is usually initiated with ammonium persulfate and N,N,N,N- Tetramethylethylenediamine (TEMED). TEMED accelerates the rate of formation of free radicals from persulfate and these in turncatalyze polymerization. The persulfate free radicals convert acrylamide monomer to free radicals which react with inactivated monomer to begin

the polymerization chain reaction. The size of the pores with in the gel matrix is depends on the polymerization condition and monomer concentrations. The sieving properties of a gel are established by the three dimensional network of fibers and pore which is formed as the bifunctional bisacrylamide cross-links adjacent polyacrylamide chains. The acrylamide concentration of the gel increases, its effective pore size decreases.

Sodium dodecyl sulphate (SDS) is an anionic detergent, with denatures proteins by “wrapping around” the polypeptide backbone and SDS binds to protein fairly specifically in a mass ratio of 1.4:1. SDS confers a negative charge to the polypeptide in proportion to its length. It is usually necessary to reduce disulfide bridges in proteins before they adopt the random-coli configuration necessary for separation by size: this is done with 2-mercaptoethanol or dithiothreitol.

Determination of protein concentration

Many methods for estimating protein concentration are available and the appropriate choice of methods on five major criteria: (1) the amount of protein available to assay (2) the concentration of the protein, (3) the specific of the assay, (4) the presence of chemicals which may interfere with the assay, and (5) the reliability of performing the assay (Garfin, 1990). An approximate range of sensitivity is given for each assay. It should be emphasized that this range is approximate since the sensitivity of each assay id highly dependent on the type of protein being measured and assay volume.

Bradford method (Dye-binding)

This dye-binding phenomenon can be readily exploited for quantitative analysis. The procedure is very sensitive, working in the range 0.01-0.05 mg of protein for the standard assay procedure. It exhibits a significant dependence on protein amino acid composition and this has recently been shown to be a consequence of the Coomassie blue dye binding primary to basic and aromatic amino acid residues, especially arginine. The binding of the protein causes a shift in

the absorbtion maximum of the dye from 465 nm (red form) to 595 nm (blue form) due to stabilization of the anionic form of the dye by both hydrophobic and ionic interactions. The dye is prepared as a stock solution in either phosphoric or perchloric acid, the latter reagent being more stable. Commercial preparations of this reagent are available from Bio-Rad and Pierce. However, the main problems are technical; the dye adsorbs to glassware and to cuvettes, making it a rather unpleasant reagent (Harris and Angal, 1989; Stoscheck, 1990; Ballog *et al*., 1996).

UV absorbtion

A rapid method of determining whether sample solutions contain protein because no additional reagents or incubations are required. No protein standard need be prepared. Most commonly, absorbance is used for generating a protein elution profile after column chromatography, or any time a quick estimation is need and error in protein concentration is not concern. Most proteins exhibit as absorbtion maximum at 280 nm, which is attributable to the aromatic rings amino acid. Secondary, tertiary and quaternary structures affect absorbance. Therefore, factor such as pH, ionic strength, and etc. can alter the absorbance spectrum. The relationship of absorbance to protein concentration is linear, because different proteins and nucleic acids have widely varying absorption characteristics. There may be considerable error, especially for unknowns or protein mixtures. Any non-protein components of the solution that absorbs ultraviolet light will interfere with the assay. Cell and tissue fractionation samples often contain insoluble or colored component that interfere. This is especially true if compounds containing purine and pyrimidine rings (nucleic acid and nucleotides) are present, in which case the absorbance of the sample is measured at both 280 nm and 260 nm and a correction formula applied:

$$\text{Protein (mg/ml)} = 1.55\ A_{280} - 0.76\ A_{260}$$

An absorbance assay is recommended for calibrating bovine serum albumin or other pure protein solutions for use as standard in other method (Harris and Angal, 1989; Scopes, 1982).

Enzyme kinetics

Enzyme kinetics (kinetein = to move) are primarily an attempt to analyze the data obtained from an enzymatic reaction and to use the data to optimize the reaction. This, of course, provides a detailed description and characterization of the enzyme as well.

Enzymatic reaction

An enzyme reaction, in the simplest case, is the unimolecular reaction. The enzyme-substrate interaction is considered to occurs as shown in the following equation:

$$E + S \underset{k2}{\overset{k1}{\rightleftharpoons}} ES \xrightarrow{k3} E + P$$

Where E = free enzyme; S = substrate; ES = enzyme-substrate complex; and k1, k2, k3 = rate constants for the formation of ES, release of S, or release of P, respectively.

From the observation of the properties of many enzymes it was known that the initial velocity (Vo) at low substrate concentrations is directly proportional to [S] (first-order reaction), while at high substrate concentration the velocity towards a maximum value that is the rate becomes independent of [S] (zero-order reaction). The maximum velocity is called Vmax (μmol/min).

Michaelis-Menten derived an equation to describe these observations, the Michalis Menten equation:

$$Vo = \frac{Vmax .[S]}{Km + [S]}$$

The equation describes a hyperbolic of the type shown for the experimental data in Figure 2.1. In deriving the equation, Michalis and Menten defined a new constant, Km, the Michaelis constant

$$Km = \frac{k2 + k3}{k1}$$

Km is a measure of the stability of the complex, being equal to the sum of the rates of breakdown of ES over its rate of formation. For many enzymes k2 is much greater than k3. Under these circumstances Km becomes a measure id the affinity of an enzyme for its substrate since its value depends on the value of k1 and k2 for ES formation and dissociation, respectively. A high Km indicates weak substrate binding (k1 predominant over k2). Km can be determined experimentally by the fact that its value is equivalent to the substrate concentration at which the velocity is equal to half of Vmax.

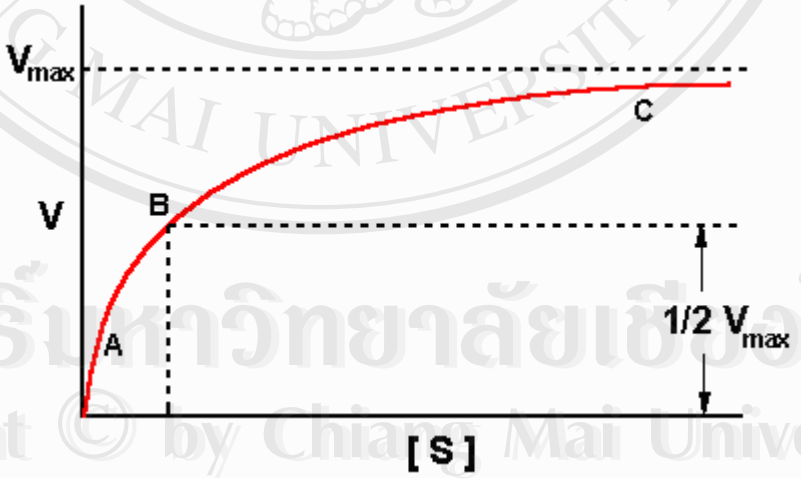

Figure 2.1 Reaction velocity (V) as a function of substrate concentration [S]

Source: www.indstate.edu/mwking/enzyme-kinetics.html

Lineweaver-Burk plot

Because Vmax is achieved at infinite substrate concentration, it is impossible to estimate Vmax (and hence Km) from a hyperbolic plot. The linearization according to Lineweaver-Burk plot of 1/Vo against 1/[S] is made (Figure 2.2). This plot is a derivation of the Michaelis-Menten equation:

$$\frac{1}{Vo} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{[S]}$$

A plot of $1/V_0$ over 1/[S] yields a straight line. The y-axis intercept corresponds to 1/Vmax, the x-axis intercept corresponds to –1/Km. The slope of the line is equal to Km/Vmax. The use of this graph for a reliable determination of the units in question is limited because various substrate concentrations may be weighted unequally. The result obtained with lower substrate concentrations has a greated influence on the slope of the straight line than that achieved with higher concentrations.

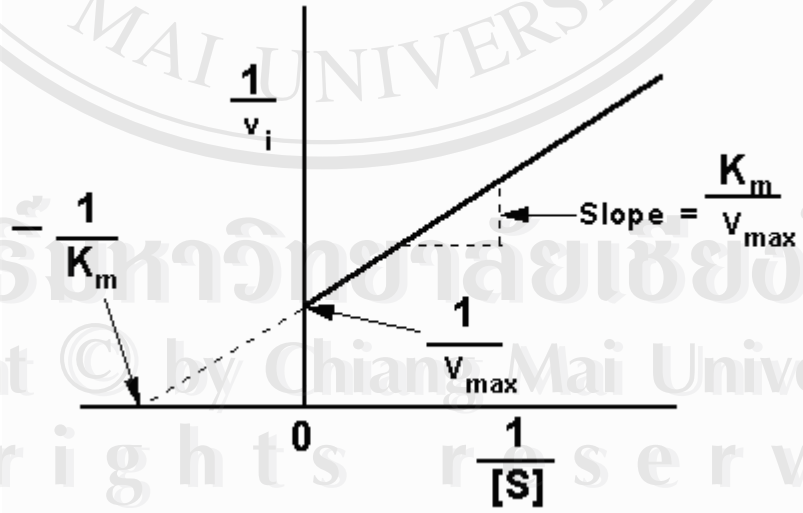

Figure 2.2 Lineweaver-Burk plot

Source: www.indstate.edu/mwking/enzyme-kinetics.html