MICROBIAL PROTEASES

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INTRODUCTION

Microbial proteases are important enzymes used in the food industries. But, the commercially improtant proteases are extracellular enzymes which essentially are excretions of the living organisms into the surrounding medium (Yamamoto, 1975). Fungal proteases are used in the United States for the modification of wheat proteins in bread doughs (Barrett, 1975), in meat tenderizing (Bernholdt, 1975), and in several less-important applications (Nout & Rombouts, 1990 and Gerhartz, 1990). In the Orient, fungal and bacterial proteases are used in the production of various types of traditional foods (Yamamoto, 1975), i.e., to make miso (fermented soybean paste) in Japan. Today, alkaline protease is formulated into most commercially available detergents. It has been observed that protease promotes removal of natural soil which contains protein stains (Minagawa *et al.*, 1978; Fujii *et al.*, 1986; Price & Stevens, 1989 and Shady, 1997).

In a similar manner, the proteases of fungi play a vital role in the production of cheeses. Fermentation processes initiated by *Penicillium roqueforti* and *P. camemberti* are well known examples (Böing, 1982).

Bacterial proteases are not used widely in food processing. They are used to a minor extent in chillproofing, in the production of protein hydrolyzates (as condiments), in the production of condensed fish solubles, and as feed supplements. Bacterial proteases are used on a much larger scale in nonfood applications, as in textile desizing and in the recovery of photographic film (Yamamoto, 1975; Monsan *et al.*, 1978; Kalisz *et al.*, 1986 and Sutar *et al.*, 1986).

When microbial proteases are used in food processing, the microorganisms must be nonpathogenic, and must not produce any type of toxins. *Aspergillus flavus*

produces a potent carcinogen, aflatoxin. In the U.S.A., the Food and Drug Administration has approved only those enzymes as generally recognized as safe (GRAS) which are produced by *Bacillus subtilis, Aspergillus orzae*, or *A.niger* (Reed, 1975; Yamamoto, 1975; Böing, 1982 and Singh *et al.*, 1994).

The specificity of the enzyme is of great importance in choosing a protease for use in food processing, but other considerations such as pH optimum, heat stability, presence of activators of inhibitors, price and availability affect the choice, e.g., in the use of rennin for the formation of milk curds in cheesemaking. On the other hand, the production of protein hydrolyzates for flavor purposes requires enzymes of broad specificity which lead to extensive hydrolysis into low molecular weight peptides and amino acid. Bitter taste can be attributed to perptide fractions that have specific amino acids at the carboxyl terminus (Yamamoto, 1975; Gerhartz, 1990; Singh *et al.*, 1994).

Papain and other plant proteases, e.g., bromelain and ficin are used in the tenderization of meat, in the chillproofing of beer, and for the production of protein hydrolyzates. Fungal proteases are used mainly in the baking industry to modify wheat gluten. They are used to a lesser extent in meat tenderizing and in the production of protein hydrolyzates. Trypsin (or Pancreatin) is used, to some extent, in the production of protein hydrolzates. Rennin and some pepsin are used in cheesmaking for the precipitation of the casein curd. Recently, fungal acid proteases (so-called microbial rennets) have been used for the same purpose. Bacterial proteases are used to a lesser extent in the production of protein hydrolzates and in the chillproofing of beer. They are also used to prevent gelatinization of condensed fish solubles. All of these enzymes are used as digestive aids, an application that represents the borderline between food and pharmaceutical usages. These are the major areas of application at the present time (Yamamoto, 1975; Kalisz *et al.*; 1986, Sutar *et al.*; 1986; and Shady & Adbel-Razik, 1996).

Proteases used in the proteolysis are presently achieved by fermentation or by autolytic processes, e.g., the curing of cheese, the aging of meat or the production of oriental foods, these enclude soy sauce, miso, tempeh and the fish sauce of southeast Asia (Yamamoto, 1975).

2. PROTEINS

Proteins are polymers of amino acids. The general formula for amino acid is $R-CHNH_2-COOH$ and for the simplest amino acid, glycine, R is a hydrogen atom.

While all of the amino acids are needed for building and maintenance of body proteins, some of them may be synthesized in the body. Those that cannot be synthesized and which must be supplied in the diet are called essential amino acids. In addition to those amino acids hydroxylysine and hydroxyproline are encountered in some proteins, for instance, elastin. Proteins are macromolecules of such chains of amino acids that are linked to each other by peptide bonds (Yamamoto, 1975 and Robwell, 1996).

Figure 1. General structure of amino acids chains.

In the above scheme the side chains are designated with the letter R. The peptide chain is linear, and in that sense the primary structure of proteins is less complex than that of the polymeric carbohydrates. A greater degree of complexity is, however, introduced because of the large number of different amino acids that may be linked linearly in innumerable sequential arrangements. The following scheme (Fig.2) shows such a sequence for a protein, the enzyme Papain (Yamamoto, 1975 and Davis, 1985).

Figure 2. Amino acid sequence of papain (From Yamamoto, 1975).

The protein has a relative low molecular weight of 23, 400 and the number of amino acid residues is 212. The scheme also indicated by the S-S three places where a cysteine molecule is bounded to another cysteine by a disulfide linkage that straddles the parallel portions of the folded polypeptide chain. This is shown in more details in Figure 3.

The scheme also indicates the end of the chain carrying a free amino group, (Ile) and the other end with the free carboxyl group of asparagine. These amino acid residues are called the amino terminals and carboxyl terminal amino acids, respectively.The SH group indicated in the scheme by the thick SH plays an important role in enzymatic catalysis. Such an SH group is present in some plant proteases, such as bromelain and ficin (Yamamoto, 1975; Davis, 1985 and Rodwell, 1996).

Amino acid contain both acid and alkaline groups. They are amphoteric molecules and act as anions in alkaline soultions and as cations in acid solutions. Consequently, they migrate toward the cathode in acid solutions and to the anode in alkaline solutions. The pH value at which the effective charge of the acid is zero and at which it will migrate neither to the cathode nor to the anode is called the isolectric point. The amphoteric properties of amino acids and the differences in their isoelectric points permit their separation through electrophoresis (Yamamoto, 1975; Barrett, 1985 and Davis, 1985).

The amphoteric properties of amino acids are also present in dipeptides, tripeptides, polypeptides and proteins. In addition to free amino acid and carboxylgroups at the end of the chains, the basic amino acids (Lys, Arg, His) and the dicarboxylic acids (Asp., Glu) contribute to the polarity of proteins. The balance of alkaline and acidic groups can readily be calculated from the known composition of proteins (Yamamoto, 1975; Barrett, 1985; Branden & Tooze, 1991; Darby & Creighton, 1993 and Rodwell, 1996).

3. PROTEASE PRODUCING MICROORGANISMS, PRODUCTION AND SOME PROPERTIES OF THESE ENZYMES

During the last three decades, microorganisms have been continuously investigated for their protease activity. Thus, the literature is rich with information on the finding, isolation and characterization of numerous microbial proteases (Ohto et al., 1966; Fukumoto et al., 1967; SarDinas, 1968; Somkuti & Babel, 1968; Arima et al., 1970; Eklund et al., 1971; Fackrell & Robinson, 1973 and Federici, 1982). In fact, protease producing microorganisms have been found to be very widely distributed among bacteria, fungi and actinomycetes (Wieland, 1972). However, the yeasts have been studied in less detail (Abou Hamed, 1995a & b and Abdel-Rahim et al., 1996).

A very large number of various species of bacteria, streptomycetes and fungi produce and excrete proteases. In view of the very low specificity of proteolytic enzymes towards their substrate, it is virtually impossible to select organisms producing the desired protese. During the last years, microorganisms have been continuously investigated for their protease activity and for the production of a technical grade of protease (Tabel, 1987; Ishak et al., 1990; El-Kotry et al., 1992, Shady & Abdel-Razik, 1996 and Shady et al., 1997).

The production of enzymes by microorganisms is one of the best means of sup-plying enzymes of uniform quality at low cost. Extracellular proteases have been studied in a wide variety of microorganisms including *Micrococcus* (Mc Donald & Chambers, 1966), *Aeromonas* (Litchfield & Prescott, 1970), *Vibrio* (Reid *et al.*, 1980), *Pseudomonas* (Boethling, 1975; Amrute & Corpe, 1978; Jensen *et al.*, 1980; Vilu *et al.*, 1980; Mckellar, 1982 and Azcona *et al.*, 1989) and *Bacillus* (Priest, 1977; Chopra & Mathur, 1984 & 1985; El-Bieh *et al.*, 1991; El-Fadaly & Afify, 1995 and Shady & Abdel Razik, 1996).

3.a Enzyme production:

It is now well established that production of enzymes, is greatly influenced by both nutritional and environmetal factors. Among these factors are, composition of nutrient media (Hafiz and Qadder, 1986; Fujiwara and Yamamoto, 1987), hydrogen ion concentration of the fermentation media (Chopra & Mathur, 1985; Durtham *et al.*, 1987 and Fujiwara & Yamamoto, 1987), incubation period of the growing organisms (Nomoto *et al.*, 1984 and Okita *et al.*, 1985) and temperature of incubation (Okita *et al.*, 19985 and Fujiwara & Yamamoto, 1987).

El-Beih *et al.* (1991) studied the effect of different pH values on protease production by *B. amyloliquefaciens* (A-6) and (A-17) and showed that pH ranging from 10.0 to 10.6 and from 9.6 to 10.6 were the most favourable for the yield of enzyme produced by cultures of *Bacillus amyloliquelaciens* strains (A-6) and (A-17), respectively. Also, Nomoto *et al.* (1984); Fujiwara (1986) and Fujiwara & Yamamoto (1987) found that the optimum pH values for *Bcilluis sp.* for protease production are similar range.

Shady and Abdel-Razik (1996) found that the optimum pH range for protease production was 6.0 to 7.0 for *Bacilluis Subtilis* (17) and *Pseudomonas fluorescens*, respectively. Optimum pH for this enzyme production has been observed to be 7.0 (Chahal & Nanda, 1975 and Abd El-Nasser, 1995).

Other factors influencing the production of extracellular bacterial proteases were studied. Skim milk when used as nitrogen sources in the production medium, supported enzyme synthesis which strongly induced the protease formation (Mckellar, 1982 and Shady & Abdel-Razik, 1996). In the same time, production of these enzymes was maximum at $30^{\circ}C$ for 3 days incubation. Organic nitrogen sources, e.g., casein and peptone as well as certain inorganic salts, (ammonium sulphate and nitrate), increased the enzyme production when added as nitrogen sources. Lactose or soluble starch also, enhanced the enzyme formations (Abd El-Nasser, 1995).

The formation of extracellular protease by fungi has been reported frequently and in many cases the enzymes have been purified and characterized, often being of some significance in an industrial process (Nakagawa, 1970, Matsubara & Feder, 1971 and El-Shafei, 1991).

Overall enzyme yields were reported to be produced with higher amounts by *Humicola lutea* (Aleksieva *et al.*, 1981) *Penicillium expansum* (Abdel-Fattah & Amr, 1987) Rhizopus oligosporus (Farley & Ikasari, 1992 and Jaya Rajy & Ayyanna, 1993) *Aspergillus oryzae* and *A.Flavus* (Karuna & Ayyanna, 1993 and Shady *et al.*, 1997) and *Aspergillus niger* (Singh *et al.*, 1994).

The ability to produce some extracellular protease appears to be a general property of most, if not all, growing fungi under suitable conditions (El-Shafei, 1991; Singh *et al.*, 1994 and Shady *et al.*, 1997), although the extent to which protease is formed varies considerably even between strains of the same species of organisms. It is well known that nutritional factors influencing growth and enzyme production. Glucose and peptone were the best carbon and nitrogen sources, respectively. Surcose and cheap nitrogen sources, e.g., corn steep liquor, also gave satisfactory enzyme yields. Supplementation of groundnut meal to the basal medium enhanced enzyme production (Singh *et al.*, 1994). Shady *et al.* (1997) found that skim milk is the best nitrogen source for *Aspergillus oryzae* and *kluyveromyces lactis*. At 5% and 2% concentration, enzyme production was enhanced for both organisms, respectively. Casein was found in the second order to induce the protease production. Molasses and peptone (0.5%) were found as the best nitrogen and carbon sources, respectively for enzyme production by *Saccharomyces cerevisiae* (Abou-Hamed, 1995 b).

Environmental factors also influence enzyme formation. Acid proteases, which are often prouced by fungi, are known to have quite low pH optima for activity and stability. Thus by adjusting the pH of this crude enrichment culture to pH values between 3 and 4, the chances of favoring the development of such organisms is increased. Analogous results might be obtained for alkaline protease (Meyrath and Volavsek, 1975).

Singh *et al.* (1994) found that optimum pH range for protease production by *Aspergillus niger* was 3.5 to 4.0, beyond which the enzyme production reduced significantly. Optimum pH of acid protease production was 5.5 for *Humicola Lutea* (Aleksieva *et al.*, 1981); 3.0 to 7.0 for protease A and B, respectively produced by *Phanerochaete chrysosporium* (El-Shafei, 1991) and 6.0 for *Aspergillus oryzae*

(Karuna and Ayyanna, 1993). Shady *et al.* (1997) found that pH 6.0 is the pH optimum for *Aspergillus oryzae* and *kluyveromyces lactis* (1), respectively. Maximum enzyme production was at 30°C after 3 to 5 days.

Yeasts are reported as protease, e.g., *Saccharomyces cerevisiae* (Abou Hawed, 1995a). Optimum pH for maximum enzymes production was 5 and the optimum temperature necessary for maximum proteolytic activity was 30°C and the suitable incubation period was 5 days.

3.b. Some Enzyme production:

The effects of temperature and pH on enzyme activity are distinct specially in food processing. The pH-activity curve is affected by the temperature used for enzyme activation. An example is shown in Fig. (4) for the neutral protease of *Bacillus polymyxa* (Reed, 1975). The optimum pH is shifted from about 7.2 at 20°C to about 6 at 45°C. This reflects the poorer stability of an enzyme at higher pH levels.

The properties (pH and temperature optima) of protease are summarized in Table (1). Properties are different with different microbial strains. These diversities between proteases is of greatest important in choosing the appropriate protease for the suitable processing and effective use.

4. CLASSIFICATION OF PROTEASES

The earliest classifications of proteases were based on the origin of the enzymes: Papain, ficin, and bromelain from plants; typsin from the pancereas; pepsin and rennin from stomach. The names of these enzymes end in "in" which reflects the earlier method of designating enzymes. A later classification and one which is still used today is based on the scheme Citied from Yamamoto, (1975). They divided proteases into exopeptidases and endopetidases. The term exo refers to enzymes that split terminal amino acids from one end of the chain by hydrolysis of the peptide bond. Endopeptidases are proteases that act on peptide bonds in the interior of peptide chain (Yamamoto, 1975). Individual endopeptidases do not split all or even a majority of the peptide bonds in a protein, but their specificity is not so narrow that a complete picture of their action can be obtained.

Another way of classifying proteases is by the chemical nature of their active site. Proteases were classified into four groups based on this concept. The first group contains the serine proteases, which have a specific seryl residue in their active site. These enzymes are strongly inhibited by DFP, which reacts with hydroxyl

Table 1. pH and temperature optima of some microbial proteases.

Character of protease Enzyme source	Temp. °C	pH	Reference
Aspergillus niger	60	3.0 -4.0	Singh et al. (1994)
Aspergillus saitoi	30	3.0-4.5	Gerhartz (1990)
Rhizopus oligosporus	40	3.3	Farley & Ikasari (1992)
Apergillus oryzae	40	7.0-8.5	Shady et al. (1997)
Kluyveromyces lactis	40	7.0	Shady et al. (1997)
Bacillus subtilis	40	8.0	Shady & Abdel-Razik (1996)
Pseudomonas fluorescens	30	6.0	Shady & Abdel-Razik (1996)
Bacillus stearothermophilus	70	8.0	Chopra & Mathur (1985)
Bacillus coagulans	60	7.4	El-Hawary & Ibrahim (1988)
Bacillus stearothermophilus	55	7.5	El-Fadaly & Afify (1995)
Pseudomonas flourescens	35-36	6.5	Alichanidis & Andrews (1977)
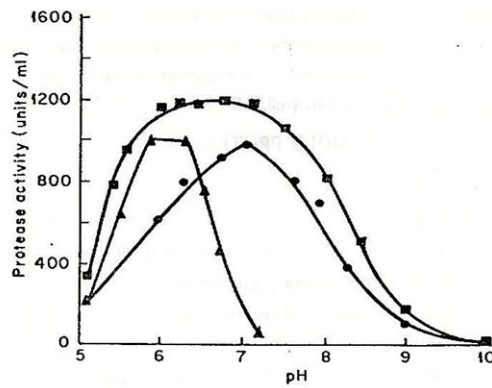
Fig.4. Influence of temperatue on the pH-activity curve of the *Bacillus polymyxa* neutral protease. The incubation temperaure was as follows: ● 20°C; ■ 37°C; ● 45°C. (From Reed, 1975).

group in a specific seryl residue. These enzymes are all endopeptidases. Trysin, chemotrypsin, elastase and subtilisin fall into this group (Yamamoto, 1975, and Rodwell, 1996).

The second group contains sulfhydryl enzymes, whose activity depends on the presence of one or more sulfhydryl groups at the active site. Oxidizing agents, alkylating agents and heavy metal ions inhibit such enzymes by binding with the thiol group. Plant proteases and some of the microbial proteases belong to this group.

The third group contains metalloenzymes, whose activity depends on the presence of a metal, usually in a stoichiometric relationship with the protein molecule. Such metals may be magnesium, zinc, cobalt, iron, mercury, cadmium, copper or nickel. The metal may be strongly bound and difficult to remove or loosely bound and removed by short dialysis against EDTA. Removal of the metal removes the enzymatic activity and readdition of the metal usually restores it. Enzymes are strongly inhibited by cyanides and other metal poisons. Carboxypeptidase A, some of the aminopeptidases and some bacterial proteinases belong to this group.

The fourth group contains the acid proteases. The presence of two carboxyl groups at the active site is suggested from the fact that enzymes are inhibited by P-bromophenacylbromide or diazo reagents. Pepsin, rennin, and many fungal proteases active at acidic pH belong to this group. The enzymes are active at the low pH range from 2 to 4 (Yamamoto, 1975 and Rowell, 1996).

5. MOLD PROTEASES

The proteolytic enzymes of fungi have usually been classified on the basis of their pH optima. These groups are referred to as acid, neutral, and alkaline proteases. Many of the organisms (*Aspergillus oryzae*) excrete all three kinds of proteases. However, several peaks in the pH-activity curves of casein and hemoglobin suggested the presence of several enzymes. The three enzymes have been designated as protease I for the alkaline, protease II for the neutral, and protease III for the acid protease (Yamamoto, 1975). Protease III is not stable at alkaline pH values, and consequently assay of its activity at pH 7.4 showed no hydrolysis, nor could the enzyme be obtained by electrophoresis at a pH of 8.5.

The pH-activity curves of the three proteases with gelatin viscosity as a criterion of activity are shown in Fig (5). A similar separation could be obtained with hemoglobin. With casein only protease I and II appear on the curve because of the poor solubility of casein at pH 4 to 5 (Yamamoto, 1975).

40°C is the temperature optimum for *Aspergillus oryzae* protease III on the

hydrolysis of casein as substrate and at a pH of 6.0 (Fig. 6). Temperature stability curves for the three enzymes in solution at pH 6.0 and over a 30-min. period indicate complete stability at 30°C and 40°C and a fairly rapid loss of activity at 50°C (Yamamoto, 1975).

Aspergillopeptidase A, *Aspergillus oryzae*, (EC 3.4.4.17) was prepared from these enzymes complex, was three times as active as trypsin in the hydrolysis of gelatin. Similar to other mold and bacterial proteases, the enzyme shows a wide specificity against peptide bonds in protein chains (Ichishima, 1970; Nakagawa, 1970 and Yamamoto, 1975). Pepsin-like acid proteases are widely distributed among the molds of *Aspergillus, Penicillium* and *Rhizopus* species. Among them an acid protease of broad specificity from *Aspergillus saitio*, which resembles pepsin in many respects, i.e., it is stable in the acid pH range from 2 to 5 and has a pH optimum between 2.5 and 3 with casein and soy bean protein (Ichishima, 1970).

6. BACTERIAL PROTEASES

The production of proteolytic enzymes from bacteria usually starts with the strain of *Bacillus subtilis*. The extracellular enzymes produced by this organism are potent endopeptidases with a pH optimum in the neutral to slightly alkaline range. This enzyme was later called subtilisin Carlsberg (EC 3.4.4.16). Besides subtilisin Carlsberg, two slightly different proteases, subtillisin novo and subtilisin BPN have been isolated (Yamamoto, 1975).

Subtilisin Carlsberg has an isoelectric point of about 9.4 and stable in the pH range from 5.3 to 6.5. It is inactivated in the pH range from 8.1 to 9.5 through self-digestion. It can be stored for long periods in the lyophilized state or in glycerol solution (Yamamoto, 1975).

Subtilisin BPN is rather different from chemotrypsin in its amino acid sequence, its active site is formed by Asp (32), His (64) and Ser (221), and an electron transfer system formed by these amino acid residues performs its catalytic in a manner similar to trypsin and chemotrypsin. These structural characteristic indicate that the enzyme is a serine protease. It is inhibited by neutral inhibitors in potato and broad been but not the specific soy bean trypsin inhibitors (Yamamoto, 1975).

The optimum pH for casein digestion is in the range from pH 10 to 11. Subtilisin BPN is about two to three times as active as trypsin in hydrolyzing casein, hemoglobin or gelatin (Ottesen and Svendsen, 1970; Smith *et al.*, 1970 and Markland & Smith, 1971).
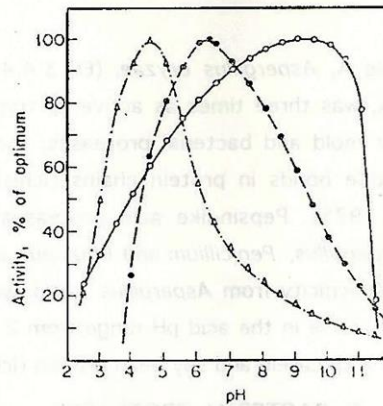
Fig.5. The pH-activity curves of three proteases from *Aspergillus oryzae*. O-O, pro-
tease I; ●● protease II; △ △ , protease III. Protease activity was deter-
mined from the reduction in relaive viscosity of a 5% gelatin solution of the
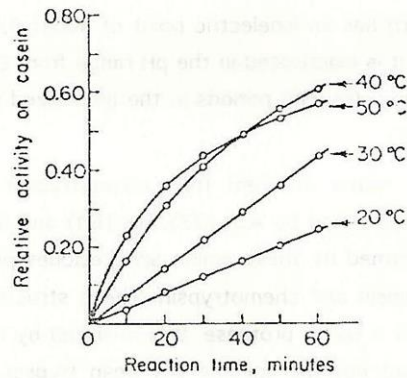appropriate pH, after a digesion period of 5 min. at37°C.

Fig.6. Effect of temperature on the hydrolysis of casein by a purified protease
(protease III) from *Aspergillus oryze*. The activity of proteases III in hydrolyzing
casein is shown for the temperatures indicated pH was 6.0.

7. USES OF MICROBIAL PROTEASES IN FOODS

The application of proteases for the processing of various foods will be summarized here. Fungal proteases are used mainly in the baking industry to modify wheat gluten. They are used to a lesser extent in meat tenderizing and in the production of protein hydrolyzates. Rennin and some pepsin are used in cheesemaking for the precipitation of the casein curd. Recently, fungal acid proteases (so-called microbial rennets) have been used for the same purpose. Also, they play an important role in the production of fermented foods by moulds from soybean, rice and other cereals. Bacterial proteases are used to a lesser extent in the production of protein hydrolyzates and in the chillproofing of beer; and to prevent gelatinization of condenses fish solubles. All these enzymes are used as digestive aids, an application that represents the borderline between food and pharmaceutical usages. These are the major areas of application at the present time (Yamamoto, 1975; Stepaniak & Fox, 1985; Sutar et al., 1986; El-Hawary & Ibrhim, 1988; Gerhartz, 1990; Nout & Rombouts, 1990; Singh et al., 1994; Shady & Abdel-Razik, 1996 and Shady et al., 1997).

One should include those applications where proteolysis is presently achieved by fermentation or by autolytic processes, such as the curing of cheese, the aging of meat, or the production of oriental foods (soy sauce, miso, tempeh, and the fish sauce of southeast Asia).

7.1. Effect of protease on bread properties:

Addition of protease to doughs improves the handling properties of the doughs and the elasticity and texture of the gluten and increases loaf volumes substantially. The fungal protease are most widely used and they have been studied extensively. Two major types of fungal proteases are currently available. One of these has negligible amylase activity, while the other one has a moderate amylase activity (Barrett, 1975).

The use of fungal proteases has assumed a more important role than the amylases. There are several reasons for this. Flour contains some native α-amylase and other sources of α-amylase are readily available. But, the native proteases of flour play no part in bread making and therefore, proteolytic supplementation must be supplied from other sources. Proteolytic enzymes are used on a large commercial scale, and it is estimated that perhaps two thirds of the white bread baked in the United States is treated with enzymes derived from Aspergillus oryzae (Barrett, 1975 and Price & Stevens, 1989).

Proteases are used during the fermentation stage to permit contact with the flour proteins (gluten) for an extended period of time. The proteases hydrolyze and shorten the protein chains and allow them to realign into sheets of protein film. This action of the enzymes modifies the protein so that shorter mixing times (and a lower energy input) are required to the point of maximum extensibility. The use of fungal protease permits a reduction of mixing time by as mush 30% without producing a deterimental effect on the dough. When used at the proper level fungal protease improves the handling and machining properties of a dough. Excessive amounts of the enyzme make the dough too slack and extensible, even to the point of undesirable stickiness of the dough (Barrett, 1975 and Price & Stevens, 1989).

The aroma (but not the taste) of bread is improved by the use of proteolytic enzymes. Presumably this improvement is due to an increase in carbonyl compounds by enzyme action (Barrett, 1975).

7.1.a. Use in cracker doughs:

Both fungal and bacterial proteases are used in cracker doughs. The enzymes mellow the gluten during the long fermentation period to provide the right balance of extensibility and strength in the dough. Such doughs can be rolled out very thin without tearing. Equally important is the need to condition the dough so that it lies flat in the oven and does not bubble up or curl at the edges. Proteases from the plant sources have not been found to be suitable for the commercial production of crackers. Proteases are useful in the production of various snack foods and crackers (Barrett, 1975).

7.1.b. Sources of proteases:

7.1.b.1. Fungal proteases:

Similar to the the α-amylase the fungal proteases are produced by growing suitable strains of *Aspergillus oryzae*. Within the past years, enyme concentrates with very high proteolytic activity and with negligible amylase activity have become available. These together with α-amylase preparations with negligible protease content permit excellent control of enzymatic supplementation at the bakery (Barrett, 1975 and Price & Stevens, 1989).

7.1.b.2. Bacterial proteases:

Enzymes isolated from *Bacillus subtilis* are approved for use in general food production and in the production of baked goods, such as crackers, cookies and many snack foods. The production, isolation and marketing of bacterial proteases is quite similar to that for bacterial amylases (Barrett, 1975 and Price & Stevens, 1989).

7.2. Rennet and cheesmaking:

Milk is a complex biological fluid, secreted by the mammary glands in lactating mammals and comprising fat, protein, carbohydrates, water, minerals, enzymes, vitamins, etc. An average composition for cow's milk is given in Table (2). The unique composition and properties of milk make it an ideal food. Cow's milk, is a starting point for many food products (Cheeseman, 1981).

Table 2. Average composition of cow's milk (Cheeseman, 1981).

Constituent	g/100g milk
Water	87.6
Fat	3.8
Protein	3.3
- Casein	2.6
- Whey proteins	0.7
Non-protein nirogen	0.03
Lactose	4.7
Minerals	0.9
- Calcium	0.12
- Phoshorus	0.09

7.2.a.1. Rennin:

1. Occurrence and formation from prorenin:

Rennin (EC 3.4.4.3) is a gastric acid protease, like pepsin, which is used extensively in cheesemaking and to some extent in the preparation of rennet puddings, in which the texture of the pudding is due to the precipitation of casein in situ. The enzyme is present in the juice of the fourth stomach of the calf. Rennin is the proteolytic agent of milk clotting found in rennet, but other enzymes may be present in the crude commercial extracts. This enzyme is formed from the inactive precursor, prorennin. It is converted to rennin through partial hydrolysis, as indicated by the reduction of molecular weight from 36,000 to 31,000 (Yamamoto, 1975 and Price & Stevens, 1989).

7.2.a.2. Mechanism of milk clotting enzymes:

The effect of rennin is catalyzes the precipitation of casein and requires special consideration. The nitrogenous materials in cow's milk are about 78% casein, 5.1% α-lactalbumin, 8.5% B-lactoglobulin, 1.7% immuneglobulin, 1.7% peptone and 5% of nonprotein derived substances. It is assumed that rennin converts casein into P-casein, which precipitates in the presence of calcium ions. Removal of calcium or

addition of citrate or phosphate as practiced in the production of evaporated milk decreases or eliminates the tendency toward coagulation. The two reactions can actually be separated if rennin is permitted to act on solutions of sodium caseinate at relatively high pH values (Yamamoto, 1975). One can also separate the two reactions by carrying out the enzymatic hydrolysis at very low temperatures because the secondary precipitation reaction occurs only at higher temperatures. Figure (7) shows the liberation of nonprotein nitrogen when crystalline rennin (0.4 mg/ml) acts on milk at a pH of 6.7 and at a temperature of 2^oC. Subsequent heating of the milk to 40^oC causes precipitation. As shown in the figure, the time required for precipitation to begin at 40^oC depends on the extent of hydrolysis at 2^oC. After a 45-min. treatment with the enzyme, the time of coagulation (at 40^oC) is practically zero (Price and Stevens, 1989).

These results indicate that the process of casein coagulation can be separated into two steps (Fig. 8). At the first step, casein is converted enzymatically to P-casein by the action of rennin (primary phase), and at the second step, P-casein is coagulated nonezymatically by heat in the presence of Ca^{+2} (secondary phase). Decrease in pH also increases the rate of coagulation. However, the nature of the gel formed also changes. Lower pHs tend to give rise to a coarse type of coagulum, while additional calcium yields a harder gel. The tertiary stage of rennet action involves a slow proteolysis of the coagulated casein molecules (Cheeseman, 1981).

7.2.b. Microbial rennets:

Milk-clotting enzymes from other sources have been sought for a long time which can replace calf rennin and be supplied at cheaper price. Recently, mold acid protease produced by *Mucor* and *Endothia species* have been developed as partial or complete substitutes of rennin. The action of microbial rennets from *Mucor miehei*, *M. pusillus* and *Endothia parasitica* on several casein fractions, which are important components in cheesemaking, has recently been compared with that of calf rennin with respect to the electrophoretic pattern of hydrolyzates and the formation of non-protein nitrogen (Vanderpoorten and Weckx, 1972). As shown in Figs. (9 and 10) microbial rennets from *Mucor miehei* and *M. pusillus* lindt produce a similar pattern of hydrolyzed products as calf rennin. Various types of cheeses prepared with microbial rennets were judged satisfactory by organoleptic tests (Arima *et al.*, 1970). Microbial rennets have been reviewed earlier (Arima *et al.*, 1970; Ottesen & Richert, 1970 and Yamamoto, 1975).
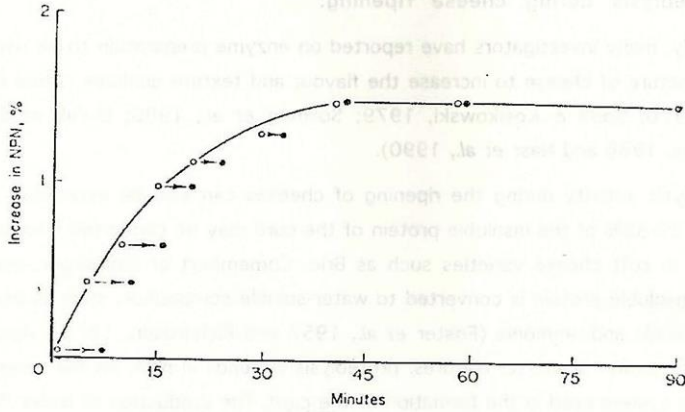
Fig. 7. The formation of nonprotein nitrogen (NPN) in milk by rennet and its relation-
ship to clotting time. The curve (o-●) respresents the formation of nonprotein
nitrogen in milk at pH 6.7 by the action of rennet. The reaction mixture is
maintained at 2°C in order to prevent curd formation. The distance between the
open circles and the full circles (o-●) represents the time required for curd
formation after the milk has been heated to 40°C. After 60 min of rennet ac-
tion clotting is almost instantaneous at 40°C (Yamamoto, 1975).
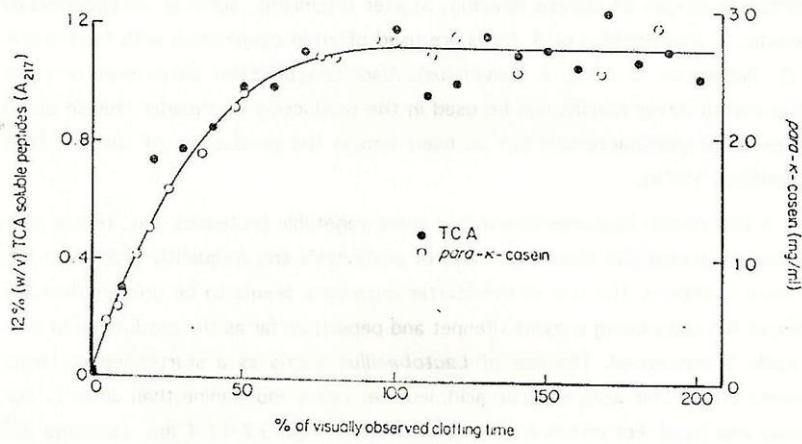
Fig. 8. Enzymic stage of milk coagulation.

7.2.c. Proteolysis during cheese ripening:

Recently, many investigators have reported on enzyme preparation to be used in the manufacture of cheese to increase the flavour and texture qualities (Abou El-Ella *et al.*, 1976; Sood & Kosikowski, 1979; Soliman *et al.*, 1980; El-Fak *et al.*, 1982 Hefnawy, 1986 and Nasr *et al.*, 1990).

Proteolytic activity during the ripening of cheeses can also be extensive. In hard cheese, 25-35% of the insoluble protein of the curd may be converted into soluble protein. In soft cheese varieties such as Brie, Comembert or Limburger, over 80% of the insoluble protein is converted to water-soluble compounds, such as peptides, amino acids and ammonia (Foster *et al.*, 1957 and Richardson, 1975). Apart from the participation of starter cultures, proteolysis depends in part, on the coagulation enzyme system used in the formation of the curd. The production of bitter flavors is generally attributed to the formation of bitter peptids, which are formed faster than they can be broken down further by proteolytic enzymes of the starter organisms (Price and Stevens, 1989).

It is impossible to treat the subject of fat and protein hydrolysis in cheese with any degree of coherence. This is due to the fact that most of the hydrolytic activity is derived from the action of enzymes produced by the numerous species of microorganisms in cheese and the enzyme coagulant used. The microflora of cheese is complex and changeable because different types of bacteria or molds predominate at different stages of cheese ripening. Starter organisms, such as *Streptococcus cremoris*, *S. thermophilus* or *S. lactis* are used often in conjunction with *Lactobacillus* (*L. bulgaricus*, *L. lactis*, *L. helveticus*). Also, *Leuconostoc citrovorum* or other citrate metabolizing species can be used in the production of cheeder cheese and a species of propionibacterium can be used also in the production of swiss cheese (Richardson, 1975).

It has already been mentioned that some vegetable proteases, e.g., rennet substitutes and pancreatin show high rates of proteolysis and frequently produce a bitter taste in cheese. The role of the starter organisms seems to be greater than the effect of the coagulating enzyme (Rennet and pepsin) as far as the production of amino acids is concerned. The use of *Lactobacillus lactis* as a starter led to larger amounts of glutamic acid, aspartic acid, leucine, valine and alanine than when *L. bulgaricus* was used. For instance, *L. lactis* provolone had 5.7-11.4 mg. (average 9.2 mg) of glutamic acid per gram of cheese solids while *L. bulgaricus* provolone showed 2.9-5.4 mg (average 3.8 mg) (Richardson, 1975).
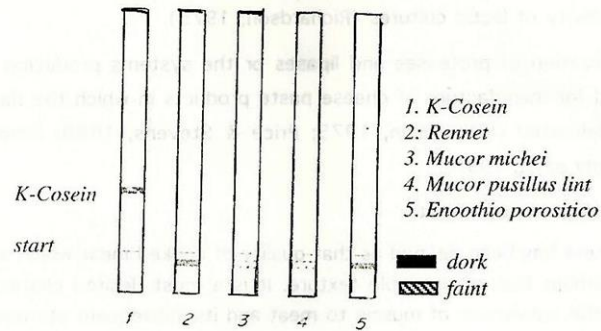
1. K-Cosein
2. Rennet
3. Mucor michei
4. Mucor pusillus lint
5. Enoothio porositico

■ dork
▨ faint

Fig. 9. Polyacrylamide gel electrophoretic patterns of K-caseim before and after treatment with calf and microbial rennets (From Vanderpoorten and Weckx, 1972).
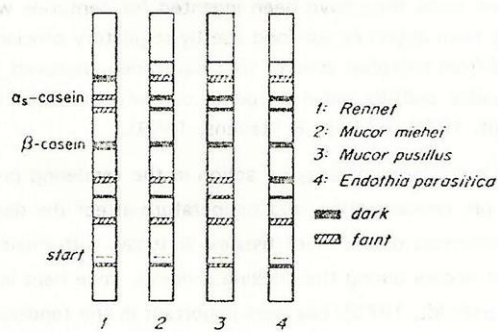

1: Rennet
2: Mucor miehei
3: Mucor pusillus
4: Endothia parasitica

▨ dark
▨ faint

Fig. 10. Polyacrylamide gel electrophoretic patterns of 4-week-old gouda cheese prepared with calf and microbial rennets (From Vanderpoorten and Weckx, 1972).

Deaminases, decarboxylases, aminases, transaminases and other enzymes are active in cheese processing and lead to the production of a variety of amino acids, amines, keto acids, aldehydes, ammonia and other derivatives (Richardson, 1975).

In an acid-coagulated product, such as cottage cheese, protease activity would not be expected to play a role, but found that direct acid cottage cheese required the proteolytic activity of lactic cultures (Richardson, 1975).

The utilization of proteases and lipases or the systems producing them has been reported for manufacture of cheese paste products in which the flavor is significantly accelerated (Richardson, 1975; Price & Stevens, 1989; Ammar et al., 1994 and Shady et al., 1997).

7.3. Tenderization of meat:

Tenderness has been defined as that quality of cooked meat which are of easy chewability without loss of desirable texture. It is a most desired characteristic of meat. During the conversion of muscle to meat and its subsequent storage, endogenous enzymes activity is the main factor contributing to the development of tenderness (Bernholdt, 1975 and Dransfield & Etherington, 1981).

Today enzymes are widely accepted by the food industry as well as by the housewife. Enzymes important to meat tenderization are, of course, the proteolytic enzymes. Most of those used commercially in the tenderization of meat are from food sources, and since they have been ingested for centuries without deleterious effect they have been approved for food use by regulatory officials. There are a few enzymes derived from microbial sources that have been approved for meat (e.g., enzymes form *Bacillus subtilis* and *Aspergillus oryzae*), but their commercial use is limited (Bernholdt, 1975 and Price & Stevens, 1989).

The exact mechanism of enzyme action in the tendering process is unknown. Factors such as pH, concentration, and temperature affect the degree of action and the resulting tenderness of the meet tissues. Actually, with most enzyme systems, the tenderization occurs during the cooking process, since heat is required for optimum activity (Bernholdt, 1975). Enzymes important in the tenderization of meat are those having the ability to break down the connective tissue proteins as well as the proteins of the muscle fibers, and as a result the meat become more tender. These enzymes also have the ability to hydrolyze, or break down, the soluble beef proteins as well as the proteins of the connective tissue. Panel studies have shown a close relationship between the enzymatic break down of the tissue structure and tenderness response of taste panels (Dransfied and Etherington, 1981).

Proteolytic enzymes are applied to the meat by sprinkling the enzyme powder

on the meat, by dipping the meat in the enzyme solution, or spraying an enzyme so-lution on or into the meat cut by means of an aerosol or other spray or injection systems. In recent years, a method has been developed in which the enzyme is intro-duced directly into the circulatory system of the animal shortly before slaughter (Bernholdt, 1975).

8. USE OF MICROBIAL PROTEASES IN DETERGENTS

Detergents containing enzymes were developed extensively during the 1960s. So that, by 1969 about half of the detergents marketed containing enzymes. Pro-teases are capsulated of enzyme particles using non-ionic surfactants and this has reduced dust in halation problems with powdered enzymes. The principal enzymes used in "biological" detergents are mixtures of amylase and neutral and alkaline pro-teases that are active in the pH range 6.5 to 10.0 and at temperatures from 303 k (30°C) to 333 K (60°C). Detergents often contain oxidizing and chelating agents and so the enzymes must with stand these also. *Bacillus* neutral protease and *Bacillus* al-kaline protease are the most suitable to date. But, others from thermophilic organi-sms having high thermal stability are also being developed (Price and Stevens, 1989 and Abdel-Rehim *et al.*, 1996).

9. MISCELLANEOUS USES OF PROTEOLYTIC ENZYMES IN PRO-TEINACEOUS FOODS

While the tenderization of meat is probably the most significant application of proteolytic enzymes in food processing, there are several other food applications worthy of note.

Many investigators point out that condiments, such as soy sauce and protein hydrolysates, may be prepared by enzymatic hydrolysis of plant, meat, fish, and milk proteins. While the basic process calls for hydrolysis by acid under pressure, the use of enzymes results in a much simpler process and reduces the destruction of amino acids. These hydrolysates are often used commercially as flavoring agents in food products (Bernholdt, 1975; Gerhartz, 1990 and Nout & Robouts, 1990).

In the fish indstry enzymes have been employed in processing of inedible fish and scrap fish to produce oil, meal and fish solubles. As in the case of protein hdy-rolysates, enzymes are used to speed up the process. However, the use of enzymes, if not properly controlled, will lead to bitter off-flavors. These off-flavors are probably due to the presence of specific polypeptides that have a naturally bitter flavor. Therefore, it is necessary to not only select the proper proteolytic enzymes,

but to use them in a process that will provide for control of their activity. This often requires utilizing a processing step wherin the enzymes are destroyed before the bitter off-flavors are produced.

In the preparation of fish protein for human consumption it is found that by treating the fish protein with enzymes derived from the genus *Aspergillus* they were able to eliminate much of the off-odor and fishy taste. The resulting product therefore is bland and more acceptable for human consumption (Bernholdt, 1975, and Gerhartz, 1990).

There are several unique applications of enzymes that can be noted. For example, it is reported that the enzymatic digestion of soy bean protein can be used to produce "soy bean sake", a Japanes wine. The resulting product is reported to compare very favorably with "natural" sake. Enzymes have also been used to produce a condiment by peoples of Thailand, the Philippines, and Viet Nam. Prepared from fish, which has been salted, the product is normally fermented for 6 to 12 months. By adding a fungal protease the process can be shortened considerably (Bermholdt, 1975).

9. CONCLUSION

Proteases are important enzymes used in the food industry. But, the commercially important are extracellular enzymes which are excretions of the living organisms into the surrounding medium. Fungal proteases are used for the modification of wheat protein in bread doughs, and play avital role in the production of cheese. Bacterial proteases and fungal ones are used in the production of various types of tradional foods to make Sou-Sauce, tempeh and miso (fermented soybean paste) and in the production of protein hydrolyzates, condensed fish solubles, as well as feed supplements. Also, they are most commercially available in detergents and as in textile as well as meat tenderization.

Several microbial strains have long been known as strong proteolytic activities. But, must be non-pathogenic and do not produce mycotoxins. However, commercial preparations of enzymes includes *Aspergillus niger, Asperillus oryzae*, some yeasts and *Bacillus subtilis & Bacillus licheniforms* have approved as generally recoginized as safe.

Protease producing microorganism have been found to be very widely distributed among bacteria, fungi, yeast and actinomycetes. Thermophiles are an important group of microorganisms which cause spoilage in the dairy industry. Also, psychrotrophic bacteria are allowed to grow in row milk they produce extracellular protease, which reduce the shelf life of the finished product.

The pH-activity curve is affected by the temperature used for enzyme activation. For the neutral protease of *Bacillus polymyxa*, the optimum pH is shifted from about 7.2 at 20°C to about 6.0 at 45°C. This reflects the poorer stability of an enzyme at higher temperatures.

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