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review

Microbiology and Industrial Biotechnology of Food-Grade Proteases: A Perspective

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Summary

Recent developments in industrial biotechnology have resulted in the exploitation of new and undiscovered microorganisms and the devising of improved methods for enzyme production, which have led to increased yields of the enzyme, thus making a viable industrial process feasible. This review tracks the developments in the field of acidic and neutral protease production with regard to the producers, methods of production and their improvement, the product and its applications.

Key words: neutral protease, acidic protease, fermentation, genetic engineering, purification, enzyme assay

Introduction

Proteases are one of the industrially most important enzymes. These proteolytic (protein digesting) biocatalysts have been in use for many centuries, at first in the dairy industry as milk-clotting agents (rennet) for the manufacture of cheese. Proteases are enzymes that catalyse hydrolytic reactions in which protein molecules are degraded to peptides and amino acids. These constitute a very large and complex group of enzymes, which differ in properties such as substrate specificity, active site and catalytic mechanism, pH and temperature optima and stability profile. The specificity of proteolytic enzymes is governed by the nature of the amino acid and other functional groups (aromatic or aliphatic or sulphur-containing) close to the bond being hydrolysed. Proteases are present in all living beings and play an important role in normal and abnormal physiological conditions, catalysing various metabolic reactions (*1*). They are significant in that they not only govern proteolytic reactions, but also regulate various enzymatic cascades, which ultimately lead to all metabolic reactions involving the breakdown of fats, carbohydrates, *etc.*

According to the Enzyme Commission (EC) classification, proteases belong to group 3 (hydrolases), and sub-group 4 (which hydrolyse peptide bonds). Proteases can be separated into two major groups based on their ability to cleave N- or C- terminal peptide bonds (exopeptidases) or internal peptide bonds (endopeptidases). While aminopeptidases cleave the N-terminal peptide linkage, carboxypeptidases cleave the C-terminal peptide bond. Though exopeptidases find commercial applications (such as leucine aminopeptidase, in the debittering of protein hydrolysates), endopeptidases are industrially more important than exopeptidases. Proteases are also distinguished by the presence or absence of charged groups in positions relative to the susceptible bond (*2*) and are classified on a number of bases: their pH optima (as acidic, neutral or alkaline); substrate specificity (collagenase, keratinase, elastase, *etc.*); or their homology to well-studied proteins such as trypsin, pepsin, *etc.* (trypsin-like, pepsin-like, *etc.*). Hartley (*3*) classified endoproteases in-

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Table 1. Characteristic features of the four types of proteases

Properties	EC No.	Molar mass range/kDa	pH optimum	Temperature optimum/°C	Metal ion requirement(s)	Active site amino acid(s)	Major inhibitor(s)	Major source(s)	References
Aspartic or carboxyl proteases	3.4.23	30–45	3–5	40–55	Ca^{2+}	Aspartate or cysteine	Pepstatin	*Aspergillus*, *Mucor*, *Endothia*, *Rhizopus*, *Penicillium*, *Neurospora*, animal tissue (stomach)	(*4–8*)
Cysteine or thiol proteases	3.4.22	34–35	2–3	40–55	–	Aspartate or cysteine	Indoacetamide, *p*-CMB	*Aspergillus*, stem of pineapple (*Ananas comorus*), latex of fig tree (*Ficus* sp.), papaya (*Carica papaya*), *Streptococcus*, *Clostridium*	
Metallo proteases	3.4.24	19–37	5–7	65–85	Zn^{2+}, Ca^{2+}	Phenyl-alanine or leucine	Chelating agents such as EDTA, EGTA	*Bacillus*, *Aspergillus*, *Penicillium*, *Pseudomonas*, *Streptomyces*	(*9–11*)
Serine proteases	3.4.21	18–35	6–11	50–70	Ca^{2+}	Serine, histidine and aspartate	PMSF, DIFP, EDTA, soybean trypsin inhibitor, phosphate buffers, indole, phenol, triamino acetic acid	*Bacillus*, *Aspergillus*, animal tissue (gut), *Tritirachium album* (thermostable)	(*4,12–14*)

to four groups on the basis of their active site and sensitivity to various inhibitors, whose properties are briefed in Table 1 (*4–14*).

Morihara (*15*) subdivided serine proteases into four subgroups: trypsin-like proteinases, alkaline proteinases, *Myxobacter* α-lytic proteinases and staphylococcal proteinases, of which alkaline proteases are industrially most significant. Subtilisin Carlsberg (*16*) produced by *Bacillus licheniformis*, Subtilisin BPN (name derived from a commercial enzyme preparation, bacterial protease Nagarase), and Subtilisin Novo (*17*) are some serine alkaline proteases. These are employed in the dehairing and bating of hides, meat tenderizing, cheese flavour development, treatment of flour in the manufacture of baked goods, improvement of dough texture, flavour, and colour in cookies, *etc.*, improving digestibility of animal feeds, reducing viscosity of fish press water, recovery of fish oils, silk degumming, proteinaceous stain removal in fabrics, *etc.* (*18–23*). Some of the commercial alkaline proteases used in laundry detergents are Alcalase®, Savinase®, Everlase™, Esperase®, *etc.* produced by Novozymes, Denmark. Alcalase® is also used for silk degumming. Some of the alkaline proteases used in the leather industry are Greasex, NovoCor™, NUE (Novo Unhairing Enzyme), PTN (Pancreatic Trypsin Novo), and Pyrase®. The applications of acidic and neutral proteases are detailed at the end of this review.

The estimated value of the worldwide sales of industrial enzymes is $1 million a year, with proteases accounting for about 60 % of it (*24*). Recent developments in enzyme technology have seen the usage of acidic, neutral, and alkaline proteases in their respective industry as well as the application of their recombinant forms. This article aims to review information pertaining to microbial acidic and neutral proteases – their sources, production techniques, and major applications.

Sources

Acidic proteases

These are found in animal cells, moulds and yeasts, but seldom in bacteria. The microbial rennin-like enzymes are derived from *Mucor miehei* (*25,26*), *M. pusillus* and *Endothia parasitica*. *M. hiemalis*, *M. racemosus*, and *M. bacilliformis* (*27,28*) are other species of *Mucor* that present protease activity of commercial value. Pepsin-like acid proteases are derived from *Aspergillus* spp. (*29*) and *Rhizopus* spp. (*30*). Calf rennet, composing of 88–94 % chymosin and 6–12 % pepsin, is extracted from the fourth stomach of the unweaned calf (*31*). Many of them contain aspartate as the active amino acid (*8*) and their specificity is defined by the presence of aromatic or bulky side chains at both sides of the cleaving bond. The carbohydrate content of these enzymes confers heat stability to these biocatalysts (*32*). Production of acid proteases from thermophilic *Penicillium* sp. has also been reported (*33*).

Neutral proteases

Papain (from *Carica papaya*), bromelain (from *Ananas comorus*), ficin (from *Ficus* sp.) are some of the proteases of botanical origin. These are cysteine proteases, papain being the most thermostable (*7*). The bacteria, *Clostridium histolyticum* and *Streptococcus* spp., produce clostripain and streptopain, respectively, which are also cysteine proteases. Godfrey and Reichelt (*34*) reported on a *Bacillus subtilis* neutral metalloprotease. Other *Bacillus* spp. that produce this enzyme include *B. cereus*, *B. megaterium*, *B. stearothermophilus* (*35*), *B. thuringiensis* (*36*), *B. pumilus* (*37*), *B. polymyxa* (*38*), *B. licheniformis* (*39*), and *B. amyloliquefaciens*. *B. stearothermophilus* produces a thermostable protease, thermolysin, which retains nearly 50 % of its activity after 1 h at 80 °C (*40*). *Pseudomonas aeru-*

ginosa and *Streptomyces griseus* are also producers of neutral metalloprotease (*41*).

Fungal neutral proteases are the most important component of commercial fungal protease preparations, which have applications in baking, food processing, protein modification, and in the leather, animal feeds and pharmaceutical industries. *Aspergillus oryzae* is the predominant fungal source of the enzyme (*42,43*). Its affinity for hydrophobic amino acids provides an advantage for its utilization as a debittering agent. Another fungus belonging to this species is *A. sojae*, which produces a zinc-dependent metalloprotease (*44*). EDTA sensitive proteinases have also been identified from other *Aspergillus* spp. (*45,46*). Neutral metalloproteases display specificity towards peptide linkages that contain hydrophobic amino acids to the amino side. Species of *Penicillium* are also reported producers of neutral proteases (*47,48*).

Enzyme Production

Isolation of proteolytic microbes

The first stage in the development of an industrial enzyme production process is to isolate a microbial strain that might have the potential to produce the enzyme in commercial yields. Extracellular microbial products, like enzymes, are screened using a plate assay and the organism's production capability can be related to the zone of hydrolysis (of the substrate) around the colony. Vermelho *et al.* (*49*) developed a plate assay for screening proteolytic microorganisms using different agar media containing protein substrates such as gelatin, bovine serum albumin, and hemoglobin. Some exceptions have been reported such as the protease produced by *Bacillus licheniformis*, which produces very narrow zones of hydrolysis on casein agar plates in spite of large enzyme production by submerged culture (*50*). Hence, care must be taken with enzymes such as proteases, which may be inducible or repressible, when designing a media, which will induce rather than repress production of the enzyme. After isolating the suitable strain, it is necessary to increase enzyme production by optimising process parameters, such as production media – composition, pH, volume (in case of SmF), moisture content (in case of SSF), concentration of mineral salts, age and size of the inoculum, fermentation time and temperature, carbon, organic and inorganic nitrogen supplement, *etc.*

Media formulation

Media rich in nitrogen sources, such as soybean meal, casein, gelatin (*51*), corn steep liquor, distiller's solubles, brewer's yeast, and carbohydrate sources such as starch, ground barley, or lactose are generally used for protease production (*12,50,52*). High carbohydrate concentrations repress enzyme production. Free amino acids also have a similar effect on protease production (*53*). Isoleucine and proline repress protease production by *Bacillus* sp. Peptides and proteins have the reverse effect, inducing protease production in a number of microorganisms such as acid protease production by *Mucor miehei* (*54*). Cheap nitrogen sources such as corn steep liquor have been used for the production of a thermostable acid protease by a strain of *Aspergillus niger* F2078 (*55*). Acid protease production has also been carried out using sweet potato residue (*56*). Sunflower meal is another low cost substrate which has been successfully used for protease production (*57*). Additives such as vitamins (like biotin), growth promoters (1-naphthyl acetic acid), *etc.* enhance protease production (*58*).

Fermentation

The nature of the fermentation, solid or submerged, influences various aspects of the growth of the microorganism as well as enzyme production (*59,60*). Depending on the culture conditions used, two different forms of the same enzyme are also expressed, such as the two acid proteases produced by the solid-state fermentation (SSF) of bran by *Aspergillus oryzae*, which differed in their carbohydrate content, one being a glycoprotein and the other devoid of any carbohydrate (*61*). Moulds grown under SSF produce more spores and fruiting bodies, thus contributing to a distinction in the physiology, which has a positive influence on enzyme production (*2*).

Submerged fermentation (SmF)

Submerged fermentations are usually carried out with a substrate, which is either dissolved or remains suspended in an aqueous medium. SmFs carried out with different substrates result in varying protease activities. While simple substrates such as casein and gelatine yield low enzyme units, more complex substrates such as soybean meal and wheat bran result in higher protease activities. Supplementation of a nitrogen-rich medium with glucose also enhances protease production (*62*). Many types of SmFs have been described, such as batch, fed-batch, and continuous. Continuous cultures have proven to yield better enzyme units than batch fermentations (*63,64*). Statistical optimisation of fermentation parameters results in optimum enzyme production (*65*). Use of relatively newer experimental designs such as radial basis function (RBF), artificial neural network (ANN), and response surface methodology (RSM) have been used to study the relation between various interacting parameters during the course of a fermentation for enzyme production (*66*).

Solid-state fermentation (SSF)

Solid-state fermentations involve microbial modification of a solid, undissolved substrate, in which microbial cultures are grown on a moist solid with little or no free water, although capillary water may be present (*67*). Concentration of solids is generally high, whereas water content is usually low (*68*). With regard to cost economics, SSF has been proved to be more efficient than SmF (*69*). The product can be recovered in highly concentrated form as compared to those obtained by submerged fermentations. Three methods of fermentation – drum, pot and tray method have been described by Aidoo *et al.* (*70*) for strains of *Aspergillus*, *Mucor*, *Penicillium*, and *Rhizopus* and have proved very successful for *A. oryzae* fermentations. A number of agro and agro-industrial residues have been exploited to analyse their potential to be used as substrates for protease production (*71–73*). Proteases from *Aspergillus* sp. and *Mucor pusillus* are produced in higher yields in SSF using wheat bran as the solid media with 50 % more moisture content than in SmF. Sometimes, a combination of two or even three dif-

ferent substrates gives higher enzyme yields than each of the substrates used individually. Rice hulls when used in combination with rice bran in the mass ratio of 7:3 proved effective for protease production by *Aspergillus oryzae* NRRL 2160 (*74*). Neutral protease production by *Aspergillus oryzae* NRRL 2217 was affected by the fermentation of the combined substrate, wheat bran and coconut oil cake (3:1) (*75*).

The production of the enzymes is affected at the molecular level. Te Biesebeke *et al.* (*76*) studied the transcriptional regulation of two protease encoding genes in liquid and solid-state cultivations of *A. oryzae* using wheat as a substrate and observed that while in SSF the nutrients were directly consumed by the fungus after liberation from the substrate, in SmF they were released in excess from the medium during fungal growth, and hence nutrient repression occurred, which in turn affected the expression of the enzyme. Sandhya *et al*. (*77*) observed a 3.5-fold higher enzyme production by *A. oryzae* in SSF than in SmF using wheat bran as a substrate.

Other fermentation techniques

SmF and SSF processes are generally applied for enzyme production, though various modifications of these techniques have been reported. A membrane-surface liquid culture (MSLC) method for the production of neutral protease by *A. oryzae* was developed, in which the microorganism is grown on a microporous membrane surface exposed to the air with the other side of the membrane in contact with the liquid nutrient medium (*78,79*).

Immobilisation

Immobilisation matrices include the conventional supports such as alginate, agar, carrageenan, polyacrylamide, glass beads, polyurethane foam, metal surfaces, *etc.* Cheap supports such as vermiculite have been used to immobilise neutral protease by adsorption (*80*). Immobilised enzymes have been found to retain their activity better than their free counterparts. In addition to enzymes, microbial cells have also been immobilised for enzyme production. The immobilised cells of bacteria (*81*) or the mycelia of fungus (*82*) are found to produce higher yields of enzyme than the non-immobilised ones.

Improving enzyme production

Strain improvement

Enzyme production can be increased by strain improvement. This is usually done by mutating the microorganism that produces the enzyme by techniques such as classical mutagenesis, which involves exposing the microbe to physical mutagens such as X-rays, γ-rays, UV rays, *etc.*, and chemical mutagens such as NTG, EMS, *etc.* Neutral protease production by *B. stearothermophilus* was improved 4-fold by mutating the strain using EMS (*83*).

Genetically engineered proteases

In addition to the conventional techniques of enzyme production by fermentation using both the wild type and mutant strain of microorganisms, and performing strain improvement by classical mutagenesis, genetic engineering has played a major role in substantially improving enzyme production, both in terms of quantity and quality. *E. coli* has been the most widely used expression vector in the cloning of genes for the purpose of overproduction of the gene product, mainly due to reasons such as its non-fastidious growth on any minimal medium, its rapid doubling time of about 20 minutes, the high copy number of plasmids it produces, *etc. E. coli* TG1 was transformed with an expression plasmid pAQN carrying the gene aqualysin I (AQI), which was derived from the *Thermus aquaticus* YT-1, to produce a thermophilic protease. Such a procedure resulted in an enzyme yield about 44-fold higher than what was produced by *T. aquaticus* YT-1 (*84*). A similar increase in neutral protease yield of 30 % was obtained when the *vhb* (*Vitroscella* hemoglobin) gene was introduced into *B. subtilis* (*85*). An organism that carries the gene for an enzyme might not be able to express the gene product due to the lack of a strong promoter for the gene or due to the absence of the promoter sequence itself. The gene for this enzyme can be cloned in a microorganism which possesses a strong promoter for the gene to be expressed (*86*). The filamentous fungi have also been identified as efficient systems for the expression of heterologous proteins (*87*). *Aspergillus* sp. has been widely used as a host for the expression of recombinant proteins of both fungal and non-fungal origin, and this includes the acid protease, bovine chymosin (*88*). Other hosts used for the expression of bovine chymosin include *E. coli*, *B. subtilis*, *Saccharomyces cerevisiae*, *Kluveromyces lactis*, and baby hamster kidney (BHK) cells (*89*). The expression of a recombinant protein depends on the composition of the fermentation medium. A gene for a neutral metal- (zinc-) dependent protease from *A. oryzae*, when cloned into *S. cerevisiae*, was expressed as an inactive protein and as an active enzyme, depending on the absence or presence of zinc (the metal on which the enzyme was dependent for its activity) in the production medium (*90*). Thermostability of neutral protease has been increased by site-directed mutagenesis, wherein an amino acid in the enzyme replaces another amino acid, thus positively contributing to heat stability of the enzyme (*91,92*). The successful secretion of recombinant proteins largely depends on various environmental factors. For the secretion of correctly folded proteins and avoidance of problems involved in the recovery of such recombinants, the recombinant microorganism can be made to excrete the protein in the periplasmic space, as in the case of bacteria such as *E. coli* (*93*).

Downstream Processing

A wide range of techniques is available for the recovery of the product from the fermented substrate and the choice depends on the source, *i.e.* intracellular or extracellular, scale of operation, enzyme stability, *etc.*

Intracellular enzymes

The majority of the secreted enzymes are external or peripheral membrane proteins and can be extracted into the culture filtrate by certain extraction techniques detailed below. But a small proportion might be firmly bound with the membrane (integral proteins) or may be intracellular. Repeated extraction of cells does not re-

lease the enzyme; cellular disintegration techniques liberate the enzyme into the soluble fraction. Methods such as pressure homogenisation, wherein the cells are subjected to high pressure through a narrow orifice at low outlet temperature (–20 °C) resulting in ice crystal formation, are useful for cell disruption. Grinding in the presence of abrasive materials such as alumina in a mortar and pestle (small scale) or glass or metal beads in a grinding mill (large scale) are also used for disrupting cells.

Extracellular enzymes

In the case of extracellular enzymes, the fermented solids are crumbled and extraction is done in either a batch or continuous process. Water, aqueous buffers, diluted solutions of salts (*e.g.* 0.9 % sodium chloride), 1 % glycerol (*94*), or diluted (0.1 %) solutions of non-ionic detergents such as Triton X-100, Tween 20, Tween 40, Tween 60, Tween 80, *etc.* are used to leach enzymes. As the detergents act on both cell wall and cytoplasmic membrane, making the cell permeable to certain protein materials, these are useful for the extraction of membrane-bound enzymes in addition to extracellular enzymes.

Generally about 50–60-minute contact period is allowed. The solvent-to-solid ratio ranges from 1 to 8; higher the value, more diluted the solution will be, though the leached product fraction will increase. Extraction temperature is around 30 °C, but a temperature of 4–10 °C can be maintained to avoid denaturation, proteolysis, and microbial growth. Extraction can be carried out at pH close to 7; however, to avoid precipitation, pH close to the pI (isoelectric pH/point) of that enzyme is not recommended (*95*). The optimum pH for the enzyme may not be the same as that required for optimal leaching. *Rhizopus oligosporus* acid protease is best extracted from fermented rice bran at pH=7, though it is most stable at pH=4 (*96*).

Certain novel methods of downstream processing have proven to be effective in the recovery of proteases. Acid protease has been recovered from solid bran fermented by *Mucor miehei* by semicontinuous multiple contact forced percolation method (*97*).

Enzyme preparations are generally supplied in liquid or solid form, irrespective of its source. In liquid preparations, the enzyme is stabilized against chemical and microbial denaturation and degradation by the addition of high concentrations of salts such as ammonium sulphate and preservatives such as glycerol to increase product shelf life. The pH of the liquid should be adjusted to optimise stability. Specific enzymes might be stabilised by the addition of divalent cations, which act as either oxidising or reducing agents. While some of these additives may also act as preservatives, specific anti-microbial preservatives are also added. Enzymes that show greater stability in solid form are spray-dried to obtain the powdered form of the enzyme (*2*).

Enzyme Purification

Enzyme purification is a complex process and a number of methods are usually applied in sequence for the purification of enzyme. The objectives of a scheme for the purification of an enzyme are: (*i*) high degree of purity, (*ii*) high overall recovery of activity of enzyme, and (*iii*) reproducibility. The extraction methods release the enzyme into the medium in addition to various cell components, such as nucleic acids and polysaccharides. The release of nucleic acid makes the solution more viscous. It forms the major contaminant along with the broken cell part. After the homogenisation of the original raw material, the first step in purification is separation of the cell debris by either differential sedimentation or precipitation, and it can be affected by centrifugation or filtration to obtain a more or less clear extract. Digestion with nuclease may reduce the viscosity of the solution and can be removed in later stages of purification. Thus, soluble proteins may be separated from organelle-sequestered proteins.

After the removal of nucleic acid and debris from the cell extract, a supernatant containing the enzyme can be further subjected to the process of removal of unwanted contaminants, small organic and inorganic molecules, other proteins and water by salt (ammonium sulphate) or solvent (acetone/ethanol) precipitation followed by dialysis against its corresponding buffer for removal of the enzyme-bound salts. Ultrafiltration is another alternative technique used to separate proteins by passing water and other small molecules through a semi-permeable membrane, thereby concentrating the protein molecules in the solution. This technique is faster and easier to handle than the two-step process of precipitation and dialysis.

Chromatographic techniques such as ion exchange, gel filtration, *etc.* give rise to purer fractions of the enzyme with a significant increase in its specific activity. Such purification procedures are also efficient ways to deduce the molecular mass of the enzyme protein using a mixture of proteins of known molecular mass as a reference standard.

Tsujita and Endo (*32*) characterised two molecular forms of acid protease, from a strain of *A. oryzae*, of molecular mass 60 and 42 kDa, respectively, by chromatography on Sephadex G-100 and CM-Sephadex C-50. Adsorption chromatography, such as on resins like Amberlite XAD, has been used to purify protease from the fermentation medium (*98*). Dye-affinity membrane chromatography is another technique, which has been utilised for the purification of neutral protease (*99*). Another type of affinity chromatography is purifying the enzyme by using its inhibitor as a ligand (*100*). Aqueous separation of enzymes can also be achieved using solvents such as butanol and octanol (*101*). Another aqueous two-phase extraction was described to separate a neutral protease using the solvent, poly(ethylene glycol) palmitate (*102*). A protease purification method based on treatment with activated charcoal and hydrogen peroxide, followed by acetone precipitation, was developed by Tunga *et al.* (*103*). A newer purification method for proteins is affinity tagging, which is based on the production of fusion proteins by the microorganism, wherein the protease fused with the affinity tag such as a polyhistidine residue is produced (*104*). This recombinant protease can then be purified using an immobilized transition metal, usually Ni^{2+}, to which the polyhistidine tag readily binds. Other affinity tags and their matrix for elution include calmodulin-binding peptide and calmodulin (*105*), albumin-binding protein and albumin (*106*), *etc.*

Enzyme Assay

The original casein assay was first described by Kunitz (*107*) and later modified by Detmar and Vogels (*108*). It involved TCA (trichloroacetic acid) precipitation of the undigested substrate, followed by photometric quantification of the released aromatic amino acids, using L-tyrosine as a standard.

Due to their compact conformation, native proteins are generally not very susceptible to degradation by proteases (*109*). Protein substrates for proteases are most often hemoglobin (*110*) or casein and must be completely soluble in buffer. Casein precipitates below 6, so it is used at neutral to alkaline pHs. Hemoglobin must be denatured before use, either by treatment with acid (if the assay is at acidic pH) or urea (neutral to alkaline pH assay). Peptide bonds are more exposed and liable to proteolytic attack when proteins are unfolded due to denaturation. Diazotised protein allows measurement of solubilised peptide with a visible range colorimeter.

»Hammarsten« casein is the recommended form of the substrate for enzyme assay, as it represents the most standard casein preparation available (*111*). Both the pH and temperature optimum for maximal activity vary according to the source of the enzyme. Hence, pH and temperature curves should be carried out to determine the individual requirements of the system.

Assay for proteases generally involves incubating the enzyme with its substrate for a specific time period, arresting the reaction with TCA, and measuring the absorbance of the solubilised peptide. A tyrosine standard curve can be used for this purpose, as this amino acid absorbs strongly at 280 nm. Between 0 and 75 μg/mL, the absorbance of tyrosine follows Beer-Lambert's law. However, if the method of Lowry *et al.* (*112*) is used to analyse the soluble peptides, higher protease values result (*9*). For extremely accurate measurements of protein hydrolysis, the amount of TCA soluble nitrogen can be determined by carrying out Kjeldahl nitrogen analysis. Buffered TCA gives superior enzyme linearity as compared to an aqueous TCA solution (*113*). A casein-based assay (*114*) is: to 5 mL of casein (12 mg/mL in 0.03 M phosphate buffer, pH=7.5), add 1 mL of enzyme solution and after 10-minute reaction, add 5 mL of buffered TCA. After 30 minutes, the mixture is filtered and absorbance at 275 nm is measured. For Folin-Lowry quantitation, to 1 mL of filtrate add 1 mL of alkaline buffer (1 M Na_2CO_3, 0.25 M NaOH), 0.4 mL of copper reagent (0.1 % $CuSO_4 \cdot 5H_2O$, 0.2 % Na-K tartarate), mix and allow to stand for 10 minutes. Then add 0.75 mL of diluted phenol reagent (Folin-Ciocalteau reagent diluted with 3 volumes of water), mix, wait for 10 minutes and measure absorbance at 700 nm versus an appropriate reagent blank. A plot of log absorbance versus log protein is linear over the range of 3–400 μg of protein (*e.g.* BSA standard).

Denatured hemoglobin can also be used as a substrate and is preferred over casein, since the complete amino acid sequence is known. A completely soluble hemoglobin is used for this purpose. In this method, known as the hemoglobin units tyrosine (HUT) assay, at the end of incubation of the enzyme with the substrate (here, hemoglobin), the undigested protein is precipitated with an equal volume of 10 % mass per volume ratio of TCA and filtered through Whatman #42 filter paper. The absorbance of the filtrate is determined at 280 nm and compared to that of a 0 to 75 μg/mL of tyrosine standard curve. One HUT unit is defined as the amount of enzyme that produces per minute an absorbance at 280 nm equivalent to 1.10 μg/mL of tyrosine (1.10 μg/mL of tyrosine=0.0084 *A* units).

Hemoglobin is the preferred substrate under acidic assay conditions, *i.e.* for acid proteases, the HUT assay being usually carried out at pH=4.5. The pK of hemoglobin is 6.8 and the pK of casein is 4.8. Both substrates are electrolytes and their solubility and secondary structure will vary as a function of pH.

Small synthetic molecules give a change in a spectrophotometric absorbance as they are hydrolysed, making a continuous assay possible. FAGLA [3-(2-furylacryloyl)--glycyl-L-leucinamide] is a substrate for metalloprotease (neutral protease) and Z-Gly-Phe [*N*-carbobenzoxy glycyl-L-phenylalanine] for carboxypeptidase. Derivatisation of the proteolytic products with dinitrofluorobenzene (DNFB) can be done to obtain the dinitrophenol (DNP) derivative and the protease activity can be monitored by the increase in the formation of NH_2 groups (*115*). Neutral proteases can be assayed using azocoll (ground, insoluble collagen conjugated with an azo dye) as this substrate gives an absorption maximum at 516 nm at a pH of 7.8, but is not significantly altered at acid pH (*116*). Acidic protease can also be assayed using a chromogenic substrate such as azocasein. Aminopeptidases are assayed using an amino acid derivative such as L-leucine--β-naphthylamide. For serine and/or sulphydryl proteases, the following synthetic substrates have been used for assay purposes:

1. *N*-acetyl-L-tyrosine ethyl ester (ATEE)
2. *N*-benzoyl-L-arginine ethyl ester (BAEE)
3. *N*-benzoyl-L-arginine *p*-nitroanilide (BAPA)
4. *N*-benzoyl-L-tyrosine ethyl ester (BTEE)
5. *N*-tosyl-L-arginine methyl ester (TAME)
6. *N*-carbobenzoxy-L-tyrosine *p*-nitrophenyl ester (Z-Tyr-pNP).

Certain fluorogenic substrates have been used to study enzyme kinetics. Fluorescent peptide substrates such as of the type A-Phe-Phe-B and bearing an amino terminal fluorescent probe group (such as dansyl or mansyl) have been used to investigate the rate of formation of A-Phe, *i.e.* the rate of reduction in the fluorescence of the substrate is measured (*117*).

Applications of Proteases

- The most significant property of acidic proteases is the ability to coagulate proteins, as is evidenced by their widespread application in the dairy industry for their ability to coagulate milk protein (casein) to form curds from which cheese is prepared after the removal of whey (*118*). By virtue of this property, microbial acidic proteases have largely replaced the calf enzyme (rennet), facilitating the expansion of the cheese manufacture industry whose development was hurdled by animal rights issues. A protease from *Pseu-*

domonas fluorescens R098 has been reported to hydrolyse the peptides found in cheese, which are responsible for the bitter taste, and thus finds application as a debittering agent (*119*).

- The enzymes used in the food industry include Alcalase®, Neutrase®, Esperase®, Protamex™, and Novozym® FM. These enzymes are commercially marketed by Novozymes, Denmark. These bacterial proteases are used for improving the functional, nutritional and flavour properties of proteins. Neutrase® is a bacterial protease which is used in alcohol production for improving yeast growth. In baking, it is used to degrade proteins in flour for biscuits, crackers and cookies. In brewing, it is used for extracting sufficient proteins from malt and barley and for obtaining the desired level of nitrogen nutrients. It is also involved in lactose reduction and flavour modification in dairy applications.
- Acid protease from *Aspergillus saitoi*, aspergillopepsin I is commercially marketed as Molsin F by Kikkoman Corp., Japan. The enzyme is useful for the production of seasoning materials from the foods containing various proteins, the degradation of the turbidity complex resulting from protein in fruit juices and alcoholic liquors, and the improvement of quality of protein-rich foods. Flavourzyme™ is a fungal complex of exopeptidases and endoproteases derived from *A. oryzae* used for extensive hydrolysis of proteins. Kojizyme™ is a similar complex, which finds application in the fermentation of soy sauce. These enzymes are also products of Novozymes, Denmark.
- Other applications, which exploit the hydrolytic property of proteases, include soy protein hydrolysis, soy sauce production, gelatine hydrolysis, casein and whey protein hydrolysis, meat protein recovery, fish protein hydrolysis, and meat tenderization. Proteases from *Bacillus subtilis* have been used to deproteinize crustacean waste to produce chitin (*120*). Another novel application of neutral protease is in combination with the physical method of ultrasonication for the extraction of rice starch (*121*).
- Papain and bromelain have been used to improve the nutritional value of feeds. Papain has been used to manufacture yeast extract and SCP. It has also been used in the extraction of flavour and colour compounds from plants. Papain also finds use in the manufacture of microbiological media.
- Neutral protease (dispase) is a bacterial enzyme produced by *Bacillus polymyxa* that hydrolyses N-terminal peptide bonds of non-polar amino acid residues and is classified as an aminopeptidase. This enzyme is marketed by many companies such as Invitrogen Corp., USA, BD Biosciences, USA, Worthington Biochemical Corp., USA, *etc.* Its mild proteolytic action makes the enzyme especially useful for the isolation of primary and secondary cells (subcultivation), since it maintains cell membrane integrity. Dispase is also frequently used as a secondary enzyme in conjunction with collagenase and/or other proteases in many primary cell isolation and tissue dissociation applications. It dissociates fibroblast-like cells more efficiently than epithelial-like cells, so it has also been used for differential isolation and culture applications. Other advantages are its non-mammalian (bacterial) source and its ability to be inhibited by EDTA. Collagenase, which hydrolyses native collagen, has been used for debridement of dermal ulcers and burns and also finds application in the lysis of diseased invertebral disks.
- Urokinase has been used for the treatment of clotting disorders.
- Proteases of *Aspergillus* find application as digestive aids in gastro-intestinal disorders such as dyspepsia.
- Brinase, a plasmin-like acid protease, hydrolyses fibrin and fibrinogen. This is applied on patients on chronic haemodialysis with clotted arteriovenous cannulae. Several minutes of brinase treatment restores vessel function. But the enzyme exhibits toxic side effects and is also inhibited by serum inhibitors.
- Clear-Lens Pro®, also marketed by Novozymes, Denmark, is used in contact lens cleaning formulations to remove protein-based deposits and protein films from contact lenses. This protease produced by submerged fermentation of a *Bacillus*, hydrolyses the protein in the deposits and films, making them readily dissolvable and dispersible in the cleaning liquid. This enzymic formulation is available both as a liquid preparation and as a microgranulate.
- Other uses of neutral proteases include silver recovery from photographic films by gelatin hydrolysis, in membrane and equipment cleaning processes, in biopolishing of wool fabrics, and in protein synthesis.

Conclusions

Proteases have found a wide range of applications in various industries such as food, pharmaceutical, cosmetic, *etc.* and have been widely commercialised by various companies throughout the world. Though the production of these enzymes has been improved significantly by the utilisation of hyper-producing strains of fungi and bacteria and genetically modified microbes as well, efforts are still being done to find newer sources of enzymes, better production techniques and novel applications of these enzymes in unexplored fields.

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