Student-friendly classification for proteases

Fernando Luis Garciá-Carreño*

Enzymes have been classified in a systematic code since the International Union of Biochemistry on the Nomenclature and Classification of Enzyme publication of 1964 and updated in 1972 and 1978. However, in scientific reports, proteases are frequently called by non-systematic terms which are confusing to the novice reader. The aim of this paper is (1) to introduce the proteases classification, (2) provide the key for its understanding, (3) give some current protease terminology used in research work, and (4) show examples of enzymes which have potential application in food technology.

Introduction

Proteases or proteolytic enzymes comprise 50% of the industrial enzymes. They are the subject of substantial research for new sources for biotechnologies. In particular, studies dealing with thermostable proteases from mesophilic and thermophilic organisms and enzymes working at low temperatures from marine organisms are receiving considerable attention (Kristjansson, 1989; Haard, 1991; Garciá-Carreño, 1993a). On the other hand, proteases are involved in general metabolism through the modification of proteins such as the digestion of food proteins, mobilization of tissue protein, neuropeptide, hormone and pro-enzyme processing. These processes are controlled by mechanisms involving gene control, zymogen production, enzyme activation and protease inhibitors. An understanding of them will help control protease activities in biotechnological processes, and eventually lead to the treatment of AIDS when the prevention of the dimerization of the aspartic protease from HIV becomes possible.

Fernando Luis Garciá-Carreño
Centro de Investigaciones Biológicas, PO Box 128, La Paz, BCS, Mexico, 23000. Fax: (682) 53625
*Currently a Postdoctoral Fellow at UC Davis, Department of Food Science and Technology, Davis, CA 95616, USA. Fax: (916) 752 4759

Biotechnology Education, Vol. 3, No. 2, pp. 144-146 1991.
0968-4425/91 $2.00 + 0.00.
Hertz Publishing. Printed in Great Britain.

Like the Linnean classification in organisms, the systematic classification of enzymes follows particular rules. Classification is the arranging into groups with similar activities and catalytic characteristics, while nomenclature is the naming of enzymes according to an international code of principles, rules and recommendations. Its aim is to provide precise communication among researchers, teachers and students.

Classification

Enzymes are classified in a systematic code. However, proteases are frequently called by non-systematic terms. In this paper the words nomenclature and name will be used to denote the systematic appellation and term for the trivial designation of the enzyme. Terms such as cathepsin, trypsin-like or pepsin-like, calpain, nexin, rennin, renin, or alkalase are common in the literature. While some of the terms are easily recognized, the others are confusing to the novice reader. Scientific activities are expected to be as rigorous as possible in the way in which the data is acquired, the conclusions arrived at, and the nomenclature and classification of the elements involved in a discipline of science. In each aspect, different methods of evaluation have been developed. More sensitive and accurate techniques and equipment are now available to the researchers. Statistics help to avoid jumping to conclusions and to be cautious about making generalizations. The classification of living things (Linnean binary taxonomy), the classification of the chemical elements (Mendeleyevian periodical table) and enzyme classification (EC activity grouping) are examples of scientific arrangement of elements in a logical and natural grouping and their connection to each other for the comprehension of the site and properties.

The terminology used by researchers and teachers is not always stated clearly. For example, the common word 'chemical', contrary to what one would expect after several centuries of development, is still a term of controversy (for details see Letters Section in *Chemical & Engineering News*, published by the American Chemical Society from 1989 to 1991). Protease terminology employed by researchers is not an exception.

Trivial names of enzymes are unclear and do not allow one to recognize either the substrate or the nature of the reaction they catalyse. Other trivial names are related to the behaviour of the protease in different conditions and not to its classification. Some examples

are thermophilic or psicrophilic, and acidic, neutral or alkaline proteases.

Unlike most of the enzymes, proteases lack specificity towards a substrate, *i.e.* a definite protein. However, this characteristic does not override the definition of enzyme: "a protein with catalytic properties due to its power of specific activation". Proteases recognize the amino acids involved in the neighbourhood of the peptide bonds forming the protein, instead of the whole molecule. This is the reason why protease activity usually can be assayed using small synthetic substrates. But it circumvents the use of the general EC criteria for naming.

Classification is performed according to the Enzyme Commission (EC), which was established after the 1955 international meeting. The conclusions were published in 1964 and updated in 1972 and 1978. The classification was based on a synthetic name and a four-digit code related to the type of activity catalysed by the enzyme, and not according to its molecular properties. So, enzyme molecules from different origins can be classified by the same code as long as they catalyse the same reaction. Moreover, isoenzymes—different molecules catalysing the same reaction and present in the same organism—are coded into the same four-digit number. For the publication of any enzyme research the EC recommendation suggests the use of the unambiguous systematic nomenclature which includes the classification code, systematic name, and the source of the enzyme. Trivial names may therefore may be used only after they have been properly introduced and defined. The EC classification is not perfect. Evolutionary relation, the key postulate of biology, is not considered by the current classification.

The EC system of classification codes enzyme activities into six main classes, according to the total reaction catalysed. Each enzyme activity is assigned a systematic name and a code number. The systematic name of each enzyme consists of (1) the name of the substrate, and (2) a word ending in '—ase' specifying the kind of reaction carried out by all the enzymes of the group to which it belongs (Dixon and Webb, 1979). Examples of the naming of several enzymes are provided in Table 1. The code number of each enzyme is formed by four digits, separated by a dot. The first digit shows to which main class the enzyme belongs, *i.e.* (1) oxidoreductases; enzymes which catalyse reactions involving electron transfers, (2) transferases, (3) hydrolases, (4) lyases, (5) isomerases and (6) ligases. The second digit indicates the subclass, the third, the subsubclass to which the enzyme belongs, and the fourth the serial number of the enzyme in its subsubclass.

In the case of hydrolases, to which proteases belong, the second digit shows those hydrolysing (1) ester bonds, (2) glycosidic bonds, (3) ether bonds, (4) peptide bonds and (5) C—N bonds other than peptide bonds. As will be noted, the systematic name for the proteases is peptide hydrolases. In this paper the term proteases will be used in a general sense when the subclass of the enzyme is not given. The third digit for the proteases is 11-19, 21-24 and 99. The first group, 11-19, is for those enzymes hydrolysing peptide bonds between amino acids in the amino or carboxylic end of the protein. They are named peptidases. The second set, 21-24, is for enzymes hydrolysing internal peptide bonds. They are named proteinases. The last group, 99, is employed for enzymes whose mode of action remains unknown. Some enzymes have now been reclassified (Dixon and Webb, 1979). They are included in the subclasses 11-19, 21-24, or 99, to avoid confusion with previous and obsolete numbering.

Peptidases, which are known in the scientific jargon as exopeptidases, are classified according to: (1) the site of the splitting, (2) the end of the protein attacked (aminopeptidase or carboxypeptidase), (3) the size of the peptide released (dipeptidase, tripeptidase, *etc.*) and (4) the size restrictions on the length of the susceptible peptide (Agarwal, 1990).

Proteinases, which are called endopeptides, are the major group of industrial proteases. They are classified according to the chemical nature of the amino-acid residues of the enzyme, which are responsible for the chemical activity. Four subsubclasses are recognized by the EC. The 3.4.21 proteases are the serine proteases since the distinctive chemical group involved in the catalytic mechanisms is a residue of serine. The 3.4.22 are the cysteine proteinases or thiol proteinases. The 3.4.23 are the aspartic, carboxyl or acidic proteinases. The 3.4.24 are the metallo-proteinases which are assisted by the divalent metal ion. The 3.4.99 are enzymes whose mechanism of catalysis remains unknown. However, recent knowledge reveals that the mechanism of catalysis of these enzymes can involve serine, cysteine, aspartic, and metallo groups and eventually could be reclassified in the 11-19 or 21-24 subclass. Some examples of the system involved in the arrangement of the enzymes, which include the name and code, are shown in Table 1. As mentioned above, the naming of the peptide hydrolases is complicated because of the lack of specificity toward a substrate from which it builds the name of the enzyme, in the same way that some trivial names have been incorporated into the EC nomenclature as 'recommended names'. Unlike all other enzymes, a systematic name is non-applicable to proteinases.

From an evolutionary point of view, which is not pondered in the EC classification, proteinases are currently grouped into six families which are broad groups of proteins for which there is evidence of an evolutionary relationship and whose sequences have about half of their amino-acid residues in common. New families are arising such as the astacin family, a metallo-peptidase family in which a crayfish

Table 1. Examples of the nomenclature of several enzymes

Recommended name	EC Number	Systematic name
Alcohol dehydrogenase	1.1.1.1	Alcohol: NAD^+ oxidoreductase
Carboxylesterase	3.1.1.1	Carboxylic-ester hydrolase
Aminopeptidase (cytosol)	3.4.11.1	α-Aminoacyl-peptide hydrolase (cytosol)
Tyr-tRNA synthetase	6.1.1.1	L-Tyrosine:$tRNA^{Tyr}$ ligase (AMP-forming)

Table 2. Classes and families of proteases

Class/family	Example of the enzyme	Amino acids involved in the active site
Serine I (mammals)	Trypsin, Chymotrypsin*	Asp(102); Ser(195); His(57)
Serine II (bacterial)	Subtilisin*	Asp(32); Ser(221); His(64)
Cysteine	Papain*, ficin	Cys(25); His(159); Asp(158)
Aspartic	Penicillopepsin*, pepsin, chymosin	Asp(33); Asp(213)
Metallo I (mammals)	Collagenase, carboxypeptidase*	Zn; Glu(270); Try(248)
Metallo II (bacterial)	Thermolysin*, *B. thermoproteolyticus* neutral protease	Zn, Glu(143); His(213)

Data taken from Neurath (1989), Agrawal (1990) and Aunstrup (1980).
*To which enzyme the sequence corresponds

Table 3. The class, type of complex formed, mechanism of catalysis and type of inhibitor linked to proteases

Class	Type of complex	Hydrolysis mechanism	Main type of interaction with inhibitors
Cysteine and serine	Covalent	Nucleophilic groups	Electrophilic
Aspartic and metallo	Non-covalent	Acid/base catalysis	Secondary interactions

(*Astacu sp.*) protease has been the simplest and the first member of the group to be sequenced (Dumermuth *et al.*, 1991). The astacin family includes mammal proteases. Table 2 shows the classes and families of proteases.

Based on the chemical mechanism of catalysis, proteases belong to at least two groups: (1) those enzymes that form covalent enzyme complexes—the serine and cysteine proteinases; and (2) those that do not form covalent enzyme complexes—the aspartic and metallo-proteinases. This property is of major importance since their mechanism of control of the activity by inhibitors is quite different. The covalent complex forming proteases have strong nucleophilic amino acids at their catalytic site. So, their inhibitory molecules must possess highly electrophilic groups including double bonds. The non-covalent complex-forming proteases rely more upon general acid/general base catalysis of the attack of a water molecule. Table 3 shows some characteristics of the proteases such as the type of complex involved in the catalysis.

An alternative non-systematic classification of proteases has emerged (García-Carreño, 1991). Proteases are named on the basis of : (1) their origin, *i.e.* plant or microbial, (2) their traditional or trivial name, (3) their trade name, (4) their specificity toward synthetic substrates, and (5) by analogy with representative enzymes of known mechanism of catalysis. The EC classification of proteases is summarized in Table 4.

A summary of the enzymes which have a potential application in food technology, the reaction catalysed, preferential cleavage, specific substrates, specific inhibitors, systematic name, sources, some comments, and former classification is shown in Table 5. A summary of the most common trivial names used for proteases with potential application in food technology is shown in Table 6.

The meaning of classification in protease classification

The subclass of a protease is usually assessed by the of molecules which either specifically bond to the catalytic centre or which chelate the divalent cations (García-Carreño, 1993b). These molecules are usually called inhibitors

Table 4. Summary of the proteases classification (EC 3.4)

Peptidases EC 3.4.11-17 EC subsubgroup	Specificity and comments
EC 3.4.11	Single residue from N-terminus (α-aminoacyl-peptide hydrolases)
EC 3.4.13	Dipeptide substrates (dipeptide hydrolases)
EC 3.4.14	Splitting of dipeptide units from N-terminus (dipeptidyl-peptide hydrolases)
EC 3.4.15	Splitting of dipeptide units from C-terminus (peptidyl-peptide hydrolases)
EC 3.4.16	Hydrolysing single residue from C-terminus, using serine residue in active centre (serine-carboxypeptidases)
EC 3.4.17	Hydrolysing single residue from C-terminus, requiring divalent cations (metallo-carboxypeptidases)
Proteinases EC 3.4.21-24 **EC subsubgroup**	**Comments**
EC 3.4.21	Serine and histidine residue are involved in the active centre (serine-proteinases)
EC 3.4.22	A cysteine residue is involved in the active centre (thiol- or SH-proteinases)
EC 3.4.23	An aspartic residue is involved in the active centre; have a pH optimum below 5 (carboxyl or acid proteinases, the exception is chymosin)
EC 3.4.24	Anionic amino-acid residues requiring a divalent cation are involved in the active centre (metallo-proteinases)
Not yet allocated proteases	
EC 3.4.99	Those enzymes whose mechanism of hydrolysis is under study or remains unknown

Note: EC subsubgroup 3.4.12 is an ancient group and their enzymes have been transferred to other subsubgroups.

Table 5. Summary of proteases with potential application to food technology

Peptidases

EC 3.4.11. **α-Aminoacyl-peptide hydrolase**
Aminopeptidase, EC 4.3.11.1; R, aminoacyl-peptide + H_2O = amino acid + peptide; SN, α-aminoacyl-peptide hydrolase; S+C, cytosol, animal tissues, yeast, bacteria, a zinc enzyme; formerly 3.4.1.1.

EC 3.4.14. **Dipeptidyl-peptide hydrolases**
Dipeptidyl peptidase, EC 3.4.14.1; R, dipeptidyl-polypeptide + H_2O = dipeptide + polypeptide; SS, Gly-Phe-NH_2 (transferase activity), Gly-Phe-NA (hydrolase activity); SN, dipeptidyl-peptide hydrolase; S+C, animal tissues, SH-protease, known as cathepsin C; formerly 3.4.4.9.

EC 3.4.17. **Metallo-carboxypeptidases**
Carboxypeptidase A, EC 3.4.17.1; R, peptidyl-L-amino acid + H_2O = peptide + L-amino acid; SS, HPA; SN, peptidyl-L- amino acid hydrolase; S+C, pancreas (mammals) hepatopancreas (decapods); formerly 3.4.2.1 and 3.4.12.2.

Carboxypeptidase B, EC 3.4.17.2; R, peptidyl-L-Lys(L-Arg) + H_2O = peptide + L-Lys (or L-Arg); SS, HA; SN, peptidyl-L-Lys (L-Arg) hydrolase; S+C, pancreas; formerly 3.4.2.2 and 3.4.12.3.

Proteinases

EC 3.4.21. **Serine proteinases**
Chymotrypsin, EC 3.4.21.1; PC, Tyr-, Trp-, Phe-, Leu-; SS, SAAPPNA, BTEE; SI, TPCK; SN, N/A; S+C, pancreas (mammals and fish); formed from prochymotrypsinogen in mammals; formerly 3.4.4.5.

Trypsin, EC 3.4.21.4; PC, Arg-, Lys-; SS, BAPNA, BAEE, TAME; SI, SBTI, TLCK, SN, N/A; S+C, pancreas (mammals and fish); formed from protrypsinogen in mammals; formerly 3.4.4.4.

Elastase, EC 3.4.21.11; PC, uncharged non-aromatic side chains; SS, elastin; SN, N/A; S+C, pancreas, *Pseudomonas*; formed from proelastase, structural homology with trypsin; formerly 3.4.4.7.

Microbial serine proteinases, EC 3.4.21.14; subtilisin, R, proteins and peptide amides; S+C, *Bacillis subtilis*; formerly 3.4.4.16.

EC 3.4.22. **Thiol proteinases**
Papain, EC 3.4.22.2; PC, Arg-, Lys-, Phe-X-; SI; SN, N/A; S+C, *Carica papaya* latex; formerly 3.4.4.10.

Ficin, EC 3.4.22.3; PC, Lys-, Ala-, Tyr-, Gly-, Asn-, Leu-, Val-; SN, N/A; S+C, *Ficus* latex; formerly 3.4.4.12.

Bromelain, EC 3.4.22.4; PC, Lys-, Ala-, Tyr-, Gly-; SN, N/A; S+C, *Ananas comosus*, formerly 3.4.4.24.

Chymopapain; PC similar but not identical to papain; SN, N/A; S+C, *Papaya* latex, also catalyses the synthesis of hippuril-aniline; formerly 3.4.4.11.

EC 3.4.23. **Carboxyproteinases**
Pepsin, EC 3.4.23.1; R; PC, Phe-, Leu-; SN, N/A; S+C, gastric juice; formed from pepsinogen; formerly 3.4.4.1.

Chymosin, EC 3.4.23.4; R, clotting of milk; SN, N/A; S+C, calf gastric juice, known as rennin; formed from prochymosin; formerly 3.4.4.3.

EC 3.4.24. **Metallo-proteinases**
Microbial metallo-proteinases, EC 3.4.24.4, several sources vg; *Bacillus thermoproteolyticus*; PC, -Leu > -Phe; SN, N/A; S+C, zinc enzyme, known as thermolysin.

Vertebrate collagenase, EC 3.4.24.7; PC, one bond in native collagen; SN, N/A; S+C, animal tissue

R, reaction; PC, preferential cleavage at the carbonyl end of the amino acid whose symbol is given; SS, specific substrate; SI, specific inhibitor; SN, systematic name; N/A, none applicable; S+C, source and comments.
BAEE, benzoyl-L-Arg-ethyl ester; BAPNA, benzoyl-L-Arg-p-nitroanilide, BTEE, benzoyl-L-Tyr-ethyl ester; HA, hippuril-L-Arg; HPA, hippuril-L-Phe; -NA, naphthylamide; $-NH_2$, amide;
SAAPPNA, succinyl-L-Ala-L-Pro-L-Phe-p-nitroanilide; SBTO, soybean trypsin inhibitor; TAME, p-toluenesulfonyl-L-Arg-methyl ester; TPCK, tosyl-phenylalanine chloromethyl ketone; TLCK, tosyl-lysin chloromethyl ketone.

because they reduce the capacity of the enzyme to bond and hydrolyse the substrate. In general, there are three main classes of inhibitory molecules: (1) active site specific, low molecular weight inhibitors which irreversibly modify the amino-acid residue of the active centre on the enzyme; (2) natural inhibitors, which are pseudosubstrates; and (3) low molecular weight chelating agents.

Conclusions

Systematic names are long and sometimes unwieldly. Trivial names are also inevitably used in everyday situations in the laboratory, technical communications, and teaching. However, this circumstance may generate confusion, *i.e.* the term alkaline protease is used to denominate enzymes which have higher activity at alkaline pH and enzymes which have isoelectric point higher than seven. Protease classification is overridden in most of the studies of the description of new sources and mechanisms of catalysis. Non-systematic terminology is used in scientific communication such as papers in journals and conferences.

In the past, proteolytic enzymes were named according to a non-systematic pattern and the former classification which was based on the molecular characteristics of the enzyme. The current classification groups enzymes according to: (1) reaction catalysed, (2) substrate specificity, and (3) mechanisms of action. However, some characteristics of biological significance, such as evolution relations, are not considered. In spite of the fact that evolutionary relationships are implied in the mechanism of catalysis, they are not defined in the EC classification. In this circumstance the notion of family external to the current classification has arisen.

An example of evolutionary relations can be found in the group of enzymes including trypsin, chymotrypsin, and elastase, *i.e.* serine proteinases, which are closely related enzymes from the pancreas of vertebrates. They seem to evolve from common ancestral protein. In invertebrates these enzymes can not evolve into separate enzymes (Osnes, 1985). It has been suggested that the chymotryptic cleavage specificity was lost during invertebrate evolution.

However, Tsai *et al.* (1986), using highly specific substrates and inhibitors, have found the occurrence of chymotrypsins in the digestive tract of five shrimp species. The presence of these enzymes in invertebrates, mainly in decapods, needs to be addressed, because shrimp and shrimp-like animals, crabs and lobsters are important resources in the fishing industry. The wastes of the industrialisation of decapods could be a meaningful source of serine proteinases.

It is possible that more families of proteases will emerge in the near future. One example is the astacin family, which was proposed recently.

The modification of proteins is important in the processing of raw material to produce food as feed. Proteinases are the most used enzymes in food technology due to their catalytic properties. Peptidases will gain importance in the modification of proteins at the end of the protein molecule. This could help to improve the flavour of some foods. However, new and cheap sources need to be investigated.

The research into sources of accessible enzymes must be encouraged. One reasonable resource could be the microbial world. However, micro-organisms used for the production of enzymes in food technology need to be 'generally accepted as safe', or GRAS, by Regulatory Institutions such as the WHO or FDA. The approval of micro-organisms will increase substantially the cost of the product. Another diverse source of the enzymes could be marine organisms. Most of them are already used for either food or feed. Nevertheless, when new enzymes are reported, their appropriate naming and classification should be included in the research work.

Table 6. Some examples of trivial names of enzymes which have application to food technology and some comments

Cathepsins (from a Greek term Kathepsin; meaning to 'digest')	proteases from lysosomes. On the basis of their specificity toward N-benzylcarbonyl-α-L-Glu-L-Tyr, benzoyl-L-Arg-NH_2, Gly-L-Phe-NH_2 cathepsins were assigned the names A, B, and C, respectively. Currently additional cathepsins have been recognized. They are called A-N and P-T, and belong to the proteinases with the exception of cathepsins A and C. Cheap sources need to be investigated
Trypsin & Trypsin-like	pancreatic enzymes, formed from protypsinogen in mammals. They are serine proteinases. Specific substrates are TAME and BAPNA. Specific inhibitors are the synthetic TLCk and the natural SBTI.
Chymotrypsin & chymotrypsin-like	pancreatic enzymes, fromed from prochymotrypsinogen in mammals. In invertebrates the presence of chymotrypsin has not been confiemd; however the presence of the enzyme has been reported. They are serine proteinases. Specific substrate is SAAPPNA. Specific inhibitor is TPCK.
Papain & chymopapain	plant proteases from Carica papaya latex. The commercial papain preparation is usually a crude extract (the dry latex). They are Cys (SH) proteinases. Revesible natural inhibitor for SH enzymes is the cystatin; protein from seeds.
Ficin	plant Cys proteinases from *Ficus carica*. The commercial preparation is a mixture of several similar enzymes.
Pepsin	gastric juice. Acid (aspartic) proteinases. pH of activity is 2-6.
Rennin or chymosin	gastric juice from calf. The only example of Asp proteinase active at neutral pH. Inhibited by the peptide analogue pepstatin A.
Takabate™ 100 ('Enmex SA de CV', Mexico)	microbial enzyme from *Bacillus liqueniformis* with a maximal activity between 50 and 65°C and highle active at pH 6-10.
Alkalase™	microbial enzyme. Similar to Takabte (Novo, Denmark).

See Table 5 for definition of abbreviations.

Acknowledgments

Thanks to Dr. L. Dimes for her suggestions to the early draft of the manuscript. Special thanks to 'Tracy'.

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