Protease inhibition in theory and practice

Fernando Luis García-Carreño

Research on new sources of proteases with improved capabilities for the modification of proteins will expand the impact of biotechnology in food technology. The study of new proteases includes assessment of their mechanisms of catalysis, which is done by evaluating the effect of specific active site inhibitors. This paper gives an introduction to protease inhibition and an example of a practical approach for the characterization of the mechanism of catalysis.

Introduction

Proteases are enzymes which catalyze the hydrolysis of the peptide bonds forming the primary structure of proteins (Dixon and Webb, 1979). They are common to organisms, from microorganisms to plants and animals. In higher animals, proteases are involved in several processes including digestion, proenzyme or prohormone activation and defense mechanisms such as blood clotting, and complement activation. In all cases, proteases are responsible for the 'modification of proteins' [any physical, chemical or enzymatic treatment which changes the protein conformation or structure and, consequently, its physicochemical and functional properties (Casella and Whitaker, 1990)]. Proteases are primarily used in the processing of raw materials in food technology (García-Carreño, 1991), although recently they have been commercially exploited as modifiers of secondary materials (by-products) in the manufacture of high value products.

Almost all proteases for industry originate from selected hyperproducing microorganisms (Aunstrup, 1980), although enzymes from other sources are continually being researched (Haard, 1991; García-Carreño, 1991). These studies look for proteases which bear specific capabilities in their hydrolytic mechanism, specificity for a particular site, or maximum activity under particular conditions such as high/low temperature, high salt concentration, endo/exopeptidase ratio, or activity in organic solvents. The study and selection of these properties may improve operations in the food processing or waste management industries. The application of a new protease in food technology depends on the type of modification accomplished by the enzyme and the conditions required for maximum activity. Consequently, the investigation of new proteases must include the characterization of their mechanism of hydrolysis. This is done with a variety of reagents known as active site inhibitors which reduce or abolish the enzyme activity by blocking the reactive amino acids in the active center or chelating the cations involved in the splitting of the peptide bonds.

The aim of the present paper is to provide a background in protease inhibition and to present a methodology for the investigation of the class of protease under study.

Fernando Luis García-Carreño
Centro de Investigaciones Biológicas, PO Box 128, La Paz, BCS 23000, México

Current address: Institute of Marine Resources, Department of Food Science and Technology, University of California, Davis, CA 95616, USA.

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Classification of proteases

The classification of proteases differs notably from that used in enzyme nomenclature because of their low specificity for the substrate. Since proteases are hydrolases and specific for peptide bonds, they are coded in the group (EC 3.4). Proteases are further divided into two sub-subgroups: (EC 3.4.11–19) for those enzymes hydrolyzing peptide bonds between terminal amino acids (the amino or carboxylic end of the substrate protein) and (EC 3.4.21–24) for enzymes hydrolyzing internal peptide bonds. The first set of enzymes are labeled exopeptidase or peptidase, and the last set of enzymes are labeled endopeptidase or proteinase. The methodology to determine the class will identify proteases from any of the groups. However, this paper will only deal with the major industrial group; proteinases are called proteases for simplicity.

Proteases are classified according to their mechanism of peptide bond hydrolysis. This mechanism depends to a large extent on the amino acid residues involved at the active center of the enzyme. Four mechanistic classes are recognized by the International Union of Biochemistry and the Enzyme Commission (EC). Each class of protease has a representative set of catalytic or reactive amino acid residues organized in a definite configuration which forms the active center of catalysis. Serine-proteases, the most studied class of proteases (encoded in the sub-subgroup EC 3.4.21), have a serine residue in the active center, as well as histidine and aspartic acid residues. The cysteine-proteases (EC 3.4.22) are characterized by the cysteine group (SH—) involved in the catalytic center. Acid-

Table 1. Some examples of proteases showing different mechanisms of catalysis

Class	Enzyme	Origin
Serine (3.4.21)	trypsin	pancreas
	chymotrypsin	pancreas
	subtilisin	*Bacilllus subtilis*
Cysteine (3.4.22)	papain	*Papaya* latex
	chymopapain	*Papaya* latex
	ficin	*Ficus* latex
Aspartic (3.4.23)	pepsin	gastric juice
	chymosin	gastric juice (young animals)
	cathepsin D	liver,spleen
Metallo (3.4.24)	thermolysin	*Bacillus thermoproteolyticus*
	carboxypeptidase A	bovine pancreas

proteases (EC 3.4.23) are distinguished by an aspartic acid residue in the active site and display maximum activity at acid pH. The metalloproteases (EC 3.4.24) possess a glutamic acid residue in the active site and require a divalent cation such as Zn, Ca, or Mg to catalyze the hydrolysis of the peptide bond. Table 1 gives examples of enzymes belonging to the four classes of proteases.

According to its amino acid sequence, a protease is grouped into a family (closely related proteins whose sequences have about half of their amino acid residues in common and evidence has been provided for an evolutionary relationship). Six families of proteases are currently recognized (Table 2); however, newer families have been identified such as plant enzymes or the astacin family [found in the Decapoda (Dumermuth *et al.*, 1991)].

Assessing the class of proteases

Several assays are required to characterize the mechanism of catalysis of a new protease including (i) response of the enzyme activity to inhibitors: (ii) chemical modifications of the active site; (iii) pH dependence of the activity; and (iv) substrate specificity.

To assess the class of enzyme, the reduction of protease activity using inhibitors (known as enzyme inhibition) is explored. For the purpose of class identification, Salvesen and Nagase (1979) recognized that any compound that decreases the measured rate of hydrolysis of a given substrate is, in principle, an enzyme inhibitor when applied to competing substrates in a reaction.

Proteases belong to at least two groups based on the chemical mechanism of catalysis. Those enzymes that form covalent complexes between the enzyme and the hydrolysis site of the substrate are the serine and cysteine proteases. Those that do not form covalent enzyme–substrate complexes are the aspartic (or acid) and metalloproteases. This distinction is particularly important since the mechanisms of inhibition are quite different. The proteases that form covalent complexes have strong nucleophilic amino acids at their catalytic site; consequently, their inhibitors must possess highly electrophilic groups including double bonds. The proteases that form non-covalent complexes rely more upon general acid/base catalysis.

When approaching the nature of inhibitors for proteases they can be divided into two groups: (i) low molecular weight inhibitors which irreversibly block a catalytic amino acid in the active center or chelate the required cation; and (ii) high molecular weight

Table 2. Classification of proteases by family

Family	Examples
Serine I	trypsin
	chymotrypsin
Serine II	subtilisin
Cysteine	papain
	cathepsin B
Aspartic	chymosin (rennin)
	penicillopepsin
	pepsin
Metallo I	carboxypeptidase A
Metallo II	thermolysin

Table 3. Inhibitors recommended for class characterization of proteases

Class	Inhibitor; abbreviation, recommended concentration and suggested enzyme control
Serine	Phenylmethylsulfonyl fluoride (PMSF), 100 mM,* in DMSO or 2-propanol, 2mM†
	Soybean trypsin inhibitor (SBTI), 12 mM,* 240 μM†
	Tosyl-lysine chloromethyl ketone (TLCK), 10 mM,* in 1 mM HC1, pH 3, 100 μM.† Trypsin-inhibitor
	Tosyl-phenylalanine chloromethyl ketone (TPCK), 5 mM,* in MeOH or EtOH, 100 μM.† Chymotrypsin-inhibitor
	Control: trypsin and chymotrypsin (bovine or porcine)
Cysteine	Iodoacetamide (IA), 100 mM,* 2 mM†
	p-Hydroxy-mercuribenzoic acid (PHMB), 50 mM,* 1 mM†
	N-Ethyl-maleimide (NEM), 100 mM,* 2 mM†
	trans-Epoxysuccinyl-L-leucylamido-(4-guanidino)butane (e64), 500 mM,* 10 mM†
	Control: papain
Aspartic	Pepstatin A (assay at acid pH), 50 μg/ml,* in DMSO or MeOH, 1μg/ml†
	Control: cathepsin D, pepsin
Metallo	EDTA 2–20 mM†
	EGTA 2–20 mM†
	1,10-phenanthroline, 100–200 mM,* in DMSO or MeOH, 2-4 mM†
	Control: thermolysin, carboxypeptidase A

It is recommended that fresh inhibitors are prepared.
*Concentration of the stock solution.
†Concentration in the mixture of inhibition after diluting in the buffer (1:50) following the procedure suggested in Table 4.

proteins. These are naturally occurring inhibitors, many of which can behave as pseudosubstrates and join reversibly (the exception being serum α-macroglobulin). A growing number of natural and synthetic inhibitors are being used to characterize newly discovered proteases. Table 3 gives a list of the most common inhibitors for all classes of protease.

Example experiment

Preliminary assays

Assays must be carried out to determine the suitable temperature and pH. Other preparatory data are obtained by assessing the period of time in which a linear reaction arises (time vs activity). This information helps to avoid misleading conclusions about the inhibition assays.

The inhibitors

Phenylmethylsulfonyl fluoride (PMSF) is soluble in dimethylsulfoxide (DMSO) or 2-propanol; thus, a vehicle control must be used for testing it. PMSF reacts slowly with proteases, so it is advisable to incubate it for several hours (up to 3 h) when partial inhibition is observed after 1 h. This inhibitor also reacts with cysteine-proteases; however, this reaction is prevented by using dithiothreitol (DTT) in the reaction mixture. A soybean trypsin inhibitor can be used to confirm the data obtained when using PMSF, but higher levels of inhibition will be expected for trypsin-like enzymes. A differentiating assay for serine-proteases should include the use of tosyl-lysine chloromethyl ketone (TLCK) which targets trypsin and trypsin-like proteases, or should use tosyl-phenylalanine chloromethyl ketone (TPCK) to target chymotrypsin and chymotrypsin-like proteases. These inhibitors help to differentiate between the main serine-proteases. Iodoacetamide (IA), p-hydroxy-mercuribenzoic acid (PHMB), *N*-ethyl-maleimide (NEM), or *trans*-epoxy-succinyl-L-leucylamido-(4-guanidino)-butane (e64) are recommended for inhibiting cysteine-proteases with papain as an appropriate control. Pepstatin A is a highly specific inhibitor for aspartic-proteases. It is dissolved in methanol and then diluted 100 or more times in the assay buffer to reduce the inhibitory effect of the solvent. An appropriate control for the solvent will be needed, and a suggested enzyme control is pepsin. Metallo-proteases are inhibited by chelating the divalent cation with agents such as EDTA for Ca and Mg enzymes, EGTA for Mg enzymes, and 1, 10-phenanthroline. It should be noted that these inhibitors are targeted for the divalent cation and are not specific to the enzyme. A control enzyme for these could be thermolysin.

Table 4. General assay for activity and inhibitory effect on proteases

Number of tubes	1–3*	4–6†	7–9‡	10–12§
Buffer	470 µl	480 µl	480 µl	470 µl
Enzyme extract	20 µl	20 µl	20 µl	20 µl
Inhibitor solution	10 µl	—	—	—
Inhibitor solvent	—	—	—	10 µl
Incubation	37°C‖		60 min	
TCA	—	—	0.5 ml	-
Substrate+	0.5 ml	0.5 ml	0.5 ml	0.5 ml
Incubation	37°C‖		60 min‖	
TCA (10%)	0.5 ml	0.5 ml	—	0.5 ml
Centrifuge	6500 *g*		5 min	
Absorbance (440 nm)	record			

*Inhibition assay; †activity; ‡control of the enzyme extract (some crude extracts could absorb at 400 nm because of the presence of carotenoids or other yellow substances); §control for the inhibitor solvent (some inhibitor solvents could modify the activity of the enzyme); —, nothing added; +, azocasein 1.5%; ‖the temperature and longevity of the incubation will depend on the thermostability and time taken to get a linear reaction.

The inhibitory assay

Table 4 shows a diagram for a general assay used to characterize the class of a protease. The suggested substrate is azocasein (a casein coupled to a dye, the hydrolysis of which releases a yellow dye with an absorbance at 440 nm); this substrate offers high accuracy, and resolution ($E^{1\%}_{440}$ in 0.1 N NaOH = 34). A solution of substrate (azocasein 1–2%) in a buffer (50 mM) of appropriate pH (the substrate is soluble up to pH 5) could be used. The controls will not release dye after the reaction is stopped. The assay includes: (i) the evaluation of the enzyme activity; (ii) the measurement of the activity in the presence of the inhibitor (inhibitory evaluation); and (iii) the controls for the inhibitor vehicle (those inhibitors which are non-soluble in water and could modify the enzyme activity) and for the enzyme extract (when it absorbs at the same wavelength as the product of the hydrolysis). When the inhibitor is dissolved in the same buffer as the reaction, the control for inhibitor vehicle can be eliminated. Similar criteria are applied if the enzyme extracts do not absorb at the wavelength used. It is advisable to include an entire evaluation for the positive control enzyme (an enzyme belonging to the assayed class of protease) to

Table 5. Kinetic assay for slowly reacting inhibitors

	Investigated enzyme			Control enzyme		
t(h)	1	2	3	1	2	3
0	I	I	I	I	I	I
1	S	—	—	S	—	—
2	TCA	S	—	TCA	S	—
3	—	TCA	S	—	TCA	S
4	—	—	TCA	—	—	TCA
Centrifugation	6500 *g*			5 min		
Absorbance (440 nm)	record					

I, Inhibitor; S substrate; TCA, tricholoroacetic acid.

estimate the validity of the results. Triplicates must be run for each assay (13 × 90 test tubes are suggested). This procedure is described in Table 4. After stopping the reaction with trichloroacetic acid (TCA), the separation of the precipitate (the non-hydrolyzed substrate) is accomplished by transferring the contents of the test tubes to 1.5 ml Eppendorf tubes and centrifuging in an Eppendorf microfuge or similar equipment at 6500 ***g*** for 5 min.

When the assay yields a limited inhibition for those enzymes with slow reactive inhibitors, a kinetic assay is highly recommended. Table 5 gives a diagram for a kinetic assay. It is designed for 1–3 h, which should be enough time to guarantee a complete reaction between the inhibitor and the enzyme.

Results

The analysis of the data

As an example, let us use data obtained during the characterization of a new protease from langostilla (*Pleuroncodes planipes*), an abundant decapod of the Pacific Ocean of Baja California Sur, México. Spectrophotometric data (triplicates of each assay and its corresponding controls) will be evaluated to calculate the activity and percentage of inhibition. An easy way to calculate the percentage of inhibition is by using any electronic spreadsheet. Qpro is recommended but any software such as Lotus 123, Quattro or similar could be used.

The instructions for Qpro will be provided as an example, but they can be adapted for your spreadsheet, according to the following procedure: (i) open a new spreadsheet; (ii) adjust column width according to the instructions in Table 6; (iii) enter the text and formulae according to Table 6; and (iv) enter your own data according to Table 7. The spreadsheet should appear as in Table 8. This program can be applied to different inhibitors by copying the entire block. The comparison of the percentage of inhibition using inhibitors for the four classes of proteases will provide information about the class of mechanism that a particular enzyme uses.

Table 6. How to enter the formulae, texts and data in the spreadsheet (the data you will see are those from the example experiment, for the experimental and control protease)

Cell/column	Width/column	Enter text/data
A1:	[W12]	'Determination of the percentage of inhibition of activity
A2:	[W12]	'of enzyme (your enzyme name) by PMSF,
A3:	[W12]	'including trypsin as enzyme control
A4:	[W12]	'----------
A5:	[W12]	'Your enzyme name
B6:	[W6]	'---triplicates---
E6:	[W6]	^avg*
F6:	[W6]	^std
G6:	[W6]	^%std
H6:		^%inhib
A7:	[W12]	'inhibition
B7:	[W6]	0.337
C7:	[W6]	0.362
D7:	[W6]	0.367
E7:	[W6]	@AVG(B7..D7)
F7:	[W6]	@STD(B7..D7)
G7:	[W6]	(F7*100)/E7
H7:		100-((E7*100)/E8)
A8:	[W12]	'activity$
B8:	[W6]	0.525
C8:	[W6]	0.526
D8:	[W6]	0.526
E8:	[W6]	@AVG(B8..D8)
F8:	[W6]	@STD(B8..D8)
G8:	[W6]	(F8*100)/E8
A9:	[W12]	'vehicle**
B9:	[W6]	0.526
C9:	[W6]	0.525
D9:	[W6]	0.525
E9:	[W6]	@AVG(B9..D9)
F9:	[W6]	@STD(B9..D9)
G9:	[W6]	(F9*100)/E9
A10:	[W12]	'extract**
B10:	[W6]	0.0
C10:	[W6]	0.0
D10:	[W6]	0.0
E10:	[W6]	@AVG(B10..D10)
F10:	[W6]	@STD(B10..D10)
G10:	[W6]	(F10*100)/E10
A11:	[W12]	'----------
A12:	[W12]	'trypsin
B13:	[W6]	'---triplicates---
E13:	[W6]	^avg*
F13:	[W6]	^std
G13:	[W6]	^%std
H13:		^%inhib
A14:	[W12]	'inhibition
B14:	[W6]	0.009
C14:	[W6]	0.008
D14:	[W6]	0.008
E14:	[W6]	@AVG(B14..D14)
F14:	[W6]	@STD(B14..D14)
G14:	[W6]	(F14*100)/E14
H14:		100-((E14*100)/E15)
A15:	[W12]	'activity
B15:	[W6]	0.199
C15:	[W6]	0.202
D15:	[W6]	0.199
E15:	[W6]	@AVG(B15..D15)
F15:	[W6]	@STD(B15..D15)
G15:	[W6]	(F15*100)/E15
A16:	[W12]	'vehicle
B16:	[W6]	0.2
C16:	[W6]	0.201
D16:	[W6]	0.201
E16:	[W6]	@AVG(B16..D16)
F16:	[W6]	@STD(B16..D16)
G16:	[W6]	(F16*100)/E16
A17:	[W12]	'extract
B17:	[W6]	0.0
C17:	[W6]	0.0
D17:	[W6]	0.0
E17:	[W6]	@AVG(B17..D17)
F17:	[W6]	@STD(B17..D17)
G17:	[W6]	(F17*100)/E17
A18:	[W12]	'----------
A19:	[W12]	'*Abbreviations: avg, average; std, standard deviation;
A20:	[W12]	'%std, percentage of standard deviation;
A21:	[W12]	'%inhib, percentage of inhibition.

Table 7. How to enter your own data into the spreadsheet

Protease under study	
Cells	Enter
B7, C7, and D7	data of activity with PMSF
B8, C8, and D8	data of activity without PMSF
B9, C9, and D9	data of the control of the solvent of the inhibitor
B10, C10, and D10	data of the control of absorbance of the extract
Protease control (trypsin in this example)	
Cells	Enter
B14, C14, and D14	data of activity with PMSF
B15, C15, and D15	data of activity without PMSF
B16, C16, and D16	data of the control of the solvent of the inhibitor
B17, C17, and D17	data of the control of absorbance of the extract

for an enzyme of the serine class. As anticipated, a pure enzyme (reagent grade) does not absorb at 440 nm in the extract control.

Conclusions for the example experiment

The reduction in proteolytic activity in the presence of PMSF is an indication of the participation of a serine protease in the langostilla extract. Serine proteases such as trypsin, trypsin-like enzymes, chymotrypsin, and chymotrypsin-like enzymes are the foremost protein digesting enzymes in invertebrates belonging to the phylum Arthropoda. The langostilla extract zymogram (García-Carreño, 1992) shows at least four fractions bearing protease activity, one of them with an electrophoretic mobility similar to porcine trypsin. The partial inhibition is due to some fractions belonging to the serine class. The other fractions belong to the metallo class, which was demonstrated by incubating with EDTA, EGTA, and 1, 10-phenanthroline. The identification of fractions belonging to the serine or metallo class could be possible in zymograms by testing the proteolitic activity in the presence of inhibitors (for technical details see García-Carreño, 1992).

Table 8. The actual spreadsheet for estimation of protease percentage inhibition you will see on the computer screen or print-out

Determination of the percentage of inhibition of activity of enzyme (your enzyme name) by PMSF, including trypsin as enzyme control

Your enzyme name							
	---triplicates---			avg*	std	%std	%inhib
inhibition@	0.337	0.362	0.367	0.355	0.013	3.693	32.403
activity$	0.525	0.526	0.526	0.526	0.000	0.090	
vehicle**	0.526	0.525	0.525	0.525	0.000	0.090	
extract$$	0.000	0.000	0.000	0.000	0.000	0.000	
Trypsin							
	---triplicates---			avg*	std	%std	%inhib
inhibition	0.009	0.008	0.008	0.008	0.000	5.657	95.833
activity	0.199	0.202	0.199	0.200	0.001	0.707	
vehicle	0.200	0.201	0.201	0.201	0.000	0.235	
extract	0.000	0.000	0.000	0.000	0.000	0.0	

*Abbreviations: avg, average; std, standard deviation; %std, percentage of standard deviation; %inhib, percentage of inhibition; @, inhibition: activity in PMSF; $, activity without inhibitor; **, vehicle (control of inhibitor solvent); $$, extract (control of extract absorbance at 440 nm).

The inhibition example using PMSF and trypsin as an enzyme class control showed 32% inhibition for the experimental enzyme and no modification in activity for the solvent (vehicle) of the inhibitor. Moreover, the langostilla enzyme extract does not absorb at 440 nm (extract control). Trypsin is inhibited by 96%, which is expected

Discussion

Proteases are used in food technology because of the diverse ways in which they modify proteins. The modification may be: (i) an abrupt decrease in the molecular weight; (ii) a limited reduction in the molecular weight; or (iii) the elimination of terminal amino acids. Some practical examples of these modifications occur during the production of protein hydrolysates from seed meals or fish, the reduction of fish waste water (stickwater) viscosity for drying, the development of flavor in enzyme-modified cheese (EMC), and the removal of seed antinutrients such as anti-trypsin. All the above-mentioned examples of protein modification need a protease (or a mixture of proteases) with particular properties for hydrolyzing proteins.

Thermophilic bacteria are being investigated as potential sources of proteases, and new sources are being sought to meet the unfulfilled needs of industries which rely on enzymes derived from microorganisms. One of the limitations of enzymes from microorganisms is that only those labeled as "generally accepted as safe" (GRAS) can be used in food processing. Thus, proteases from new sources will have to be characterized for their catalytic properties by using inhibitors of the activity. Proteases from cold-adapted marine organisms such as fish and shellfish may find increasing commercial use in low temperature food processing.

Purification of proteases should be done to characterize catalytic mechanisms. Results obtained after their isolation may explain why some inhibitors have failed to reduce enzymatic activity and why some inhibitors are nonspecific to a particular class of protease (García-Carreño, 1992).

Further reading

For further study of protease inhibition, suggested reading includes the comprehensive books by Beynon and Bond (1989) and Zollner (1989). For classification theory see the book by Dixon and Webb.

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