Proteases in food technology

F. L. García-Carreño

Proteolytic modification of food proteins is a traditional technology, but biotechnology has expanded its industrial possibilities. This paper deals with the theory of protease application to the food industry and describes the production of fish hydrolysate in the laboratory as an illustration of this technology.

Introduction

Enzymes have always been important to food technology because of their ability to act as catalysts, transforming raw materials into improved food products. The main values of enzymes are their substrate specificity (Ward and Moo-Young, 1988), catalytic effectiveness and a rate enhancement of 10^{10} or more over chemical reactions (Burbaum *et al.*, 1989) when working under mild conditions of ion concentration, temperature and pH.

The present catalytic effectiveness of enzymes is the result of their natural selection from the evolutionary pressures on the host organisms. Researchers are constantly finding new sources of enzymes for food processing.

Food technologists select those enzymes which can improve one particular unit operation of food production. These improvements involve substituting fish protein hydrolysates for milk in calf feed (Díaz-Castañeda and Brisson, 1989), saving energy and money in production processes (Christiensen, 1989), extracting juice (Kilara, 1982) and modifying the functional properties of proteins (Adler-Nissen *et al.*, 1983).

Biotechnologists, on the other hand, are constantly improving enzyme technology. These new approaches include:

- Fermentation processes for industrial enzyme production.
- The selection of wild or transgenic (genetically improved) over-producers (which synthesize the enzyme beyond its own requirements) and excretors (those organisms that release the enzyme which is easily recovered from the media).
- Protein engineering (modifications on polypeptide structures that increase catalytic effectiveness).
- The analysis of the three dimensional structure to determine the architecture responsible for the catalytic activity of the enzyme.
- Non-aqueous enzyme activities which improve the catalytic effectiveness or change the substrate specificity of particular enzymes.
- The research of synthetic enzymes such as catalytic antibodies and non-proteinaseous catalysts (Wasserman, 1990).

Proteolytic enzymes have been used in the food industry since the fermentation industry developed techniques for low-cost, high-yield production 50 years ago. Proteases have traditionally been used for cheese-making, whipping, chill-haze prevention and brewing. But recently, food technologists have been using enzymes to extract high quality proteins from marine resources. They have been used to extract carotenoproteins from shrimps (Cano-Lopez *et al.*, 1987), and protein hydrolysates from fish (Mackie, 1982). Enzymes can also be used in the treatment of fish skins for industrial uses (Stefanson, 1988).

Classification of industrial proteases

The classification of industrial proteases is based on either their origin (animal, plant or microbial sources), catalytic mechanism (exopeptidases or endopeptidases), specificity (preferred splitting site or particular peptide bond target) or the chemical nature of the amino acid or prosthetic group involved in the catalytic activity (serine, cysteine, aspartic or metallo proteases). Despite this system of nomenclature, almost all proteases are presently called by their traditional or trade names (papain, cathepsin, alcalase, etc.).

New sources of proteases are currently being investigated. Patel *et al.* (1986) and Wasserman (1990) have described several enzymes from *Sulfurococcus mucosus, Bacillus thermoruber, B stearothermophilus, Thermomonospora fusca* and *psycrotrophic Pseudomonads*, which have maximal activity at high temperature. In contrast, Simpson and Haard (1987) and Reece (1988) have proposed the use of cold-adapted enzymes from poikilothermic organisms for particular processes such as cheese-making, prevention of copper-oxidized flavor in milk, and improvement in fish fermentation.

Industrial enzymes

There are several major companies producing proteases for industrial uses (see Aunstrup, 1980) and almost all

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send free samples and data sheets for their products upon request. The microbial enzymes for food technology must be from "GRAS" (generally accepted as safe) organisms and fulfill the recommendations of FAO/WHO (Food and Agriculture Organization/ World Health Organization), FDA (Food and Drug Administration/USA), etc. The enzyme activity must be reported in international units or in units that can easily be converted. The presence of contaminating enzymes can interfere with the actual or declared main activity, thereby reducing the benefit of the product you are paying for. It is important to know the kind of protease you are going to be using (cysteine, metallo, etc.) because the inhibitors (EDTA) and activators (cys, CA^{2+}) are quite different from enzyme to enzyme. The form of the product (liquid, granules, powder or encapsulated) and the method of packing and shipping (international restrictions, FOB, etc.) must be taken in account before purchasing. Properties such as solubility, handling precautions, stability, and storage conditions are welcome recommendations from the supplier. Other valuable information from the suppliers provides: methods for activity evaluation, some applications, kinetic properties (such as activity pH and temperature dependence) and practical inactivation conditions.

Several methods have been reported for the quantification of protease activity, but almost all use as a substrate denatured hemoglobin, caseine or some derivative (i.e. dimethyl). The extent of hydrolysis reaction can be measured as follows:

(1) The titration of released protons from carboxylic groups. This is determined by adding a base to the reaction mixture (to avoid the decrease in the pH) when using alkaline or neutral proteases.

(2) The use of chromogenic reagents for amine groups liberated in each split peptide bond.

(3) The change in the molecular weight of the hydrolysed protein (i.e. size exclusion chromatography or electrophoresis).

(4) The reduction of properties related to the length of proteins (viscosity).

Marine resources of protein

The sea provides a vast supply of proteinic resources. Out of an annual world catch of almost 100 million tons, 40% is caught specifically for reduction to fish meals. The remaining 60% is designated for human consumption which has created a large seafood industry. The waste of this industry is also used for the production of protein meals. Hence, a considerable amount of protein is available from the oceans in the form of unexploited stocks, by-catch and processing waste.

Several differences exist in the processing of fatty and non-fatty raw material. Fats must be eliminated before trying to make a protein hydrolysate because lipids tend to oxidize during drying and storage, causing a bitterness in the product. Yet, despite the problems of eliminating the fats, the production of fish protein hydrolysates is easier than other substrates.

Example experiment

The raw material

For this experiment the raw material is a protein concentrate (FPC). It is the dry product of fat–2-propanol extraction of fish flesh. In brief, the fish is ground and filtered to eliminate residual liquid. Then, the fats are extracted and some moisture eliminated using three volumes of 2-propanol. The main volume of solvent is eliminated by filtering and the residual one by drying. The solvent is recuperated by distillation and reused. Table 1 shows the typical chemical composition of a non-fatty (cod) fish flesh and 2-propanol-extracted cod flesh.

The enzyme

A good enzyme to use is Takabate* 100 ("Enmex SA de CV", México) which is a microbial enzyme from *Bacillus liqueniformis* with a maximal activity between 50 and 65°C and highly active at pH 6–10. An alternative enzyme for European laboratories could be Alkalase (a similar enzyme from Novo, Denmark).

Table 1. Chemical composition of fish flesh (g/100 g dry matter)

Sample	Protein ($N \times 6.25$)	Fat	Ash	Moisture
Cod	17.0	1.0	1.0	81.5
Cod FPC	93.0	0.03	5.0	2.0

The equipment

For the production of protein hydrolysates, a laboratory will require:

(1) A reactor with a 1 l vessel, drive stirrer with variable speed (up to 1000 rpm), thermostated water bath and wide neck inlet with stopper.

(2) A pH controller including pH-electrode, peristaltic pump and a vessel for the base.

(3) A homogenizer (v.g. blender).

(4) A dryer (freeze-dryer or spray-dryer).

Process parameters

Substrate concentration (S in %; w/v) is the protein content in the reaction mixture; in this experiment it will be 13. FPC has a protein concentration of 93% (see Table 1). The grams of FPC needed to get S are calculated as follows: g of FPC = $S \times 100/93$ = 13.97. Enzyme–substrate ratio (E/S %) is the activity or weight (w/w) of the enzyme. E/S is usually between 0.1–1, in this experiment it will be 0.8. Temperatures and pH values are those of maximal enzyme activity (9.5 and 55°C for Takabate*). The base used to keep pH constant in the reaction mixture can be NaOH or NH_4OH, and their concentration in molarity units between 4–10. The degree of hydrolysis (DH) is the percentage of peptide bonds cleaved and is closely related to functional properties of the hydrolysis product such as: solubility, water binding, viscosity and organoleptical properties such as bitterness (see Alder-Nissen, 1982).

The procedure

The raw material is homogenized in a blender with the addition of water until it reaches a substrate concentration of 13%. Homogenize 139.7 g of FPC and add distilled water up to 800 ml. Pour out the substrate into the reaction vessel and set the temperature of hydrolysis in the reaction mixture to 55°C. Check the pH and add distilled water up to 1000 ml. If the pH of the substrate deviates from the pH of the

process (9.5), a volume of water will need to be replaced with concentrated base (NaOH or NH_4OH) or acid (HPO_3 or HCl) until the required pH is reached.

When the mixture is at pH 9.5 and thermal equilibrium, begin by stirring (500 rpm). A few minutes later, check the pH and temperature for any change resulting from the agitation.

Proceed by pouring a known quantity (in ml) of base (4 M) into a vessel. Eliminate the air from the base pump tubing in the pH controller by pumping the liquid into its own vessel for a few minutes.

Weigh the required quantity of enzyme for an E/S ratio of 0.8 (1.04 g). Add the enzyme into the reaction mixture and immediately turn on the pH controller.

Inactivation of the enzyme (termination of the hydrolysis)

There are several procedures for deactivating the enzyme. The most suitable methods for food technology are heat and pH inactivation. Inactivation by pH is quicker and cheaper, but the final concentration of salt in the product is higher. When the desired degree of hydrolysis is reached, add the chosen acid until the pH value is lower than the minimum pH of activity. For Takabate*, an inactivation pH of 4–4.2 is suitable.

Further processing

Sieve in a 100 or 120 mesh size filter or centrifuge the hydrolysate at 3000 g to eliminate particulate material and dry in a freeze-dryer or laboratory spray-dryer.

Calculation of degree of hydrolysis (DH)

The use of proteases which work at neutral or alkaline pH allows calculation of the degree of hydrolysis that is reached, since, under these conditions, the ionization of carboxylic groups which result from each cleaved peptide bond liberates a proton. The base consumed to keep the pH constant is therefore an indicator of the extent of hydrolysis.

The amount of base consumed will be proportional to the hydrolysis equivalents (h = meqv g^{-1})

$$h = B \times N_b \times 1/a^* \times 1/\text{MP} \quad (1)$$

where B is the base consumption (in ml), N_b the molarity of the base (NaOH or NH_4OH), a^* the average degree of dissociation of the amino groups and MP the mass of protein (usually $N \times 6.25$, g).

DH has been defined as the percentage of peptide bonds cleaved and is given by:

$$\text{DH} = (h/h_{tot})\ 100\% \quad (2)$$

where h is the hydrolysis equivalents (the number of peptide bonds cleaved during a hydrolysis process) and h_{tot} is the total number of peptide bonds in a given protein (which can be determinated from the amino acid composition of the protein). When h_{tot} for a particular protein remains unknown, the value 8.0 can be used. In this experiment a^* will be 1.0.

If we choose, for example: a DH of 6 and the h_{tot} is 8.0, S = 13, T = 65°C, E/S = 0.8(g/g), MP = 139.7 g, and N_b = 4. Then, we can calculate the ml of base needed to get the desired DH. From (2):

$$\text{DH} = 6 = (h/8.0) \times 100$$
$$h = 0.48$$

and from (1):

$$h = 0.48 = B \times 1/a^* \times N_b \times 1/\text{MP}$$
$$0.48 = B \times 1 \times 4 \times 1/139.7$$
$$B = 0.48/[1 \times 4 \times (7.158 \times 10^{-3})]$$
$$B = 16.76 \text{ ml.}$$

To approach a DH of 6, it will be necessary to add 16.76 ml of the base throughout the process in order to keep the pH constant. When the consumption of the base is 16.76 ml, the protease activity will be stopped when a drastic change in pH or temperature occurs.

The dry product can be assayed for solubility index, foaming properties, emulsifying or gel formation. The fish protein hydrolysates can be used to give food a physical appearance, taste (related to the raw material) or nutritional improvement.

Conclusions

The introduction of the concept of degree of hydrolysis (DH) to the application of hydrolases has facilitated the control of the extent of the hydrolysis even at the industrial level. The functional properties of protein hydrolysates in this approach are dependent on DH, regardless of the parameters of process such as temperature, time or enzyme concentration (Adler-Nissen, 1982). However, the accurate knowledge of h_{tot} is essential for the perfect control of the production of a hydrolysate from a particular source of protein.

Proteases are useful enzymes in food technology and there are new approaches forthcoming. Christensen (1989) has proposed the use of enzyme technology to improve or substitute engineering technology in the food industry. Proteases which are capable of particular modifications in food processes need to be investigated from new sources. Protein hydrolysis is a promising methodology in food technology which can help to solve technical problems. One of these problems is the reduction in viscosity which increases heat transfer in evaporators.

Proteases can also be used to produce protein hydrolysates from marine resources. Because of the abundance and high protein quality of marine resources, fish protein hydrolysates can help provide (a) high quality/high protein foods for developed and developing countries, and (b) ingredients to increase functional properties in food production. Learning how to produce a protein hydrolysate at the laboratory level is a good introduction to this technology which can benefit educational and industrial institutions.

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