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Applications of Fish and Shellfish Enzymes in Food and Feed Products

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I. INTRODUCTION

Because enzymes are molecules with biotransformation capabilities, they are important tools of biotechnology. Either working alone or as part of a cell, "Use of enzymes to accomplish specific desirable changes in foods has been practiced for centuries. The techniques have been handed down from generation to generation without any knowledge, until recently" (1). The importance of enzymes as tools in biotechnology and food or feed processing is increasing (2, 3). The demand for enzymes with specific properties is high, and various enzyme sources are being investigated. Enzymes are important components of food and feed for many reasons: growth, maturation, production, processing, storage, and spoilage; consumer preferences and selection; safety and control of predators; food intake, digestion, and assimilation and disease; and as analytical tools (4). The marine environment contains the largest pool of diversified genetic material and, hence, represents an enormous potential source of enzymes. The application of seafood enzymes to food and feed products has been developed during the last 30 years. Early work revealed the presence in the marine environment of several enzymes with unique properties (5, 6). This chapter will review some of the uses of such enzymes.

II. CHOICE OF ENZYME

Food and feed processing involves many different operational variables, such as temperature, pressure, flow rate, density, pH, viscosity, chemical composition, enzyme inhibitors, activators, or inactivators. The choice of an enzyme that works efficiently in such conditions is of paramount importance. Moreover, most enzyme-driven processes have a limited endpoint. The ability to restrain the enzyme activity after the work has been done, without affecting the product quality, should also be considered.

Enzyme stability and reaction rate under diverse process variables are required to achieve catalyst compatibility with industrial processes. An enzyme should be thoroughly evaluated in the laboratory, under the intended operating conditions, prior to scaling up to an industrial process. Purity of the intended enzyme is important because of the price/activity ratio.

Of particular interest for the food and feed industry is the use of enzymes that are active at low temperature. Most aquatic organisms are poikilotherms: their body temperature is similar to that of their environment. Poikilotherms living in polar, deep-sea, or any constantly low temperature environments, produce enzymes adapted in a variety of ways. These include increased enzyme concentration, change in the type of enzyme present, and adaptability of a homologous enzyme. Cold-adapted enzymes may manifest alterations in isoenzyme distribution, substrate binding, substrate turnover rate, thermal stability, physiological efficiency, and thermodynamic properties. Digestive proteolytic enzymes from cold-adapted aquatic organisms possess unique properties compared to mammalian proteases. Two key properties of cold-adapted enzymes useful in food and feed processing are high molecular activity at low reaction temperature and thermal instability (fast denaturation at moderate temperature). As industrial enzymes, these properties are advantageous because the process can be run at low temperature, thus minimizing bacterial activity and other interfering reactions, and the enzyme may be inactivated by moderate heat treatment after reaction.

Table 1 Tips for Developing an In-House Assay for Comparing Enzymes for Strength and Price

Identify the conditions for the proposed application: pH, temperature, moisture level, etc.
Consult the methods cited by the supplier
Select a substrate that is the same or similar to the material to be modified in your application
Integrate the application conditions and the proposed substrate with the published procedure
A functional property assay may prove to be more useful
Express the observed activity as specific activity to compare enzymes

This is very important in certain food process operations in which residual protease activity is undesirable (7–10).

Comparing enzymes from different suppliers for specific activity is a commonsense approach. Developing an in-house assay to compare enzymes in the lab is an easy task yielding economic and processing benefits. Tips for developing an in-house assay were developed by Boyce in 1986 (11) and are summarized in Table 1.

III. CURRENT APPLICATIONS OF FISH AND SHELLFISH ENZYMES IN SEAFOOD PRODUCTION

The traditional applications of enzymes in the seafood industry have been limited to very few products (fish protein hydrolysate, fish sauce, or cured herring). These processes are based on endogenous proteases in the fish (5). Recently, additional applications of fish or shellfish enzymes in the seafood industry have emerged. These include the improvement of the traditional applications by using exogenous enzymes to accelerate the process, production of other products (i.e., polyunsaturated fatty acids [PUFA]-enriched fish oils) (12), and the use of fish and shellfish enzymes to improve the production processes or as alternative processes in seafood production (selective removal of skin, fish-scale removal, or riddling process in cured roe production)(13–17).

A. Specialty Products

A product whose manufacture is made possible, directly or indirectly, by the use of enzymes is called a "specialty product." These products are differentiated from "conventional products," such as fresh, frozen, or canned fish or shellfish flesh, in which use of enzymes is not necessary for production, even though it may be advantageous (18). The main specialty products and the role of both the endogenous and exogenous enzymes in seafood processing are described below.

1. Mince Products

The term surimi is derived from the Japanese verb *suru,* which means "to mince" and it refers to minced and water-washed fish muscle tissues. The term is commonly identified with crab leg analogues, a surimi-based product, in Europe and in North America. Surimi is primarily used in the manufacture of various types of Japanese heat-gelled products such as *itatsuki, kamaboko, chikuwa, hanpen,* and *satsuma-age.* Essentially, surimi is a bland, preferably white, myofibrillar protein concentrate used for manufacturing different gelled or emulsion-type fishery products or as an ingredient in processing other foods. Surimi is regarded

as a valuable, functional proteinaceous ingredient, similar to the use of soybean proteins concentrates, in a variety of food products (19, 20).

Alaska pollock (*Theragra chalcogramma*) has been the most frequently used raw material for surimi processing (21). However, other fish species, such as Pacific whiting (*Merluccius productus*) (22), arrowtooth flounder (*Atheresthes stomias*)(23), Atlantic menhaden (*Brevoorti tyrannus*)(24), and sardine (*Sardina pilchardus*)(25) are also being used.

Surimi is minced fish flesh that has been refined by leaching the soluble fraction with repeated washing with water, straining to remove any remaining connective tissue elements and certain factors that accelerate protein denaturation, and the separating of lipids and other undesirable components. The product is then dewatered, stabilized, and mixed with cryoprotectants, such as sugar, sorbitol, or polyphosphates, and frozen. Surimi forms thermoirreversible gels upon heating, constituted by a three-dimensional protein network formed mainly of actomyosin (complex of actin, tropomyosin, troponin, and myosin filaments) (19, 21).

The characteristic rheological properties of surimi are largely based on the gel-forming ability of the fish myofibrillar proteins, myosin being the most important. The textural characteristics developed during gelation are expressed as gel strength, which is the primary determinant for surimi quality and price (26). The quality of surimi products is highly affected by the species-determined properties of proteins and by the activity of the endogenous heat-stable proteinases, which have a gel-softening effect. These proteases are very important as a manufacturing variable (24, 27–28; see also Chap. 19).

An important step in the formation of a high-quality gel from surimi, as occurs in the production of *kamaboko,* is the formation of ε-γ–glutamyl-lysine[GL] crosslinks in the fish proteins (Fig. 1) (29). GL crosslinks of proteins contribute to gel strength enhancement in surimi (30; see also Chap. 6). The endogenous enzyme responsible for the GL cross-link formation in fish flesh is transglutaminase. Transglutaminase (protein-glutamine-γ–glutamyltransferase, EC 2.3.2.13) is an enzyme capable of catalyzing acyl-transfer reactions in which the γ-carboxyamide group of peptide-bound glutamine serves as the acyl donor. This reaction introduce covalent crosslinks between proteins as well as peptides and various primary amines (31). When the ε-amino groups of lysine residues in proteins act as acyl acceptors, ε-(γ-Glu)-Lys bonds are formed between proteins, both intra- and intermolecularly. Without primary amines in the reaction system, water becomes the acyl acceptor and the γ-carboxyamide groups of glutamine residues are deaminated, becoming glutamic acid residues (Fig. 1)(32).

Transglutaminase-catalyzed reactions can be used to modify the functional properties of food proteins, including surimi production. Transglutaminase has been used to catalyze the crosslinking of a number of proteins, such as whey proteins, meat proteins (myosin and actomyosin), soybean proteins, and gluten. The

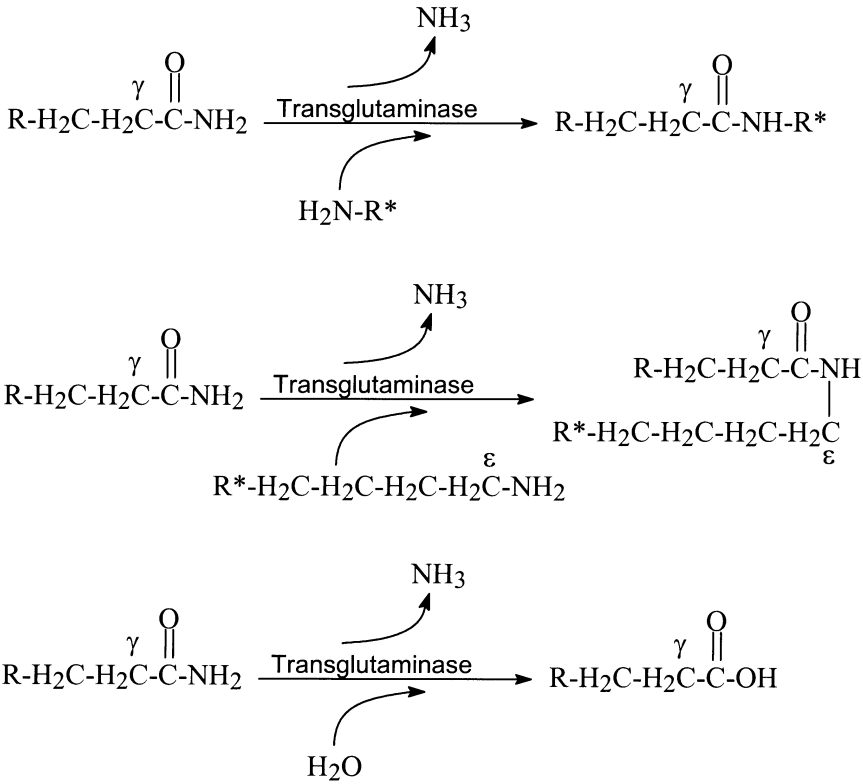

Figure 1 Transglutaminase-catalyzed reactions. When the ε-amino group of peptide-bound lysine act as acyl acceptor, ε-(γ-glutamil)-lysine bonds are formed between the proteins, resulting in their crosslinking (middle reaction). $R\text{-}H_2C^{\gamma}\text{-}H_2C\text{-}CONH_2$ = γ-carboxamide group of peptide-bound glutamine = acyl donor; $R^*\text{-}NH_2$ = primary amine group = acyl acceptor; $R^*\text{-}H_2C\text{-}H_2C\text{-}H_2C\text{-}H_2C^{\varepsilon}NH_2$ = ε-amino group of peptide-bound lysine = acyl acceptor. (from Ref. 53.)

modification of food proteins by transglutaminase may lead to textured products (hamburger, meatballs, canned meat, frozen meat, molded meat, surimi-fish paste, krill paste, baked foods, protein powders from plants, etc.), help to protect lysine in food proteins from various chemical reactions, encapsulate lipids and lipid-soluble materials, avoid heat treatment for gelation, improve elasticity and water-holding capacity, modify solubility and functional properties, and produce food proteins of higher nutritive value by crosslinking of different proteins containing complementary limiting essential amino acids. Transglutaminase use in these products has usually been from exogenous origin (33–35).

For surimi production, the role of transglutaminase and some other endogenous enzymes has been reviewed (36). The activity of endogenous fish transglutaminase decreases rapidly after catch and is almost completely destroyed by freezing. Therefore, manufacturing at sea has hitherto been required for high-quality surimi, but the cost of producing surimi at sea is considerably higher than onshore. The solution to this problem could be the addition of exogenous transglutaminase to facilitate glutamyl-lysine crosslinking (36–37).

Transglutaminase has been found in animal and plant tissues or organs (31). In the 1960s, the purification, characterization, and application of transglutaminase started with material of animal origin. Guinea pig liver has been the sole source of commercial transglutaminase for decades (38, 39). Bovine transglutaminase-enriched plasma fraction has positively been used to enhance gel strength of Pacific whiting surimi (40). The scarcity of the source and the difficulties of isolation and purification procedures for obtaining tissue transglutaminase from animals have resulted in an extremely high price of the enzyme: about $80 for one unit. It is thus not possible to use such tissue transglutaminase in food processing on an industrial scale. Fish tissue has been the source of transglutaminase in the laboratory, but not on a commercial scale. Transglutaminase from Alaska pollock was isolated and found to induce gelation in minced fish, under the same conditions as mammalian transglutaminase (Ca^{2+} dependent) (41). Recently, red sea bream (*Pagrus major*) transglutaminase was purified but the process is longer than other purification protocols from different sources and is more expensive (42). Transglutaminase from plant tissue has also been separated and purified, but is not commercialized (43). Both sources of transglutaminase are still in their infancy. Currently, extracellular transglutaminase from microbial sources has been purified from the culture filtrate of *Streptomyces* sp. (44) and *Streptoverticillium* sp. (45, 46). Microbial fermentation makes it possible to achieve mass production of transglutaminase from cheap substrates and therefore its subsequent commercial use.

The production of transglutaminase from microorganisms makes it possible to use this enzyme in a variety of food processes. Two patents report methods for manufacturing a traditional Japanese fish paste, *kamaboko,* using transglutaminase. Fish paste products are manufactured from material containing fish meat as the main ingredient and 0.1–700 U transglutaminase/g fish meat protein. A mixture of 100 parts dehydrated walleye pollock, with 3 parts NaCl, 5 parts potato starch, 10 parts water, 0.5 part monosodium glutamate, and 0.01 part transglutaminase was packed in a film, heated at 60°C for 30 min and at 90°C for 20 min, and cooled to manufacture a surimi with acceptable texture and whiteness (47, 48). The onshore manufacture of surimi can be more technically challenging than processing at sea. It is difficult owing to the inactivation of transglutaminase after harvesting. In the laboratory experiments, an extracellular microbial transglutaminase, produced by *Streptoverticillium mobaraense,* has

been added to a surimi preparation from Alaska pollock stored on ice for 2 days after catch, to obtain *kamaboko* gels. The results showed that gel strength was considerably improved through the formation of GL crosslinks by the addition of microbial transglutaminase in suwari gels (49).

In meat processing, methods for producing minced-meat products containing transglutaminase have been developed (50, 51). Minced meat and other food ingredients are mixed with transglutaminase, shaped, packed in pressure-resistant containers, and retorted to manufacture meat products such as hamburgers, meatballs, stuffed dumplings, and shao-mai (a typical Chinese food). These foods show improved elasticity, texture, taste, and flavor. Minced beef and pork, flour, onion, skim-milk powder, and condiments were mixed with water and microbial transglutaminase, packed with sauce in bags, and retorted to make raw hamburgers. Similar methods for meat and meat products treated with transglutaminase can be found in the literature (52, 53).

2. PUFA-Enriched Fish Oils

The health aspects of marine oils (lipids from fish or from marine mammals) were discovered by the Danish physicians Bang and Dyerberg in the 1970s by clinical examinations of Greenland seal hunters (54). Recently, medical and nutritional aspects of marine oils have received widespread attention, because they have been shown to have a preventive action against coronary heart diseases and to be necessary for normal brain and nervous tissue development in animal and humans (mainly young children) (55). Fish oils contain triacylglycerides and variable amounts of phospholipids, glycerol ethers, and wax esters. They contain a wide range of highly unsaturated long-chain fatty acids, with the number of carbon atoms ranging from 14 to 22 (56).

Polyunsaturated fatty acids (PUFAs) are essential components in human nutrition. For beneficial effect, the main PUFAs are the n-3 and n-6 families (also called ω3 and ω6). It is now recognized that the n-3 and n-6 fatty acids have distinct and sometimes opposing roles in human metabolism (57). Because n-3 and n-6 fatty acids are not interconverted in humans, they must be present in the diet. An appropriate ratio of n-3/n-6 PUFAs is 1:4. Nutritionists believe that the diet of many developed countries contains adequate quantities of the n-6 family, but a deficiency of the n-3 fatty acids. The dietary requirement for humans is about 1 g per day (58), but the Food and Agriculture Organization's (FAO's) Food Balance Sheet (1984–1986) indicates an average global consumption of only two-thirds of the minimum requirements. This had led to recommendations from expert committees that the intake of n-3 fatty acids be increased (59), particularly the long-chain fatty acids docosahexaenoic acid (C22:6, cis-4,7,10,13,16,19-docosahexaenoic acid [DHA]) and eicosapentaenoic acid (C20:5, cis-5,8,11,14,17-eicosapentaenoic acid, [EPA]). These desirable fatty

acids are bound into triacylglycerides, which is the natural form of fatty acids in fish oil (12). Both DHA and EPA are immediate precursors of biologically active molecules such as prostaglandins and thromboxanes, which participate in controlling a wide range of biological functions. Although DHA and EPA can be synthesized in the body by elongation and desaturation of α-linolenic acid, ingestion of the preformed molecules is usually more effective, especially for the very young or the elderly (60).

Further medical research has led to the conclusion that the beneficial effects of increasing dietary EPA and DHA can be classified into two main areas. First, these agents sustain normal healthy life through the reduction of blood pressure and plasma triacylglycerides and cholesterol, and increased blood coagulation time, because platelet aggregation decreases (61). Second, they alleviate certain diseases: blood vessel disorders and inflammatory diseases, and control of an overactive immune function resulting in alleviation of autoimmune disease, such as arthritis and some types of dermatitis (62). DHA is an important structural component in the membranes of the brain, nervous tissue, and eye, and of particular importance in the development of the fetus and the young child (63).

Dietary supplementation of long-chain polyunsaturated n-3 fatty acids is hampered because these acids are present in low concentrations in the commonly available edible oils. Microbial oils that contain high levels of either EPA and DHA can be produced by fermentation, but at a relatively high cost. Most fish oils contain only moderate levels of DHA and EPA. Cod liver oil is a well-known source of n-3 PUFAs used in the pharmaceutical industry (64). Cod liver oil is a complex mixture of more than 50 different fatty acids forming triacylglycerols, of which there is usually 8–9% each of EPA and DHA, and 22–24% of all n-3 PUFAs (12). However, fish and fish dishes currently provide an average intake of 0.2 g n-3 PUFAs per day, mainly as EPA and DHA (according to the FAO's above-cited report). To reach the proposed health requirement of 1 g per day in human nutrition requires a much greater consumption of fish or the use of fish oil in foods (apart from the possibility of using pharmaceutical concentrations). It is possible to increase the PUFA content in fish oil, or marine oils in general and use the oils in foods. There is a great commercial interest in preparing PUFA-enriched fish oil (56).

Preparation of oils in which such polyunsaturated fatty acids are enriched to high concentrations is done by various chemical methods, including using enzymic ones. It is possible to prepare triacylglycerides containing up to 30% EPA and DHA directly from fish oils without splitting the fat. Several methods are available, such as winterization, molecular distillation, and solvent crystallization. Moreover, it is possible to increase the oil concentration of n-3 PUFAs by first generating free fatty acids or esters totaling above 30%, which can then be fractionated by a variety of chemical methods, including supercritical fluid ex-

traction, complexing with urea, chromatography, or high-performance liquid chromatography (HPLC), obtaining 65–85% EPA + DHA levels according to the method (12). The PUFA concentrate can then be resynthesized into triacylglycerols using lipases from microorganisms such as yeast, fungi, and bacteria, under conditions that favor the reesterification process for triacylglycerol synthesis (12, 65–67).

The n-3 PUFAs are highly labile and may be destroyed by oxidation or *cis-trans* isomerization during processes that involve extreme pHs and high temperatures. Traditional chemical modification processes for fats and oils generally involve quite drastic conditions. Mild enzymatic modification processes are therefore necessary when heat-sensitive and labile fatty acids are involved. Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) is an enzyme that catalyzes the hydrolysis of a specific type of ester bond: triacylglycerides. They can be non-region-specific or n-1,3-specific towards triacylglycerides and they can possess specificity towards particular types of fatty acids (12). Moreover, lipases catalyze the reverse reaction: ester synthesis (from free fatty acids and alcohols) and interesterifications. There are several variations of the interesterification reaction: transesterification, in which ester–ester interchanges occur (Fig. 2), such as between triacylglycerides, triacylglycerides and monoester, monoesters, etc.; acidolysis, in which fatty acid exchange reactions occur, such as between triacylglycerides and free fatty acids; and alcoholysis, in which triacylglyceride is

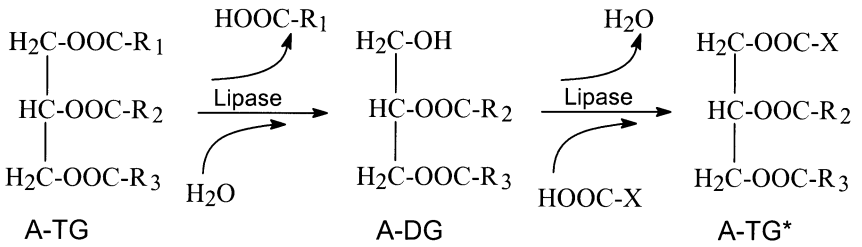

Figure 2 Schematic representation of a lipase-catalyzed transesterification to yield an acyltriglyceride enriched in EPA and/or DHA. This interesterification reaction involves sequential execution of the hydrolysis and reesterification reactions. The same enzyme catalyzes the deacylation/acylation step with different affinity by acyl positions and/or free fatty acids (PUFAs). A-TG = acyltriglyceride with native and arbitrary fatty acid residues (R_1, R_2, and R_3); A-DG = acyldiglyceride (also could be monoglyceride or glycerol, depending on the lipase specificity); HOOC-R_1 = product fatty acid; HOOC-X = new fatty acid to be incorporated. It is from a mixed of fatty acids in the reaction media, with a high proportion of EPA and/or DHA; A-TG* = acyltriglyceride with a incorporated fatty acid residue (OOC-X), which will be EPA or DHA depending on its concentration in the reaction media and the lipase specificity in the reesterification reaction. (from Ref. 224.)

allowed to react with a simple alcohol or glycerol (glycerolysis) leading to a mixture of mono- and diglycerides (12, 68).

Haraldsson surveyed the application of lipases to modify marine oils for preparing PUFA-enriched fish oil and predicted that this discovery might prove important for the marine oil industry (12), but there are only a few reports in the literature on such biotechnological applications (69). Apparently n-3 PUFAs, especially docosahexaenoic acid, are not good substrates for most commercially available lipases. This is simply a matter of fatty acid specificity and might change when lipases from fish or microorganisms, with a high affinity for long-chain polyunsaturated fatty acids, are isolated and made commercially available (68).

The discrimination of the *Candida rugosa* lipase to triacylglycerides containing different fatty acids has been reported. The C14–C18 saturated and monounsaturated fatty acids were preferentially hydrolyzed in triglycerides from capelin oil, whereas the long-chain monoenes, 20:1 and 22:1, and particularly, the PUFAs 18:4, 20:5, and 22:6, were resistant to the hydrolysis (70). Lipolytic enzymes, isolated from the Atlantic cod (*Gadus morhua*), have been shown to have preference for hydrolysis of the PUFAs over the shorter-chain acids. This specificity is completely opposite to that of the lipases commercially available at that time (mainly porcine pancreas lipase) (71–73). The specificity of purified cod lipase is in good agreement with the crude pancreas lipase of leopard shark (74) and digestive lipases of four marine fish species (75). Comparative studies of cod and human milk lipase showed that the cod enzyme has a higher reactivity for hydrolysis of very long chain polyunsaturated fatty acyl esters (76). A bile-salt-activated lipase (carboxyl ester lipase) has recently been purified and characterized from the extract of the delipidated powder of red sea bream (*Pagrus major*) hepatopancreas. The enzyme efficiently hydrolyzed ethyl esters of polyunsaturated fatty acids such as arachidonic acid and eicosapentaenoic acid, which were resistant to porcine pancreatic lipase (77). Thus, lipases from marine fish, such as red sea bream and cod, efficiently hydrolyze esters of highly unsaturated fatty acids, such as C20:4n-6 and C20:5n-3. This suggests that the fatty acid specificity of fish lipases might be a crucial factor when considering the use of lipolytic enzymes to obtain PUFA-enriched marine oils.

Different methods have been described for preparing concentrates of n-3 PUFAs, mainly EPA and DHA. However, all process described use lipases from microorganisms (*Pseudomonas* spp., *Chromobacterium viscosum, Candida rugosa* [*C. cylindracea*], *Candida antarctica, Aspergillus rhizopus, Rhizomucor miehei, Rhizopus niveus,* etc.), but not from fish, in spite of the more appropriate specificity of fish-derived enzymes. This could be because microbial lipases are less expensive and the n-3 PUFA specificity of commercially available lipases is adequate for production. These processes produce marine oils with high EPA and DHA concentration, reaching 85% of the two fatty acids (78–80). Porcine pan-

creatic phospholipase A_2 has also been used for extracting n-3 PUFA-enriched oil from cod roe and gave 24% EPA and 40% DHA in the free fatty acid fraction. In laboratory experiments, the recovery was 60%, corresponding to a yield of 6 g polyunsaturated fatty acids per kilogram cod roe (81). Although, with these results, one expects that there are industrial processes to obtain n-3 PUFAs via lipase, to the authors' knowledge there are no patents for these processes.

3. Caviar and Other Roe Production

One of the major delicatessen-type salted-fish products is caviar. The word "caviar" refers only to the riddled and cured roe of the sturgeon (*Acipenser* spp. and *Huso huso*), although in recent years it has been used to describe similarly treated roe of other, less expensive, fish species. In this way, black caviar is prepared from the eggs of sturgeon and red caviar from Salmonidae. Less expensive caviar is made *from the eggs of several fish: cod, catfish (Parasilurus astus), herring* (*Clupea harengus*), capelin (*Mallotus villosus*), lumpfish (*Protopterus aethiopicus*), and some freshwater species (20). Caviar substitute is produced by coloring lumpsucher roe (*Cyclopterus lumpus*) to mimic sturgeon caviar. The preparation of caviar starts with the riddling process, which consists of separating the roe from the roe sack (ovaries). These should be taken from the fish at the moment of slaughter. The eggs are immediately washed with cold water; salted with dry, fine salt, 3–5% of the egg weight, or brined for 8–18 min in saturated brine at egg/brine ratio of 1:3; drained; and packed in cans or jars. Some assortments of caviar are pasteurized in sealed cans at 60–70°C for 2.5–3 h. The shelf-life of pasteurized caviar at 10–18°C is 3–4 months and at -2°C it is 6–8 months (82).

The riddling process is a somewhat laborious task, which is either carried out manually or mechanically. One problem in the conventional process is that it is difficult to release the roe particles from the supportive connective tissue of the roe sac without destroying a large amount of the roe. Sometimes the yield of intact roe is as low as 50% (83). This problem has been solved using enzymes. A US patent describes a method by which proteinases (acid, neutral, or alkaline conditions) are used to release salmon roe from the connective tissue (84).

To ease the riddling process in caviar production, fish enzymes are used to achieve a gentle separation of roe from the connective tissue. Enzymatic roe rinsing is a very delicate process demanding strict control of all the variables to avoid damage of the roe cell wall. Whole or split-roe sacs are immersed in a water bath containing the egg-releasing enzyme. Several enzymes have been used. Satisfactory results have been obtained with cold-active Atlantic cod pepsin (83, 85–88) and New Zealand orange roughy acid proteinase (*Hopolostethus atlanticus*) (89). That these enzymes have high activity at low temperatures gives them unique application possibilities (e.g., it is important to run the enzymatic process

at a low temperature to keep bacterial contamination at a low level). Moreover, fish pepsins apparently split the linkages between the egg cells and the roe sack without damaging the eggs (15). After the enzyme treatment, the roe may be separated from the connective tissue by sedimentation in a flotation tank. The connective tissues are removed by flotation. By accurate adjustment of salt concentration and flotation conditions, it is also possible to remove damaged roe together with the connective tissue. The caviar yield from rainbow trout (*Oncorhynchus mykiss*) roe is about 90% compared to 70% with conventional mechanical methods (88, 90).

There is a small commercial production of cold-adapted pepsins from fish viscera (Atlantic cod) by a Norwegian company (Marine Biochemicals, Tromsø). This type of production is also beginning in Iceland at the Icelandic Fisheries Laboratories (IFL) (13, 87). Commercial production is also being started in New Zealand (89). Industrial production of caviar with these enzymes has been started in Canada, the United States, Australia, and several Scandinavian countries. Process equipment for enzymatic rinsing of roe in caviar production has been developed by Biotec-Mackzymal and by Trio Industries in Norway. So far, mainly salmon and trout caviar are produced by enzymatic methods, and the annual production is about 50 tons (14). The enzymatic production of shrimp (*Pandalus borealis*) roe caviar has also been investigated. Tests are being made at IFL (13).

Enzymatic methods, based on cold-adapted fish enzymes, can be used for other fish roe processing operations: to remove the mucoprotein layer on newly spawned eggs from walleye and catfish to improve hatching yield and survival of larvae, and the enzymatic removal of the chorion from fish eggs to facilitate observation of embryogenesis and microinjection of naked genes in research experiments (14).

4. Preparation of Cured Fish Products

Preparations of proteolytic enzymes may be used as processing aids in preparing ripened, salted fish. The annual world production of cured fish products amounts to several hundred thousand tons. Most of these are cured herring (*Clupea harengus*) (e.g., Dutch "matjes" made from immature, fatty, feeding herring) and fermented squid (*Illex illecebrosus*) (13, 91, 92). The cured products are made by a maturing or ripening process of raw materials. The sensory characteristics of cured fish result from the salting process and enzymatic changes in protein, lipids, and carbohydrates, from different interactions of the products of these enzymatic reactions, and from the added spices (20, 93).

In traditional cured fish products, a partial proteolysis occurs, catalyzed by endogenous fish enzymes during fish processing. This is caused by the activity of muscle proteases, which play an important role in muscle protein metabolism

and in tissue degradation during postmortem tenderization (82, 94–96). The activity of these muscle proteases, however, is small compared to the proteolytic activity of the gastrointestinal tract (97), especially in herring and cod (98). These fish proteases play a decisive role in the maturing or ripening process of fish meat. The ripening of these products appears to depend on endogenous digestive enzymes producing the characteristic texture and flavor (15). The possibility that components from the feed also contribute to the characteristic flavor, either directly or by participating in some reactions with the muscle components, should not be ruled out (20).

The production of herring products probably originates from Scotland in the 8th Century, and is practiced in all countries that harvest fish in the North Atlantic and the North Sea. Traditionally, whole herring, with or without the head, are mixed with salt and stored cool (5–10°C) in barrels for a few months before they are washed, filleted and packed. In many countries, concentrated salt brine is added before the barrels are closed. During storage, enzymes from the digestive tract leak out and, together with other endogenous fish enzymes, cause partial digestion of the muscle proteins. The fish meat becomes smooth and pliable and attains a pleasant rich flavor. The final salt concentration can vary from approximately 4 to 18%, depending on the recipe used. The high salt concentration and low temperature restrict excessive proteolysis, mainly due to gastrointestinal proteases, and the ripening process usually takes from 2 to 7 months. This traditional maturing procedure is time-consuming and demands large storage capacity (13, 14).

Trypsin-type enzymes from the pyloric caecum are the major contributors to endopeptidase activity in the ripening process. The concentration of these digestive enzymes is greatly affected by seasonal changes in feeding intensity, the highest being before spawning (see Chaps. 1 and 9). The North Sea herring is the only fish caught in the period from May to August that contains sufficient digestive proteolytic enzyme activity to promote satisfactory ripening (13, 99).

Another problem related to storage capacity is the use of whole herring in the ripening process. When whole fish is salted, the filleting waste becomes too salty to be used as animal feed. By filleting before salting and ripening, two advantages are obtained: the storage capacity is reduced, and the filleting waste can be used as animal feed. However, endogenous fish enzymes are necessary to achieve maturation in herring fillets or gutted fish (14). To avoid the seasonal variations and induce or accelerate the ripening process, experiments involving the addition of exogenous enzymes to the brine have been done since the early 1970s (91, 99–102). Several methods for artificial ripening of herring involving enzyme addition have been developed and patented. The nature of enzyme addition has been different. Pancreatic enzymes from pig or cattle have been used to obtain good quality matjes herring from fish caught during nonfeeding period (99), or a proteolytic mixture containing mainly trypsin or chymotrypsin (from

beef organs) has been used for the preparation of matjes herring from which head and viscera are removed (103). However, the main exogenous enzyme preparations used in the ripening process are of marine origin. By addition of exogenous fish enzymes the duration of the ripening process has been reduced and a quality similar to matjes herring obtained by traditional methods has been maintained. Nevertheless, if the enzyme preparations contain high lipase activity, the product attains an inferior flavor (99).

The first fish-enzyme addition used in artificial ripening was minced pyloric cecum from herring caught in midsummer. This preparation was added with salt to spent herring caught in October and a good ripening was obtained (100). Another artificial ripening method has been patented in which herring fillets are matured in brine containing digestive enzymes from herring. One part herring fillets is immersed in 1.3 parts brine containing 12% salt, 6% sugar, a spice mixture, and 5% minced herring pyloric cecum. After only 5 days at 3°C, the fillets attain the smooth consistency and flavor that characterize traditionally matured products. The final salt concentration is only 6%, and extensive washing before consumption is not necessary. The amount of pyloric cecum used in this method corresponds to the natural content in herring before spawning (summer-caught)(101–102). However, the industrial use of adding herring viscera enzymes to control the ripening does not appear to be widely practiced, perhaps because enzyme preparations from herring viscera are not available as commercial industrial products.

With the enzyme added as a crude extract to accelerate the ripening of herring fillets, an acceptable flavor has been obtained, but the stability of the final product is poor because of residual enzyme activity. This problem does not arise in the traditional long-term storage methods because the enzyme activity gradually declines during storage, giving a quite stable final product with little residual enzyme activity. Thus, to obtain stable products with rapid ripening methods, it is necessary to reduce the residual activity of the added enzyme as much as possible. In this sense, trypsin isolated from the pyloric cecum of Greenland cod (*Gadus ogac*) has been used in ripening experiments with herring and squid. The maturing was achieved mixing trypsin (25.5 mg) and eviscerated herring or squid mantle (1.5 kg), packed and pickled in brine, for 40 days at 10°C. The cod enzyme was more efficient than the bovine pancreas trypsin used as control (the protein solubilization rate was double). In addition, the residual trypsin activity in the brine decreased more rapidly for the cod enzyme than it did for the bovine enzyme, because of end product inhibition and enzyme instability (91). Cod trypsin has better catalytic properties than cattle trypsin at the temperature used in artificial ripening.

Squid liver has been investigated as a source of enzymes that may be used for supplementation of brined Atlantic short-finned squid (*Illex illecebrosus*) to accelerate the autolytic fermentation (92) and to tenderize squid

(*Illex argenticus*) (104). Squid liver extract has a comparatively high proteolytic activity against hemoglobin and casein at pH 2–8 and high proteolytic activity at low temperatures. It is rich in cathepsin C, an enzyme having dipeptidyl transferase and dipeptidyl hydrolase activity, optimal at pH 6–6.5, which improves the formation of tasty amino acids (glutamine, alanine, leucine, serine, lysine, arginine, and proline) in squid meat. The sweet delicious taste of brined squid may be enhanced by supplementing the brine with pure Greenland rock cod trypsin or with a neutral protease fraction from squid liver possessing cathepsin C activity during the fermentation process. Fresh squid mantles (less than 12 h postmortem) were skinned and sliced. The squid (500 g) was mixed with 350 ml brine solution including purified Greenland cod trypsin (26.5 mg, 7,000 BAPNA units) and a crude preparation of cathepsin C from squid liver (105 mg). The squid was incubated at 6°C and pH 6 for up to 120 days. The result was a brined squid with better sweetness and delicious taste than control. Cod trypsin acted to accelerate free amino acid accumulation during the first stage of fermentation, whereas cathepsin C activity resulted in a more sustained enhancement of free amino acid accumulation, mainly tasty amino acids (92).

Furthermore, most of the proteases of squid liver extract are also collagenolytic enzymes. At pH 7, the proteins of the squid sarcoplasmic and myofibrillar fractions, as well as squid collagen, are hydrolyzed to low-molecular-weight fractions after 24 h at 4°C. The high activity of squid liver enzymes, at neutral pH range and at refrigeration temperature, has been used to tenderize squid mantle meat before cooking. By soaking skinned squid mantle meat in liver extract (1:3) for 24 h at 20°C or 4°C prior to cooking 45 min in water, a significant hydrolysis of the proteins (65%) was obtained. A 40% decrease in toughness of the cooked product was obtained compared to the toughness of untreated cooked samples. The mantle cooked directly in enzyme preparations was not more tender than that of untreated samples (104).

Although artificial maturing methods have obvious advantages (reduction of storage capacity requirement and improved use of the raw material), the industrial applications of such methods still appear to be limited. One reason is that appropriate enzyme preparations are not commercially available. Their availability is complicated, especially for fish or shellfish enzymes, since both the source and purification process are expensive (15). Also, poor stability is obtained in the final products using the rapid methods as opposed to the traditional methods (14). Furthermore, the enzyme preparations should have low lipase activity, otherwise an undesirable fatty acid taste can occur in the cured product (99). The solution will be the use of purified fish or shellfish digestive enzymes, in particular cold-active proteases, because they are more suitable tools in accelerated maturing than mammalian enzymes (105).

5. Protein Hydrolysates from Fish and Shellfish

Fish protein hydrolysates (FPH) are a mixture of proteinaceous fragments and are prepared extensively by digestion of whole fish or other aquatic animals or parts thereof using proteolytic enzymes (endogenous and exogenous) at the optimal temperature and pH required by enzymes. The process of hydrolysis breaks down the protein into smaller peptides to obtain a water-soluble product (106, 107). FPH production is a means of transforming a large proportion of total landed fish, which remains underused (by-catch pelagic fish species and unconventional species such as krill), to a hydrosoluble protein concentrate with food applications (5, 107).

Liquid fish hydrolysates, prepared from small fish, were known in ancient Rome. Detailed description of how to prepare such a "liquemen" appears in *Roman Cookery Book.* Similar liquid products are popular in Southeast Asia (108). The real development of FPH started in Canada in the 1940s after World War II. Methods for the enzymatic preparation of protein hydrolysates from fish meat were developed (109). The first intended application of this amino nitrogen source was as peptone in growth media for microorganisms. Peptone was prepared by autolysis of fish viscera (110) and by a two-step enzymatic hydrolysis of whole fish, imitating in vivo digestion. The first digestion step was an autolysis of acidified minced fish with endogenous pepsins and peptidases. The second enzymatic hydrolysis step was done by proteinases added under alkaline conditions, prior to removal of the digested proteins (111). These peptones supported bacterial growth better than hydrolysates prepared by chemical hydrolysis and comparable to the best meat peptones on the market (112). At present, industrial fish peptones are an excellent substrate for biomass production in solid and submerged fermentation and are produced commercially in Norway and Japan (14).

However, the main use of fish and shellfish protein hydrolysates has been as food or feed ingredients (113). The production of FPH has been described in several reviews (113–120). The general procedure for fish and shellfish protein hydrolysate preparation could be detailed as follows (21).

1. Substrate. The raw materials are minced and mixed with an equal amount of water. The pH is adjusted to the desired level. Raw materials may include by products from filleting (waste, viscera, etc.), by-catch fish from trawlers, small underused lean fish species or krill (a collective name used for five or so species of shrimplike creatures, the most abundant of which is *Euphausia superba;* see Chap. 18).

2. Hydrolysis. Proteolysis is carried out by autolysis and/or by addition of a preparation of hydrolytic enzymes. Depending on the concentration of endogenous proteinase activity in the raw material, enzyme addition improves the hydrolysate yield. The enzymes are added at optimal hydrolytic temperature under

stirring. At the end of the incubation period, the enzymes are inactivated, and the material is pasteurized by heating to above 80–90°C.

Considering that the hydrolytic process is aimed essentially at increasing protein solubility, industrial operations use proteases with broad specificity that collectively achieve peptide cleavage at random. Commercial proteases are usually from animal, plant, or microbial source. Animal (mammalian) proteases include mostly pepsin (121), although chymotrypsin and trypsin have also been tested (122–124). Bromelain, papain, and ficin are the plant enzymes most widely used (114, 125) and patented (14). Microbial proteases of bacterial origin and low price have been evaluated (119, 123, 126). The use of proteolytic enzymes from fish itself has also been suggested (5).

3. Separation. Bones and scales are removed by sieving. Oil, sediment, and hydrolysate are subsequently separated by a three-phase separator.

4. Concentration. The hydrolysate is filtered, evaporated, and dried by a vacuum or spray-drier to prepare soluble FPH.

Type of raw material, hydrolytic power of enzyme, temperature, and incubation time are the main factors determining the protein hydrolysate yield. In enzymatic production of protein hydrolysates for food use, processes such as sterilizing the protein source, use of a bacteriostat during hydrolysis, and pasteurization of the hydrolyzed protein are necessary for good quality control (127).

Although most FPHs are produced by direct addition of an enzyme obtained from commercial sources, endogenous enzymes from the raw material are also used to manufacture different protein hydrolysate products. Thus, FPH from whole Caspian sprat (*Sprattus spracttus*), based on partial hydrolysis involving endogenous proteases, has been developed (108, 128). Krill is an underused marine shellfish with growing world production (107). It is very rich in proteases and undergoes rapid autolysis. During autolysis most of the krill protein is hydrolyzed to free amino acids after 48 h at 20°C. Because of these properties, an industrial process for production of free amino acids has been developed (129). Moreover, free amino acids are recovered as a byproduct in bulk production of krill proteolytic enzymes (a mixture of endopeptidases and exopeptidases) (130, 131), commercialized by a Swedish company (Pharmacia, Upsala) and efficiently used in debridement of necrotic animal wounds (132, 133).

The main use of FPH in food is in dietetic foods as a source of small peptides and amino acids, in products such as soufflés, meringues, macaroni, or bread, and in fish soup, fish paste, and shellfish analogues as flavoring compounds. High dispensability of such products render them suitable as a substitute for milk proteins (14, 107). The major feed applications are as milk replacers for calves (134, 135) and weaning pigs and as protein and attractants in fish feed (14). Hydrolyzed krill is now being investigated as an ingredient in starter diets for fish larvae and juveniles (136). Moreover, stimulation of the immune system

has been demonstrated in animal feeding with FPH because of certain peptide compounds (137).

The choice of enzyme preparation and the process conditions determine yield (profitability), properties (solubility, emulsifying or gelling, flavor, nutritional value, etc.), and use (food or feed) of the product (21). Bitterness is a common problem in preparation of FPHs and for this reason they are unsuitable as food ingredients (138). This is caused by the formation of peptides containing bulky hydrophobic groups toward their C-terminal. The intensity of bitterness depends on the degree of hydrolysis and the specificity of protease (i.e., the size of peptides and the kind of peptide bonds cleaved) (106, 107).

Several methods have been suggested to mask the bitterness of FPHs. These include incorporation of glutamic acid or glutamyl-rich peptides, polyphosphates, gelatin, or glycine into the products (139). Bitterness can also be eliminated by selective separation (extraction or chromatography) and by enzymatic treatment, which consists of using exopeptidases to carry out the plastein reaction. The "plastein" is resynthesized from peptides and amino acids by transpeptidation (108, 140). Plastein is a high-molecular-weight proteinlike substance with different properties from those of the original protein and devoid of contaminants having flavor. Thus far, plastein formation has been facilitated with proteolytic enzymes (trypsin, chymotrypsin, pepsin, etc.) at 37–50°C, producing a hydrophobic product (141). Cold-adapted trypsins, chymotrypsins, and pepsins from fish have been proposed to facilitate the formation of hydrophilic plasteins at low temperature to maintain the water solubility of the protein hydrolysate (15). Another method of avoiding the bitterness in FPHs is to reduce the formation of bitter-tasting peptides and excessive protein hydrolysis to peptides and amino acids. Cold-adapted enzymes from fish have a relatively narrow specificity for peptide bonds and relatively high molecular activity (8). Addition of these enzymes during the hydrolysis process has been suggested, since this improves the amount of pleasant-tasting active amino acids and reduces the percentage of TCA-soluble proteins, thus preventing the bitterness problems in protein hydrolysates (5).

6. Seafood Flavorings

Flavor enhancers or flavor potentiators are know to be chemical substances that have little flavor of their own but, when mixed with food products, have the ability to enhance the flavor of the food. Enhancers used extensively worldwide include monosodium glutamate (MSG), inosine-5′-monophosphate (5′-IMP), and guanosine-5′-monophosphate (5′-GMP). These compounds accentuate meaty flavor and have found applications as flavor potentiators in soups, sauces, gravies, and many other savory products (142).

Flavor potentiators occur naturally in sources such as sea tangle (*Lami-*

naria japonica), dried bonito tuna, and black mushroom (*Lentinus edodus*). However, flavoring agents have usually been produced by enzymatic transformations. Certain fresh seafood is rich in 5′-AMP (adenosine-5′-monophosphate), which may be converted to 5′-IMP by the action of endogenous enzymes released during processing. Several important enzymes that have applications in flavor-enhancing nucleotide and amino acid production have been identified in naturally occurring products of plant, animal, or microbial origin. These enzymes predominantly belong to the general group of hydrolases. Commercial production of extracts rich in flavoring agents is mainly from yeast extracts and hydrolyzed vegetables, both of which are rich in glutamic acid. Yeast extract is a concentrate of the soluble fraction of yeast generally made by autolysis. Hydrolyzed vegetable protein is a product composed of amino acids or peptide savory flavoring, obtained from soy bean, wheat, and other plant substances by a partial proteolysis followed by acid hydrolysis (142).

Seafood flavors are in high demand for use as additives in products such as kamaboko, artificial crab, and fish sausage, and cereal-based extrusion products such as shrimp chips (143). Seafood flavoring from various sources of raw material can be produced by enzymatic hydrolysis. Exogenous and endogenous proteolytic enzymes can aid the extraction of flavor compounds from fish and shellfish byproducts, although other types of enzymes may also be involved (5). A similar process to FPH manufacturing has been developed by a French company (Isnard-Lyraz) to recover seafood flavoring compounds from fish, shellfish, or mollusks, using whole animals or their byproducts (144). The process consists of liquefaction of the raw material by enzymatic hydrolysis, thermal inactivation of enzymes, separation of bones and shells, filtration or centrifugation, and concentration of the flavors and flavor enhancers naturally occurring in the raw materials. The purpose of the enzymatic hydrolysis is to liquefy and allow the separation of bones and shells, also facilitating concentration or drying. Moreover, enzymatic hydrolysis permits the nucleotide and protein transformation to flavor enhancers without producing additional flavor or taste. The final products consist of pastes or powders and are natural extracts with flavoring properties, but they are not flavors. Exogenous enzymes are used but endogenous enzymes are also of importance for the hydrolysis. No more details are available of this patented process (144).

Various kinds of byproducts have been used to produce marketable seafood flavoring. The production is mainly based on autohydrolysis by their endogenous enzymes. Thus the flavor from oysters (145), shrimp (146), and clam (147, 148) have been recovered.

7. Fish Sauces

Fish sauce has long been a traditional fermented fish product and is an important source of protein in Southeast Asia. It is also consumed in Europe and North

America. This seafood is a liquid product made by storing heavily salt-preserved fish material at tropical temperatures until it is solubilized by endogenous enzymes (21). Typical examples of such products are the *nuoc-mam* produced in Vietnam and Cambodia, *nam-pla* in Thailand, *patis* in the Philippines, *uwo-shoyu* in Japan, or *ngapi* in Burma (149).

The production of fish sauce has recently been reviewed (150). Traditional fish fermentation is actually a combination of salting, enzyme hydrolysis, and bacterial fermentation. The general procedure for fish sauce preparation is summarized as follows.

1. Salting fish. Small fish, both marine and fresh water, whole or minced, are mixed with sun-dried marine salt in a ratio of 3:1 for marine fish or 4:1 for fresh water fish (20–40% salt, minimum 15%). The mixture is then placed into fermentation tanks between two layers of salt.

2. Hydrolysis and fermentation. Closed tanks are stored at ambient tropical temperatures. Tissue solubilization occurs as a result of autolytic action by fish digestive enzymes. This hydrolysis provides the necessary nutrients for halophilic bacterial fermentation to begin, which plays an important role in flavor development. Protein hydrolysis is caused by trypsinlike enzymes (152) together with fish-gut peptidases. The pH of fish sauce ranges from neutral to slightly acid. Because of nonoptimal pH, partial inhibition of high salt concentration, and salt impurities, the activity of digestive proteases is at a minimum and the rate of autolysis is low (14). Under these conditions, the time needed for the full flavor of fish sauce to develop varies from 8 to 18 months. When fish sauce is made from cod viscera, mixed with salt (25% w/w) and stored at 22–27°C, only a 25 day fermentation period is needed. Moreover, this process permits the recovery of a tryptic enzyme concentrate by ultrafiltration during the fermentation process. Spray-dried enzyme concentrate has been used in the ripening of salting herring fillets (10 g kg^{-1}), obtaining a satisfactory ripening more rapidly than by the traditional ripening of ungutted herring (151).

3. First-quality fish sauce. After fermentation is completed, the liquid is drained off and saturated brine is added to the residue to extract the leftover soluble matter. The two liquids are then combined, filtered, and bottled to produce first-quality fish sauce. This amber protein hydrolysate has a high nutritional value, being rich in the essential amino acids lysine and methionine. However, the high salt concentration may be a health risk depending on the daily intake and the rate of perspiration.

4. Second-quality fish sauce. The remaining partly digested fraction is extracted several times with saturated brine until most of the proteinaceous material is recovered to obtain second-quality fish sauce. This product is cheaper and more accessible to poor people, but has an inferior nutritional value.

The long production time means that storage tanks of large capacity are required. From an economic point of view, to reduce this need it is desirable to

speed up the solubilization (21). Several methods have been proposed to reduce the production time. The first is to raise the initial temperature. A couple of weeks at 45°C in the initial storage phase reduces the total production time from 1 year to 2 months (14). The second is addition of acid combined with reduced salt content. Pepsin is inhibited by salt. Thus, an initial phase of rapid autolysis is carried out at pH 4 (obtained by addition of hydrochloric acid) and low salt concentration, at 27±2°C. After this phase (5 days), samples are neutralized with sodium hydroxide solution, and salt is added to the normal level (250 g kg^{-1}). By this method, an acceptable flavor could be achieved after 2 months. However, some endogenous enzymes that are important for flavor development during storage at neutral conditions are denatured at low pH (153–154). The third is initial alkalization at low salt concentration. If the initial pH is raised to 11 by addition of sodium hydroxide at a moderate salt concentration, alkaline digestive proteases will be very active, whereas endogenous trypsin and chymotrypsin inhibitors will be denatured. The final product has a limited storage stability and needs to be neutralized with hydrochloric acid and salt addition (to 250 g kg^{-1}) to achieve the same level and flavor as traditional fish sauce. These conditions shorten the process to 2 months (155).

These accelerated methods have achieved an improved hydrolytic rate. However, inferior flavor has also been produced, the problem in most cases being that the taste is either too weak or too bitter. Bitterness appears for the same reasons commented on in the discussion of FPHs. However, in China a method based on initial acidification is apparently used by some fish sauce manufacturers (21).

Another method used to accelerate the protein hydrolysis in traditional fish sauce is the addition of enzyme-rich components. Plant enzymes were the first to be used (156). Papain from unripe papaya, bromelain from pineapple stems, and ficin from figs have all been tested. These enzymes are cysteine proteases most active under weak acid conditions. Fish sauce recovery was obtained after 2–3 weeks of the fermentation process. However, the characteristic flavor of the finished product was inferior to the traditional, although the best results were obtained by using bromelain preparations. This accelerated method is used today in commercial fish production in Thailand (14).

The use of squid hepatopancreas tissue to accelerate traditional methods in fish sauce production has been reported (5, 15). Capelin (*Mallotus villosus*) fish sauce was prepared by mixing minced fish with salt (25% w/w) and supplementing with squid hepatopancreas tissue (SHP) (2.5% w/w). After 11 months at 20–25°C, the fish sauce was recovered by filtration and stored at ambient temperature. Fish sauce was analyzed and evaluated by a sensory panel 2–3 months after filtration. The free amino acid content and hydrolysis rate during the first month were significantly higher than for the control fish sauce or for other proteolytic enzyme supplements (fungal protease, pronase, trypsin, chymotrypsin, or

squid protease fraction). Preference analysis of capelin fish sauce showed that the product supplemented with squid hepatopancreas was highly accepted and preferred to a commercial product from the Philippines (157). The fermentation time of SHP-supplemented fish sauce can be shortened to 6 months, resulting in a free amino acid content and sensory evaluation score significantly higher than the control fish sauce. The better acceptability scores of SHP-supplemented fish sauce are caused by a higher content of free amino acids and peptides, ranging from 100 to more than 1,300 Da and rich in aspartic acid, serine, glutamic acid, and leucine. However, typical flavor has also been correlated with large peptides (the fraction greater than 10,000 Da) (158). Squid hepatopancreas proteases produce an acceptable proportion of taste-influencing free amino acids and peptides to yield fish sauce with more flavor and less fermentation time than in the traditional process.

B. Enzymes as Fish-Processing Aids

Over the last decades significant research and development have been done in seafood processing on biotechnological methods using enzymes as an alternative to conventional processing methods. This challenging task has been in progress in Iceland (13, 159) and Norway (88, 90) since the 1980s.

Mechanical processes have obvious limitations regarding selective and gentle treatment of the raw materials. In many situations, mechanical processing implies low yield and quality reduction. In some cases technical problems are not solved by mechanical means. Hydrolytic enzymes digest certain tissue structures, leaving others intact. Some normally resistant tissue structures are sensitive to enzyme digestion when correct conditions of pH, temperature, or the salt concentration are met. Because of this, enzymes can be used as specific tools in food processing, acting as gentle knives, where the aim is a selective removal or modification of certain tissue structures (14).

1. Deskinning

The common method of removing skin from fish fillets is purely mechanical: an automated machine in effect tears the skin off the flesh. The ease of deskinning varies greatly among fish species (18). The biochemical method of deskinning works because skin differs fundamentally from muscle in chemical composition and structure, and enzymes are generally very specific in their action (88). Enzymatic deskinning can be done by cold-adapted fish pepsin at low reaction temperatures (5, 15). In addition, fish pepsin at low pH breaks down fish skin rapidly but breaks down muscle protein relatively slowly (160). Proteases are usually mixed with carbohydrases to facilitate skin removal (16).

In Norway, an enzymatic skin removal method has been developed for

herring. The whole herring is treated in 5% acetic acid at 10°C, to denature the skin collagen, and is immediately transferred to the enzyme bath containing cold-active fish pepsin and 0.5% acetic acid. After 1–2 h at 20°C, the skin is partially solubilized and can be washed off. Differences in skin structure and thickness on different parts of the body make it difficult to achieve uniform deskinning (14, 83, 161).

Proteolytic enzymes from squid intestines may also be used for removing the double-layered skin of squid. The approximate collagen content in the mantle muscle and skin of squid is 4.6 and 21.9% per dry tissue and 5.4 and 28.4% per crude protein. The collagen content per crude protein of the skin is about five times as much as that of the muscle (162). The conventional mechanical deskinning method removes only the pigmented outer skin, leaving the rubbery inner membrane intact. The enzymatic deskinning process involves soaking the squid tubes and tentacles in a weak salt solution containing a squid intestine extract (including liver) at 45°C for a short time, which selectively attacks the rubbery membrane without degrading the muscle tissue (13, 163). Most of the proteases of squid offal extract are also collagenolytic enzymes (164). A complete production line for a similar process of squid enzymatic deskinning has been developed by Biotec-Mackzymal in Norway and produced by Carnitech in Denmark (14). A company from Russia and Liechtenstein (Seatec) is producing a collagenase preparation from crab hepatopancreas that may be used in deskinning of squid (20).

Starry ray (*Raja radiata*) is the most common skate species in Iceland. It is caught in a by-catch/landed-catch proportion of 16:1 every year and it is underused. Some of the skate catch is used to manufacture and export skinned skate wing (fresh or frozen). The conventional deskinning method is both laborious and difficult, since the skate wing skin is partly covered with sharp spikes. Mechanical or manual skinning leaves residual skin fragments that must be removed when the process is repeated. An enzymatic method for deskinning skate wings has been developed to yield improved product. The skin collagen is denatured by a gentle and rapid treatment, followed by incubating the skate wings at low temperature (0–10°C) in a enzyme bath for a few h (or overnight at 0°C). The final step is to rinse the dissolved skin and remaining spikes from the skate wings (13). The enzyme preparation used contains cold-active proteinases with high collagenolytic activity, possibly from fish or shellfish digestive system, and carbohydrases, that may be acting by loosening the collagenous layer or by making the access to the denatured collagen easier for the proteinase enzymes.

2. Descaling

Descaling of fish species such as redfish (*Sebastes marinus*) or haddock (*Melanogrammus aeglefinus*) by mechanical means can be a problem because

the scaling treatment is harsh and tends to damage the skin and lower fillet yield. Redfish is descaled before filleting so that the scales do not contaminate the fish flesh or the knives of the filleting machines during processing. The scaling is usually done in large rotating cylinder tunnels with a ribbed base: the scales are scraped from the skin and washed away by water. Often the skin is torn in the scaling treatment. The descaling of haddock can be difficult to accomplish without damaging the fish (especially during the summer months when the fish flesh is soft), and this process is essential to produce flesh fillets, with the skin on. Haddock is much more liable to damage than redfish and therefore it cannot be descaled in rotary cylinder tunnels. Usually haddock is descaled individually in special machines. If the fish is gutted before descaling stage, the fish flesh may be damaged, which can lead to a low yield (13, 88).

In Iceland, this mechanical process has been analyzed for replacement by a biotechnological application, using a cold-adapted fish enzyme solution for a more gentle descaling (13). The scales from redfish or haddock may be removed by the help of fish enzymes. The scales can be gently removed without affecting the skin or flesh after incubating the fish in an enzyme solution at 0°C followed by spraying with water. However, the research is still at a preliminary stage and the method is being tested as a laboratory method and has not been compared with the mechanical descaling. It is too early to predict whether enzymes can become an alternative to mechanical descaling during processing (15).

3. Membrane Removal

Salted-cod swim bladders are a product manufactured in small quantities (annual production of 30–50 tons) in Iceland for markets in Southern Europe; Italy being the major user. The fresh cod swim bladder is enclosed in a thin black membrane, but the market demands an almost white salted product. Thus, the black membrane has to be removed before the swim bladders are exported. The undesirable membrane is tightly bound to the fresh bladder and is impossible to remove efficiently by manual or mechanical means. This problem has been solved by subjecting the salted raw material to a simple enzymatic process in which the black membrane is hydrolyzed for about 20 min. This process is currently used by all salted-cod swim-bladder processors in Iceland. No details are available on this process or the origin of the hydrolytic enzyme (13). However, the source of enzyme may be from marine organisms (pepsin from cod or collagenase from crab hepatopancreas) because of the specific characteristics demanded by the process (3).

Membrane removal is a problem during the production of canned cod liver. This product is made mainly in Iceland with an annual production of about 200 tons and the principal markets are in Eastern Europe. The cod liver is sometimes infested with the seal-worm (*Phocanema decipiens*), which has to be re-

moved before canning. It is necessary to remove a thin collagenous membrane that surrounds the liver, because the seal-worm is mainly contained in this membrane. Suitable machinery is not available for this removal. Thus, the membrane is removed by hand, but only with moderate success and considerable labor cost. This produces a bottleneck in the factory during the canning of cod liver. Recently, an enzymatic method has been developed to dissolve the cod liver collagenous membrane using fish proteases. The method has been tested in canning plants and compared to the manual methods. The throughput of liver during the membrane removal stage can be increased by 20–30% when using the enzymatic method. No additional details are available about the process (13).

IV. APPLICATIONS IN OTHER FOOD SECTORS

The main use of fish and shellfish enzymes in other sectors of the food industry is in dairy technology. However, other applications have been suggested such as meat tenderizing for specific fish collagenases, and the enzymatic clarification of fruit juice. Trypsin from stomachless fish is useful because of its special ability to digest native proteins substrates compared to its counterparts derived from species with a functional stomach (5, 15, 165, 166).

A. Enzymatic Milk Coagulation

The major use of enzymes in the dairy industry is in the coagulation of milk to make cheese. The Food and Nutrition Board of the United States National Research Council uses the term "rennet" to describe all milk-clotting enzyme preparations (except porcine pepsin) used for cheese-making. The same board defines rennet as aqueous extracts made from the fourth stomach of calves, kids, or lambs, and bovine rennet as aqueous extracts made from the fourth stomach of bovine animals, sheep, or goats. Microbial rennet followed by the name of the organism is the approved nomenclature for milk-clotting preparations derived from microorganisms. Milk-clotting enzymes generally recognized as safe (GRAS) are rennet and bovine rennet. However, the United States Standard of Identity for Cheddar cheese allows the use of rennet and other clotting enzymes of animal, plant, or microbial origin (167).

The milk coagulation process has two phases: enzymatic and nonenzymatic. Milk clotting begins with enzymatic cleavage at low pH of the chymosin-sensitive bond of *k*-caseins (Phe-105—Met-106) to form para-*k*-casein and a macropeptide. The hydrolysis of *k*-casein destroys its ability to stabilize casein micelles, rendering them susceptible to coagulation in the presence of calcium. The process is done at a temperature below 10°C. Enzymatic cleavage is followed by a nonenzymatic aggregation of the altered casein micelles into a firm

gel structure (the curd) and the whey is drained off. This phase is done at 30–39°C. Both phases of milk clotting overlap (168–170).

There has been a continued interest in the search for rennet substitutes ever since a rennet shortage was anticipated in the 1960s because of a decline in the number of calves slaughtered and an increase in demand for cheese (171). Many proteolytic enzymes clot milk under appropriate conditions, and the importance of these proteases results not only from their ability to coagulate of milk but also from the relation between milk-clotting ability and the general proteolysis the enzyme may produce. Not all proteolytic enzymes are suitable for making acceptable cheese because their excessive general proteolysis leads to lower curd yield caused by excessive loss of fat and protein to the whey, and development of undesirable changes in texture (softening) and flavor (bitter off taste) during cheese aging (171–173). A few enzymes are found to be adequate when the above deficiencies are considered. Chymosin (EC 3.4.23.4) or rennin is an acid protease, from abomasa of suckling ruminants, with optimum pH, stability at pH near 7.0 (5.3–6.3), and narrow substrate specificity. This is the enzyme of choice for milk-clotting and the standard against which all others are evaluated. Chymosin converts the colloidal milk casein into a curd to give high yields and reduce proteolysis, which contributes to cheese aging (174–176).

Other proteases from microbial, plant, or animal origin have been evaluated as rennet substitutes. *Mucor miehei* rennet is the most common fungal protease preparation accepted by the cheese industry. However, fungal rennet has not been totally satisfactory since it may have a relatively broad specificity, be heat stable, cause bitterness in the cheese, and remain active in the whey (167, 177, 178). Plant proteases have been employed as rennet substitutes and appear to give rise to a softer curd than calf rennet. Milk-clotting factor from the flowers of Cardon (*Cyanara cardunculus*) is traditionally used in Portugal for making soft cheese (Serra cheese) from sheep milk, but its high proteolytic activity is not suitable in the manufacture of Edam and Roquefort cheese (179). Porcine pepsin is used as a rennet substitute but its ability to clot milk diminishes quickly above pH 6.5. This enzyme is mixed with bovine rennet and chymosin, obtaining a less expensive coagulant and avoiding low curd yield (175). Chicken pepsin has also been employed as a rennet substitute, but Cheddar cheese prepared with this enzyme can have intense off flavors (180).

Cold-adapted gastric proteases from fish and chymosinlike enzymes from marine mammals have several characteristics that make them suitable for both milk-clotting phases and that avoid the major problems with rennet substitutes pointed out above (8, 5, 15). Atlantic cod pepsin (181–182) and tuna (Atlantic tuna, *Thunnus obesus*) gastric enzyme (179) have been purified and proposed as milk-coagulating enzymes. A semipurified aminopeptidase preparation from squid hepatopancreas is currently under trial in Cheddar cheese ripening because it has potential to reduce bitterness and enhance the flavor of cheese effectively (183).

Cold-adapted fish pepsin (from Atlantic cod, Greenland cod, or Polar cod) has a lower temperature coefficient (i.e., lower Arrhenius activation energy) for milk clotting (1.4–1.7) compared to calf rennet and microbial rennet (2.0–2.8). Clotting can be done with a lower enzyme concentration, thereby conserving rennet and minimizing the presence of residual curd proteolysis (8). This pepsin also has a lower temperature optimum for hydrolysis. Moreover, gastric proteases from marine organisms are unstable at temperatures above 30°C, which make it possible to clot milk and subsequently heat-denature rennet during curd formation to inactivate proteases, and avoid softening problems and off-flavors (8, 181). This pepsin also has a high molecular activity at low reaction temperatures, which produces a cheese with much lower levels of free amino acids and bitter peptides during ripening (8).

These properties make cold-adapted pepsin an excellent rennet substitute. Atlantic cod pepsin has been used as a rennet substitute in the preparation of Cheddar cheese. The product, when aged less than 6 months, was judged acceptable by sensory panels. However, when the conventional cheddar cheese process was employed, this enzyme results in high loss of protein and fat to the whey during cheese making, and the product develops bitterness and a pasty consistency after prolonged aging (181). These problems are prevented by raising the temperature to 39°C in the initiation of the nonenzymatic phase during milk clotting (182).

Chymosinlike protease from the gastric mucosa of harp seal (*Pagophilus groenlandicus*) has catalytic properties similar to calf chymosin, and has been used successfully in Cheddar cheese preparation. Seal gastric protease is more stable than pepsin at neutral to alkaline pH, and needs lower pH (6.6) than calf rennet (pH 6.8–7) to clot milk. Cheddar cheese prepared with seal gastric proteases gave significantly higher sensory scores, and less free and peptide-bound amino acids than cheese made with calf rennet (178). Gastric mucosa from harp seals have four zymogens of acidic proteases (A, B, C, and D). Zymogen A, named pepsin A, has been isolated and is similar to calf chymosin in several physical and catalytic properties (184). Seal pepsin A appears to make a major contribution to the excellent cheese-making characteristic of the crude seal pepsins. However, the best results have been obtained with crude seal gastric proteases because the other components of the crude extract are probably responsible for the accelerated aging of Cheddar cheese made with this rennet substitute (185).

The extraction of milk-clotting enzymes from fish stomach mucosa or shellfish hepatopancreas for cheese manufacture would provide an inexpensive alternative to rennet substitutes, and could become a new food-related industry. Atlantic cod pepsin is now produced commercially in Norway (87, 88). In addition, the enzyme extraction would address a very important pollution and disposal problem, as a means of minimizing the waste associated with processed

fish and shellfish, obtaining an additional value (179, 181, 183). The need for rennet substitutes decreased considerably with the commercial introduction of recombinant bovine chymosin.

B. Prevention of Oxidized Flavor in Milk

Spontaneous development of oxidized flavors is common in dairy products. Several native milk enzymes are implicated in the process. Peroxidase (EC 1.11.1.7), which is stable at pasteurization temperatures, catalyzes oxidative reactions in milk and dairy products. Xanthine oxidase (EC 1.2.3.2), which catalyzes oxidation of xanthine or hypoxanthine to uric acid and hydrogen peroxide, also causes oxidative flavors (167).

Inhibition of milk oxidation by enzymatic treatment has been studied. Bovine trypsin can prevent oxidized flavor in milk (186). However, milk treated with bovine trypsin before packing retained certain residual tryptic activity because the enzyme was not completely inactivated by the pasteurization process. The problem is the subsequent hydrolysis of milk protein, which decreases milk quality. Trypsin from cold-adapted fish (e.g., Greenland cod) has a lower free energy of activation at reaction temperatures below 30°C and more thermal instability than bovine trypsin (7). The prevention of oxidized flavors in milk by Greenland cod trypsin has been demonstrated. When milk is treated with pure enzyme (0.005% w/v) and kept at 4°C for 4 h, no retained trypsin was present after pasteurization (at 70°C for 45 min). Concentrations greater than 0.0013% of cold-adapted fish trypsin prevent the oxidation of lipids in raw milk (8, 91).

C. Preparation of Infant Milk

Cow's milk can be "humanized" by addition of lysozyme, making it suitable as an infant milk. Lysozyme acts as a preservative by reducing bacterial counts in the milk without affecting *Lactobacillus bifius* (187).

Lysozymes are widely distributed in nature and have been found in fish, shellfish, and other animals or plants, playing a role as a defense mechanism against infectious diseases (188).

Lysozyme from Arctic scallop (*Chlamys islandica*) has been recovered and purified from scallop waste generated in shellfish factories in Norway (88, 188). This lysozyme has a particularly low activation energy at low temperature, and is active at 0°C. The specific activity of the purified scallop viscera lysozyme is nearly 300% higher than hen egg white lysozyme (widely used as a antimicrobial enzyme in the food industry) (188). Both properties, catalytic and specificity, suggest that lysozyme could be applied as a bacteriostatic and prophylactic agent in food preservation and specialty products (e.g., infant milk) without the problems caused by use of antibiotics.

V. APPLICATIONS OF FISH AND SHELLFISH ENZYMES IN FEED PRODUCTS

A. Fish Silage

Fish material not for human consumption is used for the production of fish meal, which has a world market of considerable size. However, new ways of using small pelagic fish, fish waste, and fish viscera in industry or animal feed are constantly being sought (107). Distance from a fish meal plant is a factor because of transportation cost. Pelagic fish may be caught periodically in quantities exceeding the local fish-meal processing or freezing capacities. By-catch is often thrown overboard because of its low selling price. The production of fish silage offers a convenient way of using these resources.

The product of the process of preserving and storing wet fodder in a silo is called silage. The traditional use of the word has been in conjunction with green forage, preserved either by adding acid or by the anaerobic production of lactic acid by bacteria. Fish silage has been adopted for analogous products of whole fish or fish parts (189, 190). Fish silage may be described as a liquid product made from whole fish (by-catch fish) or fish waste plus acid or, less frequently, alkali. Liquefaction is caused by the action of enzymes naturally present in the fish and is accelerated by the acid, creating the right conditions for the enzymes that are active at low pH to hydrolyze quickly most of the protein. This process yields an aqueous solution and limits the growth of spoilage bacteria (191).

Fish silage was first produced in Scandinavia in the 1930s by Edin, who treated different types of fish and fish waste with a mixture of sulfuric and hydrochloric acids to preserve and liquefy them. Production of acid fish silage on an industrial scale started in Denmark in 1948, and in 1951 its annual production was about 15,000 tons (190).

There are two methods for the production of fish silage (190). The first is acid-preserved silage. This is produced by the addition of inorganic or organic acid, which lowers the pH below 3–4, which is enough to avoid microbial spoilage. Silages made with inorganic acids require a lower pH. This pH is optimum for enzymes naturally present in the raw material used. Endogenous enzymes, mainly pepsins and cathepsins, hydrolyze the tissue structures producing an amber liquid, which is an aqueous phase rich in small peptides and free amino acids. Since no salt is added during silage, the autolysis is much faster than in fish sauce, and a high recovery of aqueous phase and an oil-rich fraction are normally obtained after a few days of storage (depending on the kind or raw material). The aqueous phase has a bitter taste and is not suitable for human consumption. This method is the most widely used.

The second method of production is fermented silage. A bacterial fermentation is initiated by mixing minced or chopped raw material with a fermentable sugar, which favors growth of lactic acid bacteria. These bacteria are usually

added as a starter culture. The lactic acid bacteria produce acids and antibiotics, which together destroy competing spoilage bacteria, and the low pH achieves the tissue hydrolysis.

The silage method is an alternative to fish meal production in animal feeding. The main advantages of fish silage production are the simple technology and low investment costs compared with fish meal production. Fish silage can be made in fishing vessels and in small isolated places where fish meal plants cannot be operated economically (191). Moreover, the energy requirements of silage production are very low compared with fish meal. Oil separation in the silage process is achieved by enzymatic hydrolysis instead of by heating and pressing as in fish meal production. The final product in fish silage is more stable and resistant to spoilage, putrefaction, development of pathogens, and fly infestation than fish meal in tropical areas, because of acid preservation. The main disadvantages are the high water content (70–80%), resulting in high transportation costs, variable storage stability, and variable chemical composition. Fish meal is less bulky and thus cheaper to transport and store. However, different considerations may determine whether fuel should be used to evaporate water or whether the product should be transported in the form of liquid silage (21, 190).

With these considerations in mind, fish meal factories are usually settled at fishing ports, where it is convenient to use the readily available waste material for fish meal. In small and isolated ports and fishing communities, supplies of fish waste may be small and irregular. Therefore it is generally not economical to produce fish meal in these places, whereas production of fish silage is a feasible option (191).

Acid-preserved silage is used commercially in Scandinavia, Denmark, and Poland. During the 1970s, the annual production in Denmark reached a level of about 60,000 tons (192). Commercial silage production has been limited because of the increasing demand for standardized feeds in modern husbandry. However, renewed interest in this technology is arising, because it is being recognized as the most useful method for solving problems with waste handling in the aquaculture industry. Moreover, new methods for silage fractionation and processing are being developed to achieve a standardized product. Recently, pilot-scale and small-industrial-scale production has been introduced in many countries without approaching the same size of the fish meal industry. The annual production of fish silage is about 120,000 tons, produced mainly by the use of formic acid, acetic acid, and mineral acids (190, 191). As yet there is no commercial production of fish silage by the fermentation method, but the method is in partial operation on a small scale to use local fish waste in developing countries (190, 193, 194). Its application in fish feeding has been demonstrated (195).

Industrial-scale processing equipment for acid-preserved silage was developed during the 1980s in Norway. The raw materials for silage production are pelagic fish, trash fish, fish wastes, viscera, and byproducts. The production

process is simple and has been developed in depth (190). Recently, the process has been reviewed to respond to several questions raised by the fish silage industry (191). The raw materials are minced into small particles (3–4 mm in diameter), to distribute the enzymes throughout the mass of fish and also to ensure thorough blending of the acid to avoid pockets of untreated fish where bacterial growth may continue. The minced fish is then mixed with acid preservatives.

The choice of acids for preservation is between mineral acids (hydrochloric and sulfuric), organic acids (acetic, propionic, and formic), or a mixture of both. Mineral acid can lower the pH to 2, but this silage is an unfinished product and must be neutralized before feeding. However, the high salt level resulting from the neutralization is nutritionally undesirable and produces an unstable product. The organic acids are more expensive than the mineral acids but their use gives stabilization at higher pH (around 4.0). Silage produced with organic acids can be used in feed without neutralization (189). Formic acid is the most used acidulant for the production of fish silage.

The mixture is stirred constantly in the first steps of digestion and the temperature is chosen for the right hydrolysis rate. During the mixing stage, silage gradually liquefies because of the hydrolysis activity of fish endogenous proteases. These enzymes have an optimal pH range of 2–4 and their activity decreases sharply above pH 4. At pH 3–4 the process is catalyzed by the exo- and endopeptidases of the muscles and digestive organs and results in a large accumulation of amino acids. At pH 2, the activity is restricted to pepsin-type proteinases, and the liberation of short peptides and amino acids is reduced (196). Hydrolysis activity is dependent on temperature, with maximum activity at 45–50°C. Approximately 80% of the protein in acid-preserved silage becomes liquified after 1 week at temperatures between 23 and 30°C (190).

After the liquefaction step, the silage is stored in tanks to precipitate insoluble tissue fragments, bone, sand, and other heavy particles to the bottom. Silage is stored for long periods, because of the acid content, but antioxidants need to be added to prevent oxidation of the fat (197). During storage it is also necessary to consider the metal corrosion in the tanks where silage is placed and the other metallic parts: a normal commercial steel tank will corrode at about 0.7 mm/year if the silage is kept at 36°C using formic acid as preservative. All silage-processing equipment should be designed in stainless steel (corrosion rate of 0.004 mm/year) (191).

During the storage, silage can be separated into three phases: a lipid–protein emulsion on top, an aqueous phase containing soluble nitrogenous compounds in the middle, and a small insoluble fraction at the bottom. Hydrolysis is terminated by pasteurization at 85°C for 15 min to inactivate proteases and lipases. After heating, the silage is deoiled by decantation and centrifugation. The liquefied protein phase is acidified to pH 4 to prevent spoilage. To reduce the transport and storage costs of fish silage, it is possible to produce concentrated

silage by vacuum evaporation to reduce the bulk by about 50%. Concentrated silage has a syrupy consistency and may be used as an additive in pellets for animal feeding (191).

There is extensive literature describing conditions in the mixing step to obtain good-quality silage in a short time. In Poland, minced fish offal is treated with 0.2% sulfuric acid, 0.2% hydrochloric acid, and 2% formic acid to decrease the pH of the pulp to 3.5–3.8. After 1 day of maturation at 40°C, the product takes on a liquid consistency and has a pleasant fishy smell. The product can be stored for months at room temperature and has a high nutritional value for poultry (149). In Norway, cod-viscera silage is prepared by adding 0.75% propionic acid and 0.75% formic acid to minced viscera. The product has a pH of 4.3 and after 17 days at 27°C, about 85% of the protein is solubilized and no further solubilization occurs. Silage retains its fresh acidic smell for at least 1 year at this temperature (198). In Iceland, viscera silage without liver was produced with 3% formic acid and pH 3.5–3.8, at 35±2°C for 6–7 days (191).

For raw materials having low acid–protease activity, it is necessary to add exogenous proteolytic enzymes or enzyme-rich raw material (14–15). This practice is common in France and the United States. The hydrolysis of minced fish offal in the presence of added enzymes take places in few hours at optimum process variables. The result is the solubilization of about 80% of the total nitrogen. Most of the soluble fraction is peptides and amino acids. A variety of proteinases of animal, plant, or microbial origin, with maximum activity at pH ranging from 2 to 8.5, and with broad range of temperatures, are commercially available. The accelerated method may be rather complicated and requires expensive equipment and accurate control (149, 191). Addition of commercial enzymes in fermented silage production to accelerate the liquefaction process has been used. Addition of bromelain (0.7–0.9% w/w) and *Lactobacillus plantarum* to minced whole fish with 15% molasses increased the proteolysis rate and decreased the liquefaction time from 15 days to 12 h (199).

Fish silage usually is better digested than fish meal and is used to feed immature (poorly developed digestive system) domestic animals, poultry (203), fish (204), and as milk replacers for young animals (weaning calves and piglets)(85, 205). When mature ruminants or fish are fed on highly concentrated feeds based on fish silage, the animal production and growth are reduced. This is probably caused by adverse effects of highly hydrolyzed protein in the digestive metabolism of these animals (206, 207). The substitution of 5–10% of the feed protein by silage protein is the highest recommended dose. This is advisable as there are indications that health, fertility, and general appearance are improved when some fish silage protein is included, for the same reasons as for fish protein hydrolysates (21, 190). Recently, fish silage has been investigated as a raw material for plastein synthesis. Fish silage with a 65–70% degree of hydrolysis is the optimum source for plastein reaction, with pepsin at pH 5.0 as the most productive enzyme (208).

B. Carotenoid Pigments

One of the outstanding features of the salmonid fishes (Atlantic salmon, *Salmo salar*) is the salmon-pink color of their flesh. This color is, in the consumer's mind, closely connected with the quality of the fish, and a correct coloring of the flesh is of great importance to the salmonid aquaculture industry. Farmed salmon with a different color that is natural for the species will have a low classification and a low price in the market. The color of the flesh of salmonids is caused by astaxanthin, which belongs to a large group of compounds named carotenoids. Salmonids are not able to synthesize astaxanthin and depend on an adequate supply through their feed to obtain the color. Wild salmonids obtain astaxanthin from small crustaceans such as krill and shrimps. In salmonid farmed production, carotenoid pigments are used in the feed for improving the attractive pinkish–red color of the fish meat. For rainbow trout (*Oncorhynchus mykiss*), this is done to produce a flesh color similar to that of salmon (209, 210).

An important source of astaxanthin in industrial production is the yeast *Phaffia rhodozyma.* A good review about the yeast culture, pigment extraction, and its use in salmonid farming has been published (210, 211). Astaxanthin is also the major carotenoid pigment in shrimp and lobster (14). Proteolytic enzyme treatment of shrimp (212, 213), snow-crab shell (214), and shellfish (215) wastes allows the recovery of the carotenoid pigment along with the protein, since about one-third of the dry matter in crustacean shell waste is protein. By this method, the carotenoid pigment is recovered in the form of a protein–carotenoid complex, which is more resistant to oxidation and gives better results than free astaxanthin in the coloring of farmed rainbow trout (5).

The proteolytic enzymes used to aid the extraction of carotenoprotein have been trypsin type proteases. Pure bovine trypsin has been used with shrimp waste. The waste was soaked in the extraction buffer 0.5 M trisodium ethylenediaminetetraacetate (Na_3 EDTA) in a proportion 1:3 w/v, mixed at 4°C, and the homogenate was added with 0.1% (w/w) bovine trypsin. The digestion was done at pH 7.7 for 24 h at 4°C. After filtration of the homogenate, the filtrate was precipitated with ammonium sulfate, recovering carotenoprotein after centrifugation. The extraction process recovered a carotenoprotein fraction containing about 80% of the protein and carotenoid pigments present in shrimp offal. The long-term stability of the astaxanthin associated with the carotenoprotein was improved by addition of protease inhibitor and antioxidant to the product. Composition and properties of this product allow its use as feed supplement for pen-reared salmonids, coloring the flesh. High temperatures speed up the yield of carotenoprotein and do not require EDTA, but the odor and taste of the product are negatively affected (212).

Because better results have been obtained at low temperature, experiments in carotenoprotein extraction from shrimp process waste have been made

that replace bovine trypsin with Atlantic cod trypsin (213). Cod trypsin is a more efficient catalyst than bovine trypsin at low reaction temperatures (7). Under identical conditions and in the same extraction buffer as above, using the same enzyme concentration, 64 % of astaxanthin and 81% of shrimp waste protein was recovered as carotenoprotein with purified Atlantic cod trypsin. However, using pure bovine pancreatic trypsin, the carotenoprotein recovered was 49% of the astaxanthin and 65% of the waste protein. Pure cod trypsin is necessary for effective extraction, because when a semipurified cod trypsin fraction has been used, a poor yield of intact carotenoprotein is recovered. This is presumably caused by other proteolytic or lipolytic enzymes, which are present in semipurified extract and act to degrade the carotenoprotein. However, at the moment the use of pure cod trypsin as an extraction aid is not feasible for commercial preparations of carotenoprotein, because purified enzyme is very expensive (213). The solution will be to find other cheaper ways of obtaining cod trypsin without residual activity, or to use cold-adapted trypsin from other cheap psychrophilic organism sources. The autolysis of crustacean waste in acid ensilaging prior to pigment extraction may be used to increase the recovery yield of astaxanthin (216).

VI. FUTURE OF FISH AND SHELLFISH ENZYMES APPLICATIONS

Exploitation of fish and shellfish enzymes for biotechnological applications in the food and feed industry is complicated by variable availability of raw material and expensive production because of the comparatively low enzyme concentration. It seems that profitable processes can only be established when the fish or shellfish enzymes have unique properties (chemical conditions, specificity, etc.) that cannot be imitated by less costly enzymes of plant or microbial origin. In the future, some of these enzymes may be produced more profitably by recombinant DNA or gene technology. With this technology, it is possible to cut out a small segment or gene from any chromosome (DNA molecules) and recombine this gene with genes from another chromosome. Genes for enzymes produced in minute quantities or from obscure sources may be transferred to high-yielding microorganisms (2, 16, 20, 88).

Recombinant DNA technology has been employed to clone the calf gene responsible for rennet activity into *Escherichia coli* and *Saccharomyces cerevisiae* (217–218). Cheddar-cheese-making trials comparing recombinant chymosin with calf rennet have found no significant differences between the two. Recombinant enzymes are now approved for commercial use, and recombinant chymosin meets some religious dietary requirements not met by calf rennet (167, 187). The amino-acid sequence, cloning, and cDNA encoding of fish and shellfish enzymes with importance as food-processing aids have been described:

transglutaminase from salmon (*Onchorhynchus keta*) liver (219) and trypsin from crayfish (*Pacifastacus leniusculus*) hepatopancreas (220).

Another approach to enzyme engineering is site-directed mutagenesis. This methodology can be used to make small modifications in the nucleotide sequence of a gene. Recently, modified genes have been used to produce enzymes with slightly different amino acid composition/sequence in determined positions, but with markedly different catalytic and stability properties. High methionine content appears as a general property of cold-adapted serine proteinases, and one position (Met-134) is present in the cod chymotrypsins as well as in cod salmon, and dogfish trypsin. The inherent mobility in methionine side-chains may contribute to the maintenance of flexibility at low temperatures. This methodology could transforms proteases (obtained by cheaper biotechnological processes) into other proteases with kinetic and thermodynamic properties similar to cold-adapted proteases (221).

Extreme environmental conditions are found in the oceans. Pressures from 1 atm at sea level up to several hundred at the sea bottom (1 atm every 10 m depth); temperature below 0°C in the Arctic and Antarctic oceans and exceeding 100°C in the hydrothermal vents in the ocean bottom; salinities from fresh water in the salt marshes up to 6N NaCl in the precipitation pits (salt mines); from almost zero concentration of organic substances to eutrophic areas are frequent. Because of the diverse habitats in the seas, there is an immense biodiversity of marine organisms and an immense genetic diversity. Each organism has a metabolism adapted to such conditions. Because metabolism is driven by enzymes, a vast diversity of enzymes with particular kinetic capabilities is expected to be discovered.

Cold-adapted enzymes from fish living at temperatures about the freezing point of seawater and thermoresistant enzymes from organisms, including crustacea, living in the hydrothermal vents have been reported. Enzymes working better at 2–4 M NaCl from organisms living in salt mines are hot topics in marine biology. Each time an enzyme with special kinetic abilities is discovered, a new potential application for food technology arises.

Enzymes help in a variety of processes, and in some examples they have become an important and indispensable part of the processes used by the modern food industry to produce a large and diversified range of products for humans and animals. The foremost advantage of enzymatic processes are the enzymes' specificity of both substrate and reaction, transforming only one molecule in a complex mixture of analogous molecules, rendering almost 100% transformation without byproducts. The transformation is done under mild conditions of temperature and pH. However, although enzymes are usually considered catalysts working at mild conditions, new discoveries show that enzymes can work at extreme conditions. In some cases, enzyme technology competes with and even substitutes for traditional engineering technology (222).

The search for new and improved enzymes is an approach appearing somewhat empirical to the new technologies such as protein engineering, gene cloning, synzymes, and reaction in supercritical carbon dioxide. However, it is a proven approach that has withstood the test of time. The screening of microorganisms and plants has become standard procedure and screening of marine organisms is at its earliest stage. Food technology is an evolving activity; new processes and ingredients will appear, so there will be a rapid growth in industrial applications of enzymes. The challenge to the enzyme technologists in food processing is to find new enzymes and to understand the kinetic and molecular basis of enzyme transformation to improve the functional and nutritional properties of foodstuff (223).

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