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Effects of temperature, pH, salt concentration and pre-heating treatment on enzymatic processes in cod and herring roe

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Gao Li

Abstract

In recent years, an increasing attention has been paid to producing high value products from fish byproduct, such as fish roe. However, the nutritional and sensory properties of the roe products can be affected by many factors during the processing, such as fish species, pH, salt concentration and temperature. The objective of the present thesis is to characterize and compare proteolytic and lipolytic activities in cod roe and herring roe under different temperature, salt concentration and pH.

The proteolytic activities of unsalted cod roe were highest at 50℃, regardless of the pH value. Cod roe extracts with salt, compared with extracts without salt, have shown a much higher proteolytic activity at pH 5, but a much lower activity at pH 7. The proteolytic enzymes of herring roe, both salted and unsalted, were less active that those of cod roe. The lipolytic enzymes of cod and herring roe were mostly found to have maximum activities at 50℃ and pH 7, and were higher in herring roe than cod roe. The present study also showed that heating at 50℃ did not completely inactivate the proteolytic and lipolytic enzymes while the enzymatic activities were inactivated after heating for 10 minutes at 60 and 70℃. From the results we conclude that fish species, salt concentration, pH and temperature all have influence on the proteolytic and lipolytic activities in fish roe. To inactivate proteolytic and lipolytic activities in cod and herring roe, temperatures above 50℃ is needed.

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1 Introduction

The importance of better utilization of marine resources has been demonstrated for long time as the world population increases and the wild stocks are fully exploited. In recent years fish industries start to realize the importance of better utilization of fish. However, today most of the fish byproducts are used for low price fish feed ingredients such as fishmeal and fish oil (Sovik and Rustad, 2005). Only 10% is made into food or other value-added product. However, these 10% accounts for nearly half of the total value of all fish byproducts. Therefore, an increasing attention has been paid to producing more high value products from fish byproduct, such as caviar made from fish roe.

Caviar is product made from eggs of aquatic animals that have been singled out and separated from connective tissue. The best-known caviar is made from sturgeon fish, which has been a popular product since the Persian and Russian Empires (Bledsoe *et al.*, 2003). Now sturgeon caviar is the only product that can be labeled as “caviar” in the U.S. However, due to low less availability of sturgeon, an increased attention has been paid to the caviar made from other fish species. Many other fish species have been used for making different kinds of caviar, such as cat fish (*Ictalurus punctatus*), salmon (*Salmo salar*), cod (*Gadus morhua*), herring (*Clupea harengus*), lumpfish (*Cyclopterus lumpus*), capelin (*Mallotus villosus*), orange roughy (*Hoplostethus atlanticus*) and many other fish or crustaceans (Bledsoe *et al.*, 2003, Body, 1985, Eun

et al., 1994, Katsiadaki *et al.*, 1999, Power and Voight, 1992). For example, in Nordic countries, the most popular fish species for making different kinds of caviars are Atlantic salmon (*Salmo salar*), Atlantic cod (*Gadus morhua*), herring (*Clupea harengus*), lumpfish (*Cyclopterus lumpus*) and capelin (*Mallotus villosus*). Caviar made from other fish species than sturgeon must be labeled with the common name of the fish used, such as "salmon caviar", "cod caviar" and "herring caviar". In this paper we will mostly focus on the "cod caviar" and "herring caviar".

Atlantic cod (*Gadus morhua*) is a demersal fish which becomes pelagic during feeding and spawning. Although the distribution of cod more depends on prey rather than temperature, large fish are normally found in cold waters (0-5℃) (Cohen *et al.*, 1990). Atlantic cod can live up to 20 years, with feeding on invertebrates during juvenile period and fish during adult period. However, during their spawning period and in winter, they feed on mixed food in coastal areas. Atlantic cod is one of the most popular commercial fishes in Europe, mostly catches from Iceland and Norway. They can be made into many products, including fillets, liver oil and fish roe.

Atlantic herring (*Clupea harengus*), along with cod, are the most important fish species in the Baltic Sea. They can live up to 20 years and normally feed on zooplankton like copepods, arrow worms and krill in the pelagic zone (Casini *et al.*, 2004). During the spawning season, the fish lay their eggs on the sea bed, where they clump to gravel, seaweeds or stones. The larvae can grow up to 10cm long in the first

year, and start to spawn in the third year. Atlantic herring (*Clupea harengus*) is an important oily fish which have a long history as a food fish in Europe. They are harvested for their flesh, which can be eaten raw, fermented, pickled and smoked. In recent years, an increasing number of herring is filleted in Norway, which makes it possible to utilize their roe for making caviar.

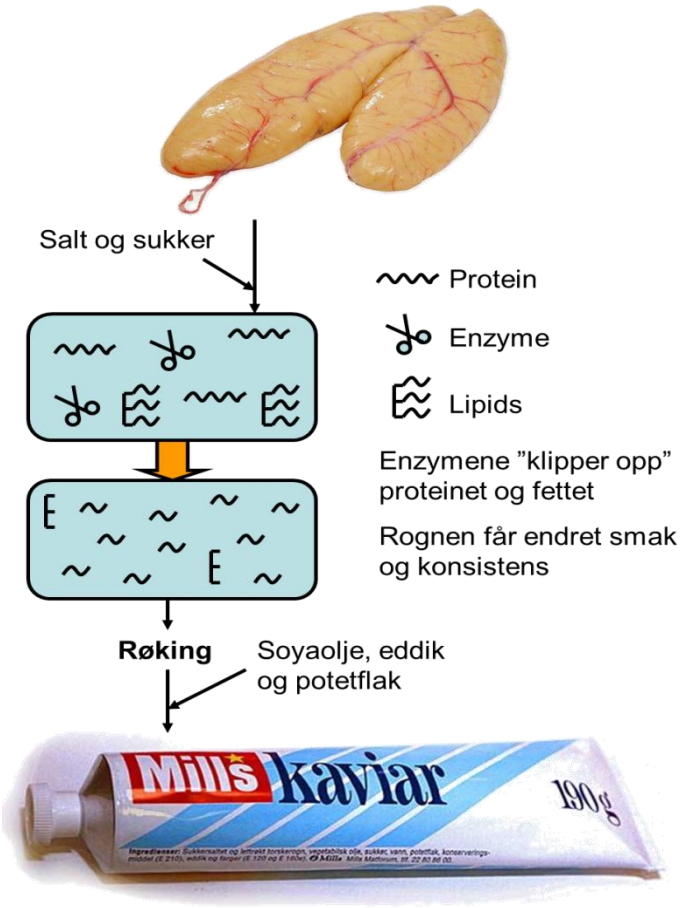

Fig. 1 Processing of cod roe into paste product.

Scandinavian countries have long tradition of making cod roe pastes. The processing of cod roe is shown in Figure 1. The roes are often harvested as a by-product during cod fisheries, salted with salt and sugar, mixed with dried potato flakes, rape seed oil

and preservatives (benzoate, sorbate) and vinegar. These cod roe pastes can be stored for up to 21 months and are often used as sandwich spread. During the salt ripening, proteins are broken into small peptides and lipids are broken into fatty acids (Fig. 1). There are also changes to the texture of the fish roe, probably because of the breakdown of chorion proteins.

It is well-known that in ripened meat products, the flavor and texture are the consequence of protein and fat degradation (Jonsdottir *et al.*, 2004, Toldra, 1998, Toldra *et al.*, 2000). Triqui and Guth (1997) have found that (Z)-1,5-octadien-3-one were potent odorants in ripened anchovy. Also Milo and Grosch (1996) have identified (Z)-1,5-Octadien-3-one, (E,Z)-2,6-nonadieanal, propionaldehyde, acetaldehyde, methional and (E,E)-2,4-decadienal as the character impact odorants of boiled salmon. More than 260 volatiles have been identified in dry-cured ham, most of which are from enzymatic oxidation of fatty acids and their interactions with proteins, peptides and free amino acids (Toldra, 1998).

Although the production of caviar has been demonstrated for many years, little detailed information is available regarding the nutritional and sensory properties of its raw materials (Katsiadaki *et al.*, 1999). The properties of the roe products can be affected by many factors, such as species, stages of maturity and processing technology. Cod and herring roe are very different as raw materials both with regard to the composition of proteins and fat, and to the activities of enzymes which

influence the sensory properties of the product. Both protein and lipids are important for the taste of a fish roe product. Also the quality of roe is often variable regarding maturity stage and processing technology. Earlier research on cod roes has shown a close relation between the maturity stages and the sensory properties of the cod (Katsiadaki *et al.*, 1999). It is known that cod roes from late in the season are better as raw material for making caviar, which may be due to the degraded state of the yolk proteins(Olsen, 1997). On the other hand, little is known on the biochemical changes during processing of fish roe such as salting and sugarsalting (Olsen, 1997).

Caviars are often made from undamaged eggs which have a proper color, a desirable mouth feed and limited fishy flavor (Bledsoe *et al.*, 2003). The processing of roe into caviar varies with different type of fish species. However, the major processes of making caviar, regardless of the type, are recovery and yield of roe, separation of eggs from skein materials (screening), salting fish eggs and curing process. In Norway, the traditional brining process of cod roe are salting and sugarsalting, by adding salt or salt and sugar to the fish roe. This also gives taste to the product and preserves it for longer storage. Fish roe can be salted as a whole skein, or separated from the egg skein (Eide *et al.*, 1999). Salt uptake can be affected by types and degree of maturity of the eggs, salt content, pH, curing time and temperature (Bledsoe *et al.*, 2003, Huang *et al.*, 2001). During the salting, many chemical and biochemical changes occur, which influence both the nutritional and sensory properties of the caviar product.

The content and composition of protein in fish roe are important for its sensory and nutritional properties, which need to be controlled during the processing. Proteolytic enzymes are present in all forms of living organisms, which associated with protein digestion and control many physiological functions, such as proteolytic cleavage of peptide bonds, activation of zymogens, blood coagulation, the lysis of fibrin clots, the release of hormones from precursor proteins and the transport of secretory proteins across membranes (Neurath, 1984). Based on a functional criterion, the proteases are separated into four classes: serine, cysteine, aspartic and metallo proteinases (Neurath, 1989). Earlier studies of fish and its byproducts have shown that the activities of these proteolytic enzymes varied with species, age, water temperature, salt concentration and pH (Khalil *et al.*, 1987, Sovik and Rustad, 2005b). Sovik and Rustad (2004) have identified maximum cathepsin B activity at 35℃ in the viscera and the liver, and 50℃ in cut off, while maximum collagenase activity at 20℃ in cut off, 35℃ in liver and 50℃ in viscera. Earlier researches have also found the optimal pH of 5 in caseinolytic enzyme, pH of 2.6 in protease and pH of 7.2 in aminopeptidasse of mullet roe (Chiou *et al.*, 1989).

Since the temperature, salt concentration and pH may change during the processing of the fish roe, the processing technology may have strong influence on the proteolytic activity of fish roe product. Earlier research of Mullet and Alaska Pollack roes has found that the proteolytic activities vary with different temperature, pH and salt

concentration (Chiou *et al.,* 1988). Sovik and Rustad (2005a) have identified the differences of proteolytic activities in cod caught at three different fishing grounds.

The lipid content and composition is one of the key factors that determine roe quality since lipid is the main energy reservoir, nutrients supplier and bioeffectors of intracellular biochemical reactions, cell–cell interactions, and various physiological processes (Sargent *et al.*, 2002, Murzina *et al.*, 2009). Lipases are digestive enzymes and involved in the metabolism of lipids and functioning of biological membranes (Brockerh, 1974). They are required as digestive enzymes which transferring lipid among different organisms, metabolizing of intracellular lipids and functioning of biological membranes (Brockerh, 1974). The control of lipase activity in fish roe is important since it can hydrolyze nutritionally valuable fat into free fatty acids and glycerol. Free fatty acids are more easily oxidized than triaclyglycerides and this may impact the sensory and nutritional properties of the roe products. Degradation products from lipids and reaction products from reactions between proteins, peptides and oxidized lipids are also important for flavor and taste of the roe products.

There has also been a desire to develop other products from cod roe and other roes, where one could use heated roe. Therefore knowledge on the heat stability of the enzymes is important. The optimum temperature for enzyme activity is the temperature that enzymes have the highest rates of reaction. When the temperature increased beyond the optimum, the rates of denaturation is increased and the enzymes

start to break down (Ashie *et al.*, 1996). Heat stability of enzymes is related to both enzyme structure and other factors in the microenvironment, such as pH, amounts of water, substrate binding, and the amount and type of salts (Adams, 1991, Ashie *et al.*, 1996). Also different enzymes in fish roes may have different pH and temperature optima. Moreover, the heat stability of enzymes in fish is also related to the living environment of the species. For example, low thermal stability is observed in cold water fish, such as cod and herring, which often has the highest catalytic efficiency of enzymes at low temperature. That is due to the decrease of activation energy is needed for the cold water fish to reach the transition state (Georlette *et al.*, 2004).

Few studies have characterized sensory and nutritional properties of fish roe product. It is not known how, for example, the properties of fish roe can be influenced by temperature, salt concentration and pH. We assume that the lipolytic and proteolytic activities in cod and herring roes can be manipulated by changing temperature, salt concentration and pH, but the optimum levels are unknown. The objective of the present thesis is to characterize and compare proteolytic and lipolytic activities in cod roe and herring roe under different temperature and pH.

2 Materials and methods

2.1 Cod and herring roe

The fresh cod roe was bought from the fish market and the frozen herring roe was supplied by SINTEF Fisheries and Aquaculture. Both roe types were used to determine proteolytic and lipolytic Activity. For determination of the heat stability of the proteolytic and lipolytic activity, frozen cod roe was provided by SINTEF Fisheries and Aquaculture. The fish roe was transferred directly to Department of Biotechnology, Norwegian University of Science and Technology (NTNU) and stored at -80℃ until used for experiments.

2.2 Citric acid-phosphate buffer

In order to be able to use the same buffer for all measurements of enzyme activity, it was decided to use citric acid-phosphate buffer. The preparation of the buffer was described by McIlvaine (1921). The buffer is made from two stock solutions – 0.1M citric acid and 0.2M di-sodium hydrogen phosphate. This buffer covers the whole pH range from 2.2 to 8.0. The amount of each solution to achieve the right pH is given in Table 1. All the pH was checked by using a pH – meter after mixing of the buffer.

Table 1 Contents of citric acid and di-sodium hydrogen phosphate needed for citric

acid-phosphate buffer

pH	0.1M citric acid (mL)	0.2M Na_2HPO_4 (mL)
3.0	80.3	19.7
5.0	49.0	51.0
7.0	19.0	81.0

2.3 Proteolytic acitivity

2.3.1 Preparation of protein extracts

Cod and herring roes were taken out from -80℃ and put overnight in 4℃ cool room. 20g of the fish roe was added directly into a centrifugation with 20mL citric acid-phosphate buffer at room temperature and the mixture was homogenized with max speed for 30sec (Ultra Turrax). After that, the mixture was centrifuged (Eppendort Centrifuge 5415R) at 13200g for 20min at 4℃. After centrifugation, the supernatant was decanted into a 100mL volumetric flask and the volume filled up to 100ml. The extract was divided into several portions and stored at -80℃. The procedure was carried out at room temperature.

2.3.2 Analysis of proteolytic activity

Proteolytic activity was determined as described by Barret (1977) with a few

modifications. Enzyme extract for proteolytic activity was taken out from -80℃ and put into water (room temperature) with bottle for about 30min. 1.2mL citric acid-phosphate buffer and 0.4mL substrate (1% haemoglobin) was added with 0.4mL extract in a test tube. The samples were incubated for 5 min in water baths before 0.4ml of suitably diluted enzyme extracts were added. Incubation time was set to 1h at 40℃, 50℃, 60℃ for pH 5, 6, 7. After that, the reaction was stopped by addition of 2.0mL 5% w/v TCA (trichloroacetic acid, Merck). Then the samples were cooled for 30 min and then filtered through glass wool, followed by the determination of the amount of acid soluble peptides in the filtrate according to Lowry *et al.* (1951). BSA (Bovine serum albumin, Sigma No. A9647) was used as standard solution. Activities are expressed as mg hemoglobin cut per g water-soluble protein per hour and are given in arbitrary units (U) based on the mean of three measurements. Activity was determined at three different pH 5, 6 and 7, and at three different water bath temperature 40℃, 50℃ and 60℃.

2.4 Lipolytic activity

2.4.1 Preparation of extract

10g of the protein extract (from 2.3.1) was weighed and added into centrifugation tube for analysis of lypolytic activity. 10mL 0.1M ice cod citric acid-phosphate buffer was added and the mixture was homogenized with max speed for 30sec (Ultra Turrax).

The Ultra Turrax was rinsed with about 10mL ice cold buffer. The mixture was centrifuged at 5900g for 30min. The supernatant was filtered through glass wool and added into a 50mL volumetric flask. The volume was made up to 50mL with buffer. The extract was divided into several portions and stored at -80℃. Blank samples were made by denaturing the enzyme extract at 80℃ for 30min. The sample was centrifuged at 800g for 10min. The supernatant was diluted in the same way as the other samples.

2.4.2 Preparation of Liposomal Dispersion

Solution of 2 mM 4-MUH (10 mg 4-metylumbelliferylheptanoate (4-MUH) in 17.3 mL chloroform: methanol (2: 1, v/v)) and 2mM soya lecithin (27.1 mg soya lecithin in 17.3 mL chloroform: methanol (2: 1, v/v)) was mixed in a round flask at 36℃ immediately before evaporation. After that, the mixture was evaporated to a volumn of 1mL solution and transferred to a large test tube where the rest of the solvents are completely removed by nitrogen gas. After drying for 1h in a desicator, 8.65 mL 0.15M NaCl was added into the mixture and the solution was mixed 20min with a whirlmixer at max speed. The solution was then treated in an ultrasound bath for 30 sec. Whirl mixing and treatment with ultrasound was repeated 3-4 time till the substance on the glass wall went into solution. The formation of liposomes can be seen in a microscope as a carpet of very small particles. The dispersion was filtered through a syringe filter with a pore size of 0.45μm and then 0.22μm with vacuum

before use.

2.4.3 Analysis of lipolytic acitivity

Lipolytic activity was determined as described by Izquierdo and Henderson (1998). The whole procedure was carried on ice. Enzyme extract for lipolytic activity was taken out from -80℃ and put into water (room temperature) with bottle for about 30min. 40uL enzyme solution (suitable diluted in citric acid-phosphate buffer at the desired pH 5, 6, 7) and 20uL ice cold liposomal dispersion was added into a test tube. The sample was covered with petrifilm and incubated in a shaking water bath for 15min at the desired temperature (22℃, 40℃, 50℃ and 60℃). After that, the reaction was stopped by addition of 3mL ice cold 1M Tris-HCl-buffer. Then the fluorescence was measured immediately in a fluorimeter (LS-50B Luminescence Spectrophotometer) with an excitation wavelength of 365nm, excitation slit of 10nm, emission wavelength of 450nm and emission slit of 5nm. 4-MU (4-Methylumbelliferone) dissolved in distilled water was used as standard solution. Activities were expressed as an intensity in fluorescence based on the mean of three measurements. Activity was determined at three different pH 5, 6 and 7, and at three different temperatures 40℃, 50℃ and 60℃.

2.5 Heat stability

Cod and herring roe were taken from -80℃ for and put into water (room temperature) with bottle for about 30min. 18 bags of 10g cod roe was incubated in water bath for desired time (10min and 20min) at desired temperature (50℃, 60℃ and 70℃). Then the cod roe was put on ice. And the extracts were made directly after heating. Then the fish roe were taken for analysis of proteolytic and lipolytic activity as described before.

2.6 Statistics

The experimental data were tested for statistical significance by using one-way analysis of variance (ANOVA) with Tukey's multiple comparison test, and differences were considered significant at the $p<0.05$ level.

All the statistical tests were performed by SPSS 19.0 for windows. All tables were made in word 2010, and figures were made by SigmaPlot 12.5.

3 Results

3.1 Proteolytic activities of cod and herring roes

3.1.1 Trial experiment

Table 1 Trial experiment of proteolytic activities of 0% salted cod roe at pH 6 (n=3). Unit: absorbance under 750nm wavelength.

	40℃	50℃	60℃
Protein[a]	1.15±0.08	1.06±0.11	0.84±0.14

[a] the absorbance of cod roe with TCA added after water incubation.

Samples of 0% salted cod roe at pH 6 were used for a trial experiment without dilution, and the results were shown in Table 1. Compared with the standard curve, the results of sample were around 2 times higher than the optimum range of standard curve, which means the fish roes extracts need to be diluted before determination of proteolytic analyzing (Table 1). Therefore, the following analysis of proteolytic activities of cod and herring roes were all 10 times diluted.

3.1.2 Proteolytic activities of cod and herring roes

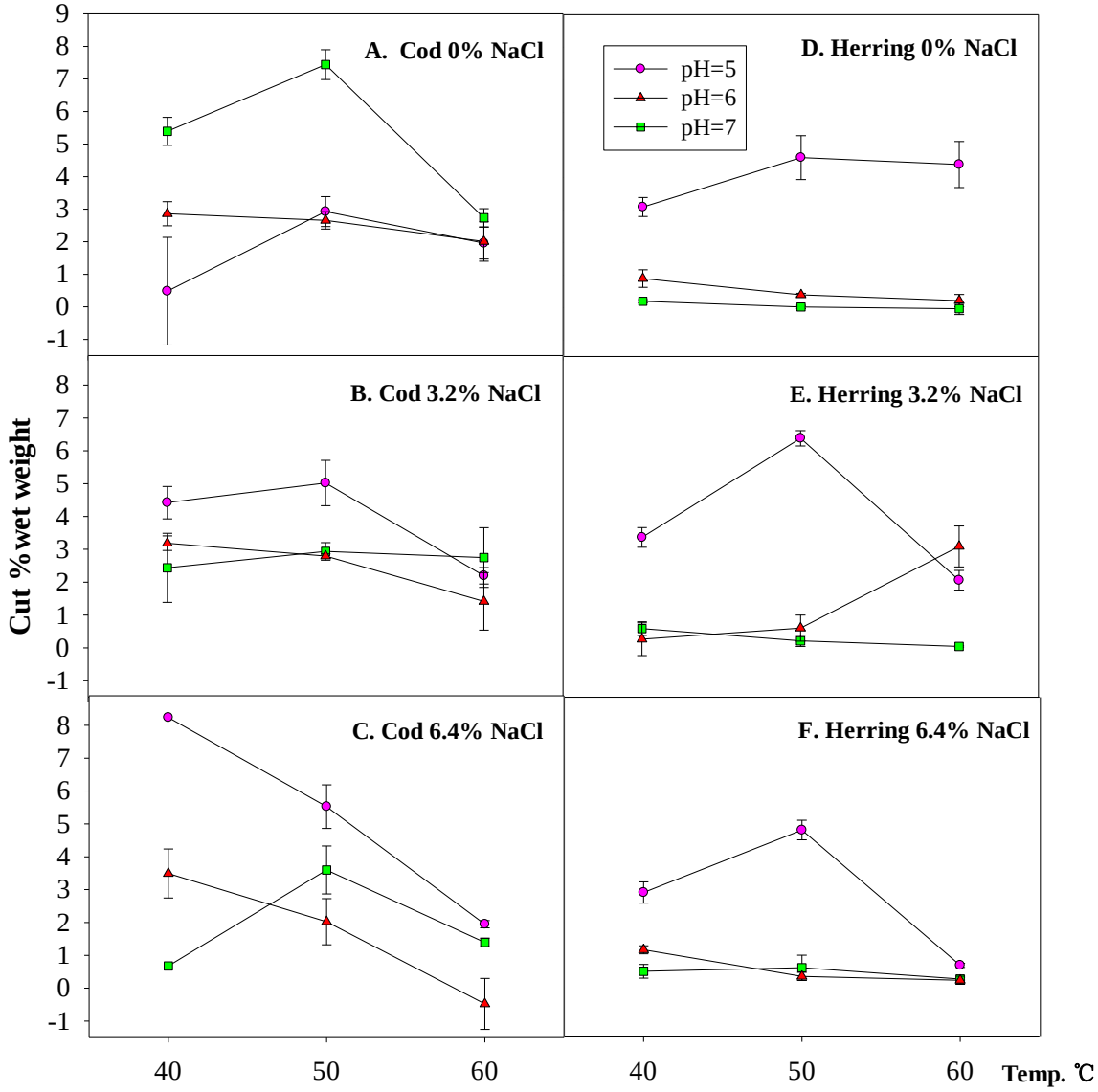

Figure 2 Proteolytic activities of cod and herring roe as a function of different concentration of NaCl (0%, 3.2% and 6.4%), different temperature (40℃, 50℃ and 60 ℃) and different pH value (5, 6 and 7). Error bars indicate standard deviations from 3 replicates analyses.

The proteolytic activities of cod and herring roe under different NaCl concentration, different temperature and different pH are shown in Fig. 2. In cod roe, the proteolytic activities of 0% salted cod roe were highest at 50℃, which significantly ($p<0.05$)

decreased when temperature increased to 60℃, regardless of the pH value (Fig. 2 A). In extracts with 3.2% salt, the proteolytic activities did not change with increasing temperature from 40℃ to 50℃, but significantly ($p<0.05$) decreased at 60℃ (Fig. 2 B). On the other hand, the activities under pH 6 and 7 kept constant between 40℃, 50℃ and 60℃ (Fig. 2 B). In extracts with 6.4% salt, the proteolytic activities at pH 5 and 6 kept decreasing from 40℃ to 60℃ (Fig. 2 C). On the other hand, the activities at pH 7 significantly ($p<0.05$) increased from 40℃ to 50℃, but significantly ($p<0.05$) decreased when the temperature increased to 60℃(Fig. 2 C). For herring roe at pH 5, regardless of the salt concentration, the proteolytic activities were highest at 50℃, which significantly ($p<0.05$) decreased when the temperature increased up to 60℃ (Fig.2 D, E and F). The enzymes activities at other pH value were mostly less than 1% cut per wet weight, except that the activity of 3.2% salted herring roe was about 3% cut per wet weight at 60℃ (Fig.2 D, E and F).

As shown in Figure 1, the proteolytic activities of cod and herring roes have both similarities and differences. For example, in most of the treatments, the activities of the two fish at pH 5 were higher than those at pH 6 and 7, regardless of salt concentration and temperature (Fig. 2 B-F). Exception was found in cod roe extracts without salt, where the enzyme activity at pH 7 was higher than at pH 5 and 6 (Fig. 2 A). Another similarity was that, for most of the salt concentrations, the proteolytic activities of cod and herring roes at pH 5 increased from 40℃ to 50℃, and decreased when the temperature increased up to 60℃ (Fig. 2 A, B, D, E and F). On the other

hand, there are a few differences between the cod roe and herring roe. In cod roe extracts without salt, the proteolytic activities in cod roe were significantly ($p<0.05$) lower than herring roe at pH 5, but significantly ($p<0.05$) higher than herring roe at pH 6 and 7 (Fig. 2 A and D). In extracts with 3.2% salt, the activities in cod roe were similar with herring roe at pH 5, but significantly higher than herring roe at pH 6 and 7 (Fig. 2 B and E). In extracts with 6.4% salt, the activities in cod roe were significantly ($p<0.05$) higher than herring roe at all pH values (Fig. 2 C and F).

3.2 Lipolytic activities of cod and herring roes

3.2.1 Trial experiment

Table 2 Trial experiment of proteolytic activities of cod roe at pH 5, 6 and 7 (n=1 for blank; n=3 for all the samples). Unit: intensity of the fluorimeter

	22℃	40℃	50℃	60℃
pH=5 (1)	20.29±0.43	40.64±2.00	44.94±3.87	34.05±3.48
pH=6 (10)	12.56±2.03	18.26±5.52	18.83±5.06	16.07±10.78
pH=7 (25)	32.22±2.15	45.21±3.30	48.89±4.62	92.60±12.52

Results are shown as the absorbance of cod roe with Tris-HCl added after water incubation. Numbers in brackets stands for the dilution ratio of the fish sample (1: 1, 1: 10 and 1:25).

Samples of cod roe were used for a trial experiment without dilution, and the results were shown in Table 2. Compared with standard curve, the results of the sample were in the range of standard curve, which means that undiluted samples can be used for analyzing of lipolytic activities (Table 2). Therefore, the following analysis of lipolytic activities of cod and herring roes were all diluted as the trail experiment.

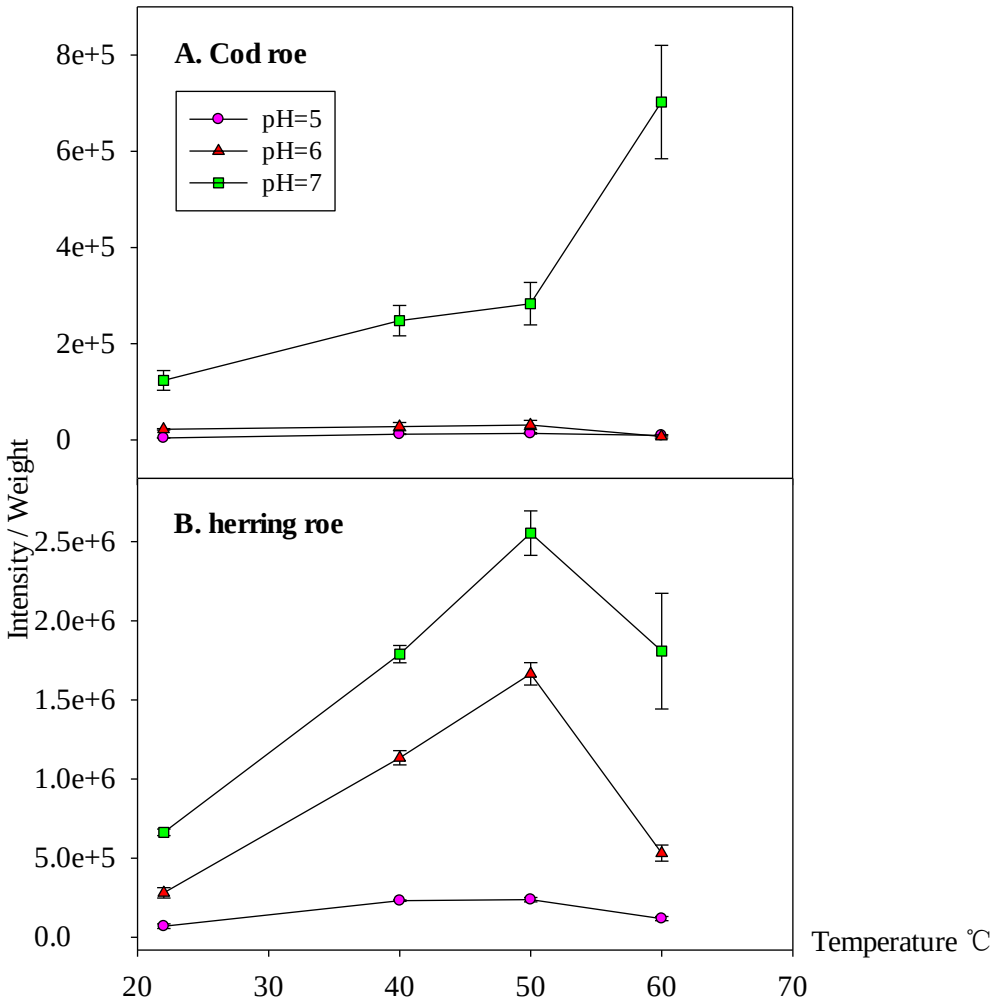

Figure 3 Lipolytic activities of cod roe and herring roe as a function of different temperature (22, 40, 50 and 60℃) and different pH value (5, 6 and 7). Error bars indicate standard deviations from 3 replicates analyses.

3.2.2 Lipolytic activities of cod and herring roes

The lipolytic activities of cod roe and herring roe under different temperature and pH are shown in Fig. 3. The lipid enzymes have much higher activities in herring roe than cod roe (Fig. 3 A, B). In cod roe, the lipolytic activities was higher at pH 7 than pH 5 and 6. Under pH value of 5, the enzyme activities significantly ($p<0.05$) increased from 22℃ to 50℃, which rapidly decreased ($p<0.05$) at 60℃ (Fig. 3 A). Similarly, under pH value of 6, the enzyme activities kept almost constant between 22℃ and 50℃, but significantly decreased ($p<0.05$) when temperature went up to 60℃ (Fig. 3 A). However, lipolytic activities cod roe kept increasing ($p<0.05$) from 22 to 60℃ when pH value was 7 (Fig. 3 A). Similar curves were observed for the lipolytic acitivities of herring roe. The enzyme activities were highest at pH 7 and lowest t pH 5. Moreover, statistics have shown that lipolytic activities under pH value of 5, 6 and 7 all kept increasing ($p<0.05$) from 22℃ to 50℃, but significantly ($p<0.05$) decreased when temperature was 60℃ (Fig. 3 B).

3.3 Heat stability

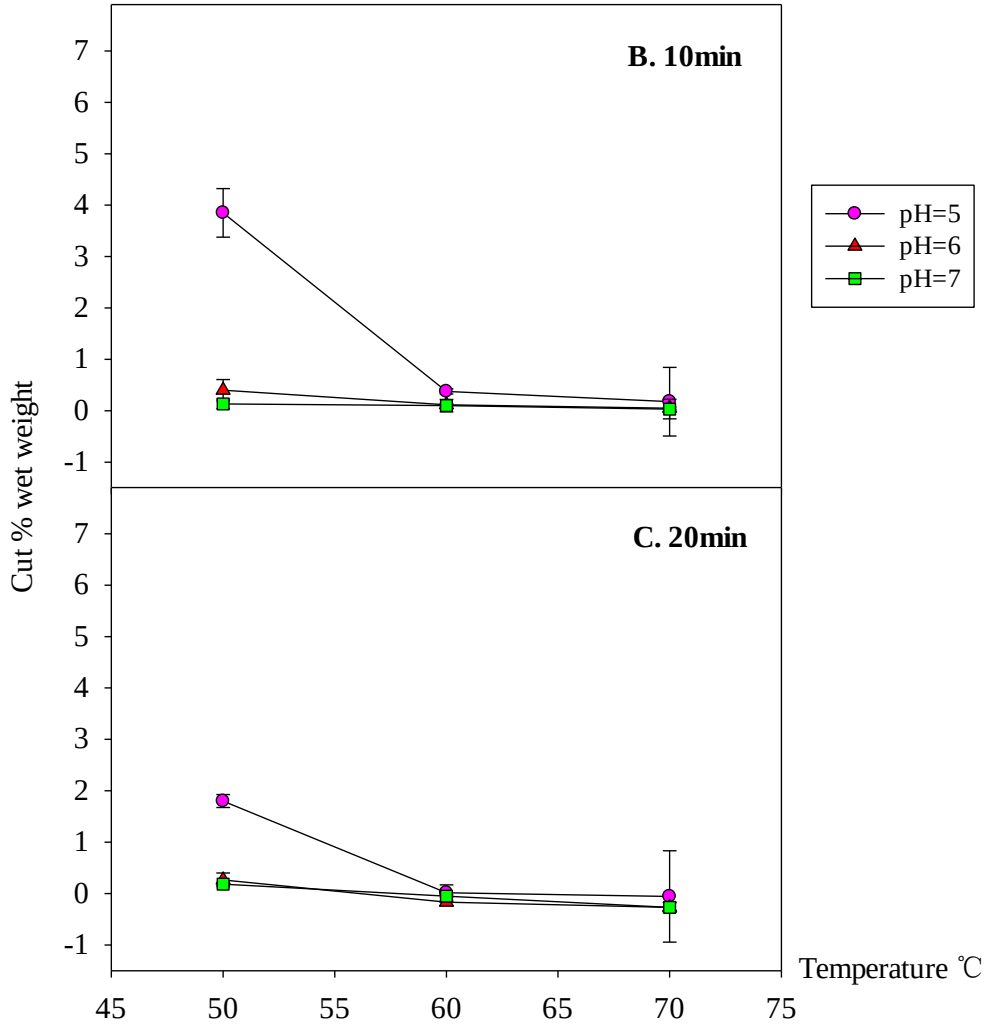

Figure 4 Proteolytic activities of cod roe as a function of different pre-heating temperature (50, 60 and 70℃), pre-heating time (no heating, 10min and 20min) and different pH value (5, 6 and 7). Error bars indicate standard deviations from 3 replicates analyses.

The proteolytic activities of cod roe pre-heated at different temperature and different pH are shown in Fig. 3. Similar curves were observed between the activities for different pre-heating time (Fig. 4 A and B). When pre-heating time was 10min, the proteolytic activities at pH 5 significantly ($p<0.05$) decreased between 50℃ and 60℃,

then they were inactivated (Fig . 4 A). Similarly, the enzyme activities at pH 6 and 7 were all inactivated at 50℃, 60℃ and 70℃. When pre-heating time was 20min, the proteolytic activities at pH 5 remaining active when pre-heating temperature was 50℃, whereas become inactive when the temperature was 60℃ and 70℃ (Fig . 4 B). However, the enzyme activities at pH 6 and 7 were almost inactive regardless of the pre-heating temperature. Compared with the pre-heated cod roe, the proteolytic enzymes in non-heated roes were always active regardless of pH value (Fig. 2 A).

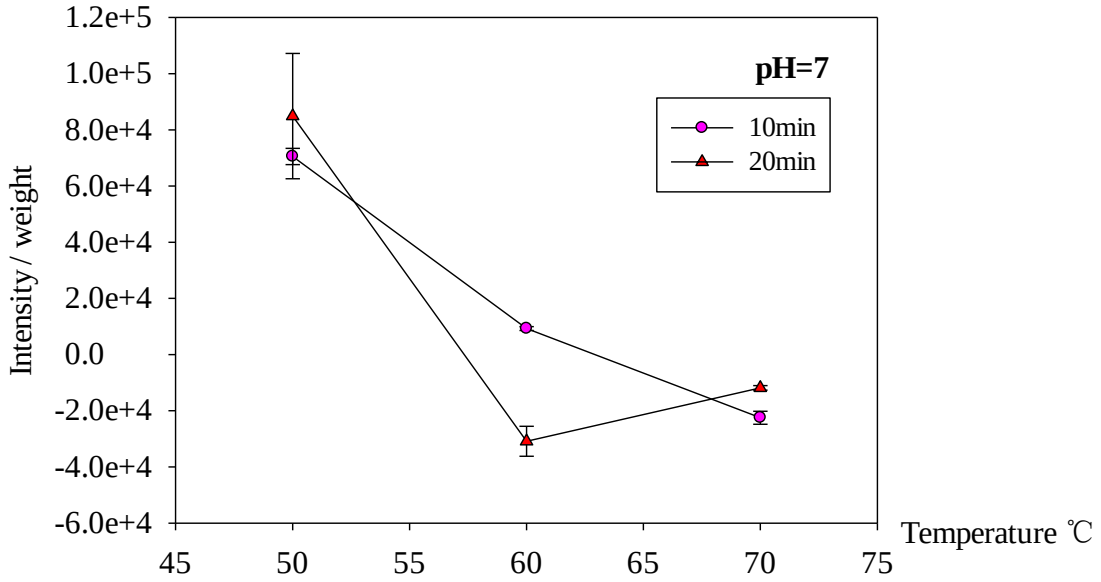

Figure 5 Lipolytic activities of cod roe as a function of different pre-heating temperature (50, 60 and 70℃), pre-heating time (10min and 20min) at pH 7. Error bars indicate standard deviations from 3 replicates analyses.

The lipolytic activities of cod roe under different pre-heating time and temperature are shown in Fig. 4. When pre-heating time was 10min, the lipolytic activities at pH 7 significantly ($p<0.05$) decreased from 7.1e+4 to -2.3e+4 (Fig. 5). However, when

pre-heating time was 20min, the enzyme activities significantly ($p<0.05$) decreased between 50℃ and 60℃, whereas increased significantly ($p<0.05$) between 60℃ and 70℃ (Fig. 5). As shown in Figure 3 A, the lipolytic activities of cod roe at pH 5 and 6 were very low. Therefore, cod roe at pH 7 was chosen for analyzing the pre-heating treatment on activities of lipolytic enzymes.

4 Discussions

4.1 Influences of temperature, pH and salt concentration on proteolytic activities of cod and herring roes

Few studies have been carried out on the proteolytic activities in fish roe, even less have been taken on the activities in salted fish roe (Sovik and Rustad, 2005b). In present study , the proteolytic activities of unsalted cod roe were highest at 50℃, regardless of the pH value (Fig. 2 A, B and C; Appendix Table 3). This is closely in accordance with the literature where the optimum temperature for the proteolytic activities in cod viscera has been reported to be 50℃ (Sovik and Rustad, 2005b). Also Munilla-Moran and Saborido-Rey (1996) has reported that the optimum temperature enzymes in intestinal extracts were about 10℃ higher than enzymes in stomach.

Salt concentration may have influence on the proteolytic activities in fish roe. However, the influence is variable with different pH value and fish species. Earlier study of Mullet and Alaska Pollack roes has shown that the caseinolytic activity increased with increasing salt concentration at pH 5, but the aminopeptidase activity decreased with salt concentration at pH 7 (Chiou *et al.*, 1989). This was in accordance with present study, which showed that the cod extracts with salt, compared with extracts without salt, have shown a much higher proteolytic activity at pH 5, but a

much lower activity at pH 7 (Fig. 2 A and C; Appendix Table 3). This was probably because the caseinolytic enzymes were mostly active at pH 5, but the aminopeptidase enzymes were mostly active at pH 7 (Chiou *et al.*, 1989, Chiou *et al.*, 1988).

The proteolytic activities of fish roe vary between different species (Khalil *et al.*, 1987). Early researches have reported that the proteolytic activities in viscera, liver and digestive tract were different according to species (Hidalgo *et al.*, 1999, Khalil *et al.*, 1987). Also recent research of Sovik and Rustad (2004) suggested that viscera from tusk and ling shown less activities of trypsin and chymotrypsin than cod, saithe and haddock. This was in accordance with the present study, which found that the proteolytic enzymes of herring roe, both salted and unsalted, were less active that those of cod roe (Figure 2 and 2; Appendix Table 3). One possible reason for this difference could be the difference between living environment of fish species, for example living depth (Sovik and Rustad, 2004). However, there could be other reasons that may have strong influence on the proteolytic activities, such as spawning season and spawning place.

4.2 Influences of temperature, pH on lipolytic activities of cod and herring roes

In the present study, the lipolytic enzymes were mostly found maximum activities at 50℃ (Fig. 3; Appendix Table 4). This was highly in accordance with the earlier findings of cod liver and cut off, which showed maximum lipase activity at 50℃

(Sovik and Rustad, 2005a). However, different optimal temperature was found by Lie and Lambertsen (1985), who showed that lipolytic activity from intestinal juice of pyloric caeca and anterior ileum from cod were highest at 37℃. Also Sovik and Rustad (2005a) have found that optimal temperature for lipase in viscera of cod was 35℃.

Species may also have influence on the lipolytic activities of fish roe. In present study, the lipolytic activities were higher in herring roe than in cod roe, regardless of pH and temperature. These differences can be also found in same fish caught in different places, such as the lipase activity at pH 5 of liver from cod caught in the Barents Sea was higher than in the Icelandic Sea (Sovik and Rustad, 2005a). The differences might be caused by different factors, such as the active swimming of the fish and the fatty acid composition in the feed (Jonas and Bilinski, 1964, Liang *et al.*, 2002).

The lipolytic activities in both cod and herring roes are found highest at pH 7 and lowest at pH 5 (Fig. 3). This difference in lipolytic activity has also been described in other fish species by other researchers. Lipase activity was found to be highest at pH 7 in viscera of cod, intestines of oil sardine, stomach and pyloric caeca of mullet, red muscle of mackerel and liver of rohu (Sovik and Rustad, 2005a, Nayak *et al.*, 2003). Sovik and Rustad (2005a) have found that the pH value in crude extracts of cod muschle, liver and viscera were all around 7. These finding may therefore indicate that the maximum lipolytic activity at pH 7 was due to the natural environment of pH

7 in fish.

4.3 Influences of pre-heating treatment on proteolytic and lipolytic activities of cod and herring roes

The pre-heating treatment has influence the content of protein and activities of proteolytic enzymes in fish roe, which might be due to coagulation or denaturation of the roe protein (Balaswamy *et al.*, 2010). The present study has found that the proteolytic enzymes were only active at pH 5 with 50℃ pre-heating temperature (Fig. 3). In other pH and temperature, the proteolytic enzymes were almost inactive. Similar result was found by Kopylenko and Rubtsova (2004) who showed that the proteinase activity in pink salmon roe was highest at 35℃, but dropped to 5% of the baseline level at 65℃. Sternin and Dore (1993) also reported that the preheating temperature of roes should not be over 70℃, which was similar to the result from the present study. The proteolytic activities of cod roe at pH 5 were 2 times higher in the 10min heated treatment than in 20min heated treatment (Fig. 4). That was in accordance with the fact that the pre-heating time has influence on the denaturation of proteolytic enzymes. However, 20min of pre-heating seems not enough for denaturation of all the enzymes in cod roe at pH 5.

The present study has shown that lipolytic enzymes of cod roe at pH 7 were active at 50℃, but became inactive when temperature increased up to 60℃ or 70℃ (Fig. 5).

The results were highly in accordance with earlier research of cod cut off, liver and viscera, which showed that the lipase became inactive when pre-heating temperature increased up to 60℃ (Sovik and Rustad, 2005a). No difference was observed between the lipolytic activities in cod roe with different pre-heating time (Fig. 5). That was probably because the pre-heating time was not long enough. Sovik and Rustad (2005a) have found that lipases of cod were still active after 40min of pre-heating at 40℃ and pH 7.

5 Conclusions

The proteolytic activities of unsalted cod roe were highest at 50℃, regardless of the pH value. Cod roe extracts with salt, compared with extracts without salt, have shown a much higher proteolytic activity at pH 5, but a much lower activity at pH 7. The proteolytic enzymes of herring roe, both salted and unsalted, were less active that those of cod roe. The lipolytic enzymes of cod and herring roe were mostly found to have maximum activities at 50℃ and pH 7, and were higher in herring roe than cod roe. The present study also showed that heating at 50℃ did not completely inactivate the proteolytic and lipolytic enzymes while the enzymatic activities were inactivated after heating for 10 minutes at 60 and 70℃. From the results we conclude that fish species, salt concentration, pH and temperature all have influence on the proteolytic and lipolytic activities in fish roe. To inactivate proteolytic and lipolytic activities in cod and herring roe, temperatures above 50℃ is needed.

6 References

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Appendix

Table 1 Proteolytic activities of cod and herring roe

		22℃	40℃	50℃	60℃
Cod Roe	pH=5	4278±165	12063±764	13708±1481	9542±1331
	pH=6	22110±1539	27781±8633	31170±9493	7321±3343
	pH=7	123716±20568	248003±31569	283165±44135	702292±117772
Herring Roe	pH=5	70415±14911	231687±4674	238105±13654	118482±13218
	pH=6	280932±32714	1134361±45162	1665205±70708	532296±50969
	pH=7	663325±20683	1789284±54459	2553943±140397	1808460±365261

Table 2 Lipolytic activities of cod and herring roe

		pH=5			pH=6			pH=7		
		40℃	50℃	60℃	40℃	50℃	60℃	40℃	50℃	60℃
Cod	0%	0.48±1.65	2.93±0.46	1.96±0.49	2.86±0.37	2.66±0.27	2.01±0.61	5.39±0.43	7.44±0.46	2.74±0.28
	3.2%	4.42±0.49	5.02±0.69	2.2±0.25	2.44±1.05	2.94±0.27	2.75±0.91	3.19±0.22	2.8±0.07	1.42±0.88
	6.4%	8.24±0.00	5.53±0.66	1.95±0.11	3.49±0.75	2.02±0.7	-0.48±0.77	0.68±0.00	3.6±0.73	1.39±0.13
Herring	0%	3.09±0.29	4.61±0.67	4.4±0.71	0.9±0.27	0.4±0.05	0.22±0.19	0.2±0.05	0.02±0.05	-0.03±0.18
	3.2%	3.39±0.3	6.41±0.23	2.09±0.3	0.62±0.21	0.25±0.17	0.07±0.02	0.29±0.5	0.63±0.4	3.12±0.62
	6.4%	2.92±0.32	4.83±0.3	0.69±0.06	0.51±0.21	0.62±0.39	0.27±0.03	1.17±0.12	0.35±0.12	0.24±0.12