Use of Two-Dimensional Electrophoresis To Evaluate Proteolysis in Salmon (*Salmo salar*) Muscle As Affected by a Lactic Fermentation

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Two-dimensional electrophoresis was used to study proteolysis in salmon fillets inoculated or not with the starter culture *Lactobacillus sake* LAD. Protein fragments appeared increasingly with time in both samples, indicating that the main quantitative changes were due to endogenous enzymes. In the most acidic zone (pI = 4−6.20) particularly, proteolysis was overall independent from processing. In contrast, fermentation had a significant effect in the pH range 6.20−8.35, suggesting a specificity of the bacterial proteases of *L. sake* toward alkaline to slightly acidic proteins. Furthermore, fragments surrounding tropomyosin (apparent pI = 4.70) appeared in fermented samples, indicating that the protein may be a suitable substrate for the metabolism of *L. sake* LAD.

Keywords: *2-D electrophoresis; salmon; proteolysis; tropomyosin; Lactobacillus sake*

INTRODUCTION

Changes in proteins influence to a very large extent the quality of fresh or processed fish products, particularly texture attributes. Thus, denaturation of protein under extreme conditions of salt concentrations or temperatures can induce excessive toughening (Sikorski and Sun Pan, 1994; Tejada et al., 1996). On the other hand, softening of fresh fish flesh was attributed to many factors including the fragmentation of myofibrils (Jiang et al., 1990), cleavage of titin (Seki and Watanabe, 1984; Tsuchiya et al., 1992), degradation of the extracellular matrix (Ando et al., 1991), or release and subsequent proteolysis of α-actinin (Papa et al., 1996).

In a previous paper, Morzel et al. (1998) showed that protein pattern as observed on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) changed extensively during processing and storage in salmon muscle fermented with various lactic acid bacteria. However, the origin of enzymes (endogenous or bacterial) responsible for such changes was not investigated. The affinity of the two types of enzymes, for example, toward specific proteins or proteins of given isoelectric points, was therefore also unknown. Two-dimensional (2-D) electrophoresis allows visualization of both molecular weight and isoelectric point of proteins. Since the technique was first reported by O'Farrell (1975), 2-D electrophoresis has received many improvements. A limitation to the technique, particularly in immobilized pH gradient (IPG) technology, is the poor resolution of large proteins (Humphery-Smith et al., 1997; Celis and Gromov, 1999). However, within the lower molecular weight range (<100 kDa), 2-D electrophoresis is now described as providing the highest resolution in protein analysis (Celis and Gromov, 1999). The technique has found a number of applications to food analysis, including the characterization of raw material such as wheat flours (Dougherty et al., 1990), bovine caseins (Zeece et al., 1989), or fish white muscle (Martinez and Christiansen, 1994). Furthermore, it proved to be successful in studying the course of events followed by cod proteins during surimi-making (Martinez et al., 1992) or in monitoring proteolysis during Cheddar cheese ripening (Chin and Rosenberg, 1998).

The present study was undertaken to determine the effect of a lactic fermentation on proteolysis in salmon muscle, using 2-D electrophoresis. Fermented salmon samples were compared to a control noninoculated sample to evaluate the specific changes, if any, induced by the proteolytic activity of a strain of lactic acid bacteria.

MATERIALS AND METHODS

Raw Material. Farmed Atlantic salmon (*Salmo salar*) originating from Norway was delivered in the Ifremer as whole eviscerated fish stored on ice, 5 days after slaughtering. Salmon was filleted immediately prior to processing. All processing and sampling steps were carried out under stringent hygienic conditions to avoid contamination by environmental microorganisms.

Processing of Salmon Samples. Processing of fermented salmon samples was performed as follows: salmon fillets were trimmed of visible lateral fat, weighed, and stored at 2−4 °C until required for experimental use on the same day. Sucrose (1% w/w), NaCl (2.5% w/w), and freeze-dried cultures (0.4% w/w) of *Lactobacillus sake* LAD (Gewürzmüller, Stuttgart, Germany) were weighed, and the mixture was applied evenly by rubbing onto the visceral side of the fillets, which were placed on a rack at 12 °C overnight to allow the diffusion of the ingredients in the flesh.

The following morning, the undissolved ingredients remaining on the surface of the fillets were removed by scraping them off with a knife. Fillets were further prepared by removing pin-bones. Each fillet was vacuum-packed in polyamide−polethylene bags (Grace Multiflex GmbH, Flensburg, Germany). Samples were incubated at 12 °C for 72 h and subsequently stored at 4 °C for a further 18 days.

Noninoculated (control) samples was produced in the same manner, omitting the starter cultures in the processing.

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Between the addition of the ingredients and packaging, the samples were covered by large polystyrene boxes to prevent or reduce aerial contamination by starter cultures used in the processing of fermented samples. Furthermore, samples were placed at 4 °C immediately after packaging. The incubation at 12 °C for 3 days, part of the processing of fermented fillets, was not retained as it would represent a major risk of bacterial growth. The resulting growth of either lactic acid bacteria or pathogens would render the comparison of noninoculated and inoculated salmon fillets impossible.

Two whole fish were used in the study. Two fillets obtained from the same fish were used to process one fermented (F) and one control (C) sample. Each treatment was therefore produced in duplicate, and the samples received the labels F1, F2, C1, and C2. Times of processing and storage were labeled as follows. RM corresponds to the day of reception of the raw material (fresh salmon). Day 0 corresponds to the step of vacuum packing. Samples were also taken on days 3, 7, 14, and 21.

Enumeration of Bacteria. Enumeration of microorganisms was performed by the surface spread plate technique. Total viable counts were determined on plate count agar (Oxoid, Basingstoke, Hampshire, U.K.) on RM for all samples and on day 3 for control samples, whereas lactic acid bacteria (LAB) were enumerated on MRS agar (Oxoid) on RM and day 0 for all samples and on day 3 for fermented samples. Incubation of all plates was performed at 30 °C for 24−48 h.

Determination of pH. Salmon flesh (1.5 g) was homogenized in 10 mL of distilled water. Readings were taken on a Consort p600 meter (Bioblock Scientific, Illkirch, France).

Two-Dimensional Electrophoresis. The extraction buffer consisted of 8 M urea, 5 mM Pefabloc-SC (Interchim, Montluçon, France), 4% (w/v) CHAPS, and 3.5 mM TCEP-HCl in 40 mM Tris base. The final pH of the solution was brought to 9.4 with 10 N NaOH. White muscle (0.15 g) was homogenized in 3 mL of extraction buffer in a Potter RGL 100 (Heidolph, Kelheim, Germany) for 2 min and 30 s. The extracts were centrifuged at 10000 rpm for 10 min, and the clear supernatant was collected. The protein content of this final extract was measured according to the Bradford method (Bradford, 1976).

3-10L Immobiline DryStrips, 7 cm (Pharmacia Biotech, Uppsala, Sweden), were rehydrated overnight following the manufacturer's indications in a solution consisting of the extract diluted in extraction buffer, supplemented with 15% (w/v) glycerol, 0.8% (v/v) preblended pH 3.5−9.5 ampholytes (Pharmacia Biotech), and Orange G (Merck, Darmstadt, Germany) as dye. The protein load applied to each strip was adjusted to 10 μg. Isoelectric focusing was performed at 20 °C using a Multiphor II flat-bed unit connected to an EPS 3500 XL power supply (Pharmacia Biotech), programmed in gradient mode at 200 V for 1 Vh followed by 3500 V for a total of 6300 Vh. Gel strips were subsequently equilibrated 2 × 10 min in 0.5 M Tris-HCl, pH 6.8, supplemented with 1% (w/v) dithiothreitol (DTT) and 2.5% (w/v) iodoacetamide, respectively. Orange G was added to the second equilibration solution to tint the strips.

SDS−PAGE was performed in a Mini-protean dual slab cell (Bio-Rad, Richmond, CA) according to the method of Laemmli (1970), using discontinuous 12% resolving−3% stacking polyacrylamide 1 mm thick gels. Electrophoresis was carried out at 5 mA per gel for 10 min followed by 15 mA per gel until the dye front reached the end of the gel.

Gels were silver-stained using a protocol slightly modified from that of Blum et al. (1987), with the exception of the fixation steps performed in 40% ethanol−10% acetic acid for 1 h followed by 5% ethanol−5% acetic acid overnight. Development was stopped with a 0.5% glycine solution.

For each individual sample (one duplicate, one type of processing, one time of sampling), three gels were produced.

Western Immunoblotting. After electrophoresis, proteins were transferred overnight at 15 °C onto a 0.45 μm nitrocellulose membrane in a buffer consisting of 0.4 M Tris base, 150 mM glycine, 20% (v/v) methanol, and 0.01% (w/v) SDS. Transfer was performed at 20 V in a Trans-Blot cell (Bio-Rad). Membranes were saturated in 8% skimmed milk powder

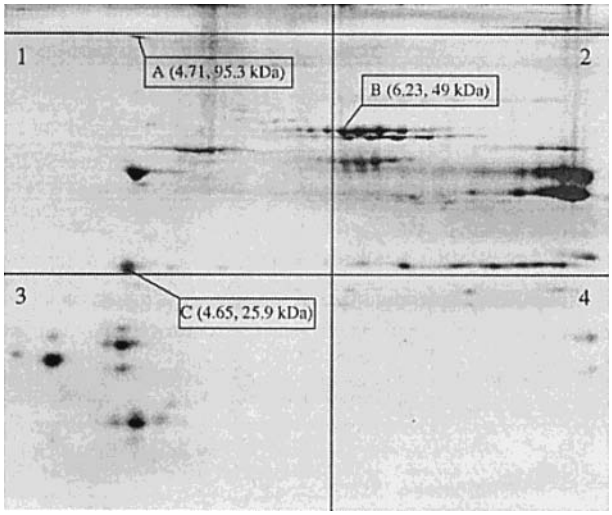

Figure 1. Location of the three spots A−C used for dividing a gel into four zones. Spots are labeled with their MW and p*I* (mean value for the 22 computer-generated gels). The presented gel corresponds to a sample of raw material (fresh unprocessed salmon). Ten micrograms of protein was loaded onto a 7 cm linear 3-10 Immobiline Dry strip.

reconstituted in 0.9% NaCl and incubated with a 0.5% solution of the primary polyclonal antibody diluted in 0.9% NaCl, for 3 h at room temperature. Three types of primary antibodies, all produced in rabbit, were used. Anti-actin was produced in-house, whereas anti-desmin and anti-tropomyosin were purchased from Sigma Immuno Chemicals (St. Louis, MO). After a washing in a PBS−Tween buffer [0.137 M NaCl, 0.027 M KCl, and 0.01 M phosphate buffer, supplemented with 0.05% (v/v) Tween 20], membranes were incubated in anti-rabbit IgG antibodies labeled with phosphatase, diluted in 5% bovine serum albumin (BSA) in 0.9% NaCl. Specific proteins were subsequently revealed using BCIP/NTB as a substrate.

When the location of the immuoreaction in comparison to surrounding features needed to be better visualized, membranes were stained immediately after transfer and prior to immunoblotting with a colloidal gold solution (Protogold) following the manufacturer's instructions (British Biocell International, Cardiff, U.K.).

Image Analysis. Gels were first visually examined, and outstanding consistent differences were recorded. Gel images were acquired through an Epson GT-12000 scanner (Seiko Epson, Nagano, Japan). Analysis was performed using the software Melanie II 2-D PAGE (Bio-Rad, Hercules, CA). Because of the very large number of gels produced, analysis of individual spots proved to be impossible. For brevity purposes, a synthetic gel was therefore generated for each individual sample from the three replicates by selecting features present in at least two of the three gels. In the case of raw material, two synthetic gels were created from the six gels corresponding to the same fish. Subsequently, computer-generated gels were analyzed by dividing them into four zones, as shown in Figure 1. The limits of each zone were set by choosing three polypeptide spots present in all gels and drawing contiguous lines to these spots. The upper limit was placed directly above spot A, corresponding to the largest protein consistently observed. Additional spots were sometimes present above this line, but entry of high molecular weight proteins into IPG strips has been reported to be difficult (Görg et al., 1998), which results in a poor reproducibility of patterns in the high molecular weight range. The line contiguous to spot B separated the gel into two zones of p*I* with a limit of ∼6.20, and spots were further separated according to their molecular weight with a cutoff point of ∼25.5 kDa. In each zone, polypeptide spots were enumerated. The effects of time, type of processing, and fish were tested for statistical significance by ANOVA, using Statsgraphics 6.0 (Sigma Plus, Paris, France).

Determination of Molecular Weight (MW) and Isoelectric Point (p*I*). Determination of the MW and p*I* for spots of interest was achieved by comigration of a ready-to-use 2-D SDS−PAGE standards mix (Bio-Rad) with a sample of raw material. Experimental MW and p*I* were deduced by the Melanie software after construction of internal scales based on the standard values.

RESULTS AND DISCUSSION

Bacterial Flora and pH. Total viable counts in the raw material were 2.5×10^3 CFU g^{-1} in the fish used for processing samples F1 and C1 and 1.3×10^4 CFU g^{-1} in the fish used for processing samples F2 and C2. No LAB were detectable at the 10^{-1} dilution in the initial flora. This result is in accordance with microbiological reports on fresh fish: although several authors (Mauguin and Novel, 1994; Gram and Huss, 1996) reported the isolation of *Carnobacteria* or *Lactobacilli* from fresh fish, LAB represent only a minor part of the flora (Valdimarsson and Gudgjörnsdóttir, 1984).

On day 0, counts of LAB (i.e., of the starter cultures) in inoculated samples F1 and F2 were 2.7×10^8 and 7.4×10^8 CFU g^{-1}, respectively. No LAB were detected in the control samples, indicating that no aerial contamination between samples occurred. The inoculation level in *L. sake* LAD was higher than in previous production. However, on day 3, LAB reached levels of 1.9×10^9 and 1.1×10^9 CFU g^{-1} in F1 and F2, which is comparable to levels previously reported (Morzel et al., 1997). No further enumeration was performed as the main objective of the microbiological analyses was to verify that the usual fermentation conditions were reproduced. The initial pH of ∼6.2 dropped to 5.3 on day 21 in the two fermented samples, which is consistent with values previously reported (Morzel et al., 1997). The pH remained almost constant during processing and storage (final value = 6−6.1) in the control samples.

General Characteristics of Gels. A total of 72 gels were produced. The total number of polypeptides varied from 141 to 530, whereas the number of polypeptide spots on computer-generated gels varied from 171 to 335. Proteins were separated on the *X*-axis according to their p*I* in the range 4−8.35 and on the *Y*-axis according to their MW in the range ∼8−100 kDa.

Immunoblotting with specific antibodies allowed location of actin, tropomyosin, and desmin on the gels, as shown in Figure 2. Actin and tropomyosin were always represented on all gels, whereas desmin could be seen on only 10 of the 22 synthetic gels and appeared inconsistently as a single spot or as an array of several spots. It was therefore difficult to calculate a mean value for the p*I* of desmin, and it was preferred to show results as a range of values. In contrast to desmin, actin appeared consistently as an array of five to six spots. The average p*I* values corresponding to the extreme spots in the array are noted in Figure 2. Tropomyosin also had a specific shape. It was difficult to decide whether the spot was homogeneous or composed of two very closely located polypeptides. However, because only one type of tropomyosin is theoretically present in salmon fast muscle (Martinez et al., 1993; Heeley and Hong, 1994), the spot was invariably considered as a single polypeptide.

The apparent MW and p*I* (mean values of 22 synthetic gels) of actin and tropomyosin and the apparent MW of desmin (mean values of 10 synthetic gels) are presented

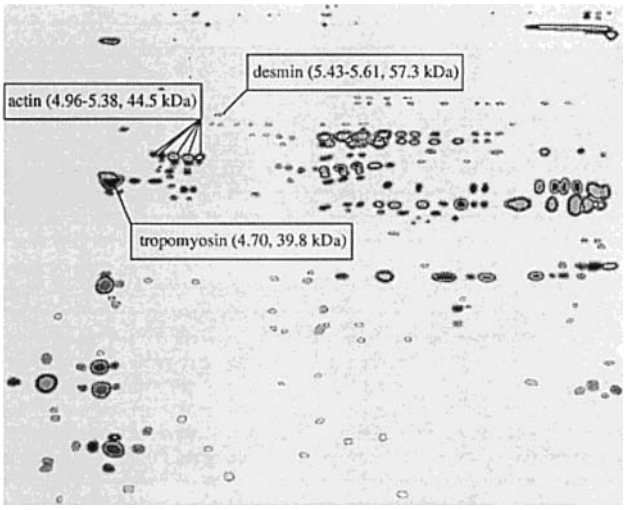

Figure 2. Location of actin, desmin, and tropomyosin on a 2-D computer-generated gel. The presented gel corresponds to a control sample taken on day 7. The three proteins are labeled with their MW and p*I*, as described in the text.

in Figure 2. It has to be noted that the experimental values of MW of actin, desmin, and tropomyosin (44.5, 57.3, and 39.8 kDa, respectively) are slightly higher than those previously calculated from one-dimensional gels. For example, a MW of 43 kDa was measured for actin (Stefansson and Hultin, 1994). Desmin showed apparent MW values of 55 kDa in mammalian muscle and 53 kDa in avian muscle (O'Shea et al., 1981). Tropomyosin was reported to appear either as a single 35.5 kDa band (Stefansson and Hultin, 1994) or as a 35−37 kDa doublet band (Tsuchiya et al., 1980). Despite the use of internal standards, calculated values of MW and p*I* may vary considerably for a similar protein. To give only one example, Kovalyov et al. (1995) reported a p*I* of 4.92 for the α-chain of tropomyosin extracted from human heart, whereas values of 4.4−4.5 and 4.7−4.8 are reported in the databases HSC-2DPAGE (Heart Science Centre, Harefiled, U.K.) and HP-2DPAGE (Max Delbrück Centre for Molecular Medicine, Berlin, Germany), respectively. In the present study, further calibration may be required to verify the validity of the internal MW and p*I* scales. Nevertheless, on the overall performance of the gels, it can be concluded that the resolution and reproducibility achieved were very satisfactory.

Proteolysis. Visual observation of the gels allowed the pinpointing of clear differences in the area surrounding the spot identified as tropomyosin. As seen in Figure 3, small polypeptide spots appeared in this area. These polypeptides, slightly more acidic than the tropomyosin spot, were visible from day 7 or 14 in fermented samples. It is most probable that appearing spots were not of bacterial origin, because bacterial proteins represent only a minute fraction of total proteins in the samples. Furthermore, any spot corresponding to bacterial proteins should be detected by day 3 because no further bacterial growth occurs in fermented samples at 4 °C (Morzel et al., 1997). No identification of these new spots was performed, but it seemed likely that they were tropomyosin fragments.

Proteolysis observed by electrophoretic techniques may be evidenced by the appearance of newly formed bands/spots. A simple and rapid method to evaluate proteolysis, although not exhaustive, is therefore to monitor the counts of detected polypeptides as a function

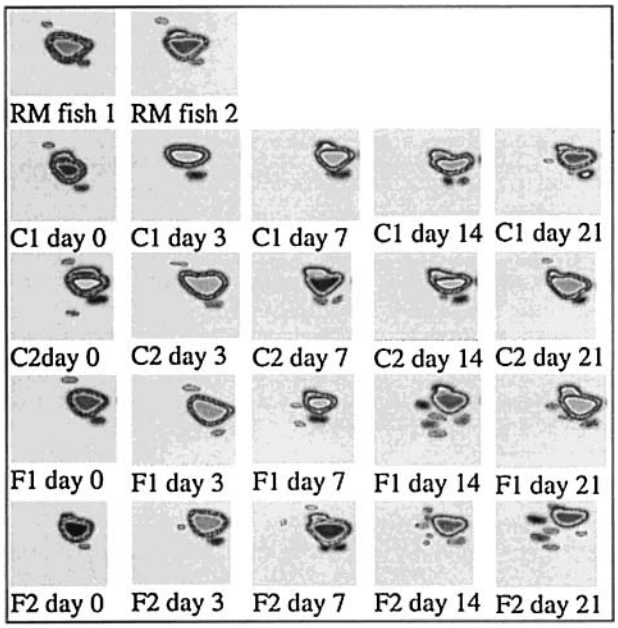

Figure 3. Tropomyosin-containing window for all 22 computer-generated gels. Samples are labeled as follows: F1 and F2 represent duplicates of fermented samples; C1 and C2 represent duplicates of control samples. RM is the raw material (fresh unprocessed salmon).

Table 1. Number of Features Recorded for Each Sample and for Each Zone for All Samples at the Different Times of Sampling

time of sampling	sample code	zone number			
		1	2	3	4
RM	C1/F1	42	92	11	23
	C2/F2	49	103	23	14
day 0	C1	44	84	23	8
	C2	66	96	30	15
	F1	49	110	21	11
	F2	36	81	22	14
day 3	C1	56	89	27	11
	C2	60	94	21	11
	F1	32	90	30	13
	F2	48	95	31	16
day 7	C1	59	114	34	18
	C2	95	135	38	38
	F1	84	131	35	23
	F2	103	140	37	31
day 14	C1	90	120	50	39
	C2	78	117	49	34
	F1	101	149	58	45
	F2	114	133	53	42
day 21	C1	63	89	36	17
	C2	85	103	49	30
	F1	86	115	42	52
	F2	95	94	48	41

of time. Table 1 shows the counts of polypeptide spots enumerated on computer-generated gels, for each sample and for each zone on the different times of sampling.

Time effect was highly significant ($p < 0.1\%$) whether for the whole area or for each zone individually, with an overall increasing trend in the counts. Treatment effect was nonsignificant, but the probability value was 5.37%. As to the fish effect, it was clearly overall nonsignificant, indicating that numbers of spots did not differ significantly between the two fishes used in the study. When the total amount of features was studied for each zone, a somewhat different picture appeared. Thus, treatment had a significant effect ($p < 1\%$) only on zone 4 (pI = 6.20−8.35, MW < 25.5 kDa) and more generally over the whole most alkaline zone. Levels of significance associated with the fish effect were all superior to 5% but approached very closely this value for zones 1 and 3 (pI < 6.20) with 5.13 and 5.12%, respectively. When these two zones were combined, the fish effect became significant ($p < 5\%$).

Time Effect. The very significant effect ($p < 0.1\%$) of time on protein degradation was fully expected. Numerous investigations have shown degradation of specific proteins under refrigerated storage conditions. For example, progressive proteolysis of α-actinin (Tsuchiya et al., 1992; Papa et al., 1996), titin (Seki and Watanabe, 1984; Astier et al., 1991), or desmin (Verrez-Bagnis et al., 1999) was evidenced in muscle of various fish species. However, in the case of salmon, little information is available concerning proteolysis occurring at refrigerated temperatures. Salmon fillets have been reported to become soft-textured during processing of gravlax (sugar−salt cured salmon noninoculated with starter cultures), which may presumably be linked to a proteolyic activity. Alteration of the SDS−PAGE pattern of muscle proteins in fermented salmon was also observed (Morzel et al., 1998), and the hypothesis that the recorded proteolysis was due to bacterial enzymes was rejected.

Fish Effect. The overall nonsignificant fish effect demonstrated the limited influence of individual characteristics on further proteolysis, in the particular case of the two fishes used in the study. However, analysis performed per zone indicated that this was verified only in the most alkaline pH range. In the most acidic zone (pI = 4−6.20), numbers of spots were significantly lower ($p < 5\%$) in the samples with the lowest initial bacterial load. Nevertheless, it cannot be concluded that the initial flora was the only causative agent of the enhanced proteolysis. For example, Sigholt et al. (1997) observed an influence of stress on Atlantic salmon texture, which they linked to the degree of protein denaturation and subsequent enzymatic activity. Apart from the initial flora, many factors such as proximate analysis, condition of the animal before slaughtering, or stress during landing may be involved in the phenomenon of acidic protein degradation.

Treatment Effect. In the present study, the level of significance associated with the treatment effect was very close to the 5% value. This suggests that, although most of the protein degradation was not dependent on bacterial enzymes, fermentation probably influenced proteolysis in a limited proportion. This was confirmed, for example, by the specific alteration of the tropomyosin area.

Tropomyosin is an integral constituent of the thin myofibrillar filaments (Harrington, 1979). In a myofibrillar extract of whiting or carp, this protein was sensitive to the action of cathepsin L (An et al., 1994; Aranishi et al., 1998) but was unaltered by cathepsin B (Hara et al., 1988). However, cathepsin L in salmon was unable to degrade tropomyosin in physiological conditions (Yamashita and Konagaya, 1991), and this protein was not affected during post-mortem of rainbow trout (Tsuchiya et al., 1992). The two latter studies suggest that tropomyosin is not easily proteolyzed in salmonids by endogenous enzymes. In salmon muscle, decrease of a 35 kDa protein was observed during fermentation with different strains of LAB including *L. sake* LAD (Morzel et al., 1998). This protein may presumably correspond to tropomyosin. In salmon muscle fermented with *L. sake* LAD, pH reached 5.2−5.3 after

21 days. This low pH may have stimulated catheptic activity and facilitated tropomyosin proteolysis. However, degradation observed in the present study could also be induced by bacterial enzymes of *L. sake* LAD. Egan et al. (1989) studied the metabolism of *L. sake* in a vacuum-packaged meat and showed that H_2S could be produced from nitrogenous compounds after depletion of the carbohydrate source. Furthermore, Montel et al. (1994) reported that *L. sake* showed a proteolytic activity, observable by the release of phenylalanine, lysine, glutamic acid, alanine, valine, and isoleucine. In salmon tropomyosin, glutamic acid, lysine, and alanine are the three most common amino acids (Heeley and Hong, 1994), which may explain partially how tropomyosin could be a suitable substrate for bacterial enzymes of *L. sake* LAD.

The influence of fermentation on proteolysis was further evidenced by the analysis of counts per zone. Thus, higher numbers were recorded in fermented samples for zone 4 and more generally over the pH 6.20−8.35 range, indicating a more pronounced occurrence of alkaline to slightly acidic low-protein fragments. Such a specificity of LAB proteases has not been reported to date despite the abundance of research conducted on bacterial proteolytic activity. In most tests in vitro, casein or gelatin was used as substrate (Niemand and Holzapfel, 1984; Molina and Toldrá, 1992; Mauguin and Novel, 1994). Studies using myofibrillar or sarcoplasmic muscle proteins were also conducted (Molina and Toldrá, 1992; Fransen et al., 1997). Nevertheless, such tests do not allow the specific study of the orientated proteolysis toward neutral or alkaline proteins.

Conclusions. As previously described in this paper, large proteins cannot easily be observed by 2-D electrophoresis performed using IPG strips. In salmon muscle subjected to a lactic fermentation, intensities of the myosin heavy chain and of a 95 kDa component decreased (Morzel et al., 1998). These major changes were not confirmed by 2-D electrophoresis and, more generally, results obtained via SDS−PAGE alone or 2-D electrophoresis seemed to be difficult to correlate. In contrast, 2-D electrophoresis provided other valuable types of information on proteolysis occurring in fermented salmon. For example, the technique enabled the study of very fine changes in defined pH ranges or in even more limited areas corresponding to a single protein.

ABBREVIATIONS USED

SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; 2-D, two-dimensional; LAB, lactic acid bacteria; RM, day of reception of raw material; CFU, colony-forming unit; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TCEP-HCl, tris(2-carboxyethyl)phosphine hydrochloride; DTT, dithiothreitol; BSA, bovine serum albumin; BCIP/NTB, 5-bromo-4-chloro-3-indolyl phosphate/Nitro Blue Tetrazolium salts; IPG, immobilized pH gradient.

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