Watson, R. R. The role of selenium and retinoids in immunomodulation and cancer prevention. *Food Nutr. News* **1986** (National Livestock and Meat Board, Chicago, IL).

Watson, R. R.; Moriguchi, S. Cancer prevention by retinoids: role of immunological modification. *Nutr. Res.* **1985**, *5*, 663–675.

Zechmeister, L. *Cis-trans* isomerization and stereochemistry of carotenoids and diphenylpolyenes. *Chem. Rev.* **1944**, *34* (2), 267–344.

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Influence of pH on the Proteinase Complement and Proteolytic Products in Rainbow Trout Viscera Silage

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Nine classes of proteinases were screened for their stability and activity in silages of trout viscera, ensiled at pH 2 and 3. Both endo- and exo-peptidases were active in the pH 3.0 silage (silage A), quickly breaking down the protein nitrogen to amino nitrogen. In the pH 2.0 silage (silage B) only acid endo-peptidase and a weak exo-peptidase activity were present, slowing the formation of amino nitrogen during autolysis. The additional presence of formic acid in pH 2.0 silage (silage C) limited the increase in pH during autolysis and prevented any increase in amino nitrogen, indicating the complete suppression of exo-peptidase activity in such silages. Correspondingly, the amounts of short peptides and amino acids were highest in silage A and smallest in silage C.

The conversion of fish processing wastes and underutilized fish species into fish silage produces a versatile feed ingredient for many farmed animals and fish raised by aquaculture. Although the production of fish silage has become commercialized, the proteolytic enzymes participating in the autolytic process have not been clearly elucidated. Acidic and alkaline proteases have been recovered from silages (Reece, 1988), and the effects of many proteolytic enzymes have been observed in conventional silages (pH 4.0), breaking down proteins to short peptides and free amino acids (Stone and Hardy, 1986). The physical and chemical changes occurring in fish silage during autolysis are well documented (Freeman and Hoogland, 1956; Tatterson and Windsor, 1974; Backhoff, 1976; Raa and Gildberg, 1976; Gildberg and Raa, 1977; Reece, 1980; Stone et al., 1984; Hall et al., 1985; Haard et al., 1985; Stone and Hardy, 1986), but conclusive evidence linking the nature of autolysis to the various proteinases participating in the process has been lacking.

In this study, nine different classes of proteinases were screened for their stability and activity in high- and low-pH silages. Physical and chemical changes occurring in these silages during autolysis were monitored to understand the relationship between the type of proteinases present and the nature of autolysis.

EXPERIMENTAL SECTION

Preparation of Silages. Rainbow trout (*Oncorhymchus mykiss*), 1–2 years of age and weighing between 500 and 800 g (25–40 cm long) were harvested from the aquaculture ponds of the Saskatchewan Research Council, Saskatoon, during Oct 1986. Visceral contents of 100 fish were immediately chilled and later (~5 h) passed through a meat mincer. Minced viscera were thoroughly mixed, frozen in small lots (1 kg) at −40 °C, and stored at −70 °C. Silages were prepared by slowly acidifying the thawed viscera while rapidly mixing with a paddle-type agitator. In silages used for enzyme assays, viscera were initially diluted with water (1.33×) before acidification, to facilitate better extraction and pH adjustment. One group of silages (silage A) were acidified with formic acid (3% w/w) to pH 3.0, while lower pH silages (silage B) were acidified to pH 2.0 with 12 N sulfuric acid. A third type of silage (low-pH buffered silage or silage C) was prepared by further acidifying silage A to pH 2.0 with 12 N sulfuric acid.

Assay of Proteinases. The aqueous phases of silages A and B were used for enzyme assays after 12 h of ensilation at 30 °C. Aqueous phase was obtained by centrifugal separation (2000*g*, 30 min) and filtration of the siphoned aqueous phase through Whatman No. 3 and 42 papers. The pH and protein content (Gornall et al., 1949) of the aqueous phases were measured, and the remaining aqueous phase was stored in aliquots at −70 °C. Aliquots were thawed and then held on ice during the proteinase assays.

General proteolytic activity was assayed at the initial pH of the silages, pH 2 and 3, with azocoll (this and all other proteinase substrates were obtained from Sigma) by a modification of the method of Dean and Domnas (1983). The substrate (5 ± 0.1 mg) was suspended in 2.3 mL of buffer (the compositions of this and other buffers are given in Table I) at 30 °C (all assays were conducted at 30 °C) and the reaction initiated by addition of 0.2 mL of silage. The reaction was terminated after 50 min by adding 1 mL of 35% trichloroacetic acid (TCA) and filtered. Absorbances of filtrates were measured at 520 nm against a water reagent blank. Pepsin-type activity in silage was measured by an identical procedure at pH 1.7, along with a standard pepsin preparation (Sigma; 3× crystallized, 1 mg/mL), and activity expressed as pepsin equivalents. Activities of other pro-

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Table I. Composition of Buffers Used for the Assays of Proteinase in Fish Silage[a]

enzyme	opt pH	buffer used at opt pH	buffer used at pH 3.0	buffer used at pH 2.0	molarity	other components in buffers
pepsin	1.7	KCl–HCl	NA[b]	NA	0.1	
chymotrypsin	7.8	Tris–HCl[c]	citrate	KCl–HCl	0.1	0.1 M $CaCl_2$
trypsin	8.2	Tris–HCl	citrate	KCl–HCl	0.05	0.02 M $CaCl_2$
elastase	8.0	Tris–HCl	citrate	KCl–HCl	0.1	
carboxypeptidase A	7.5	Tris–HCl	citrate	KCl–HCl	0.025	0.5 M NaCl
carboxypeptidase B	7.65	Tris–HCl	Gly·HCl	KCl–HCl	0.025	0.1 M NaCl
aminopeptidase	7.0	phosphate	citrate	KCl–HCl	0.06	
cathepsin B	6.4	phosphate	citrate	KCl–HCl	0.08	0.8 mM EDTA + cysteine, 1% DMSO[d,e]
cathepsin C	5.3	citrate	citrate	KCl–HCl	0.1	0.5 mM MEA·HCl[f]
general proteolytic act.			citrate	KCl–HCl	0.1	

[a] All assays were conducted at 30 °C. [b] NA = not assayed. [c] Tris = tris(hydroxymethyl)aminomethane. [d] EDTA = ethylenediaminetetraacetic acid. [e] DMSO = dimethyl sulfoxide. [f] MEA·HCl = mercaptoethylamine hydrochloride. Buffer compositions according to Dawson et al. (1986).

teinases were measured against the following substrates: BTEE, chymotrypsin-like activity (Walsh and Wilcox, 1970); BAPNA, trypsin-like activity (Dean and Domnas, 1983); $Suc(Ala)_3$–NA, elastase-like activity (Kasafirek et al., 1976); Hip-Phe, carboxypeptidase A like activity (Folk and Schirmer, 1963); Hip-Arg, carboxypeptidase B like activity (Folk et al., 1960); Leu–NA, aminopeptidase-like activity (Pfleiderer, 1970); BAPNA, cathepsin B like activity (Otto and Riesenkonig, 1975); Gly-Phe–NA, cathepsin C like activity (McDonald et al., 1966). Activities of all proteinases were corrected against substrate blanks (apparent activity in the absence of substrate) in addition to the normal reference blanks (without enzyme sample).

Physical and Chemical Changes in Silages during Autolysis. Silages A–C prepared as described earlier were dispensed into capped jars (30 g) and test tubes (6 g), which were allowed to autolyze at 30 °C. At various intervals, a jar of each silage was removed and homogenized and duplicate samples were withdrawn for total nitrogen (1–2 g) and nonprotein nitrogen (8–10 g) analyses. Silages in test tubes (in duplicate) were also removed at similar intervals, and 5-g aliquots were centrifuged at 1400*g* for 30 min. After centrifugation, the aqueous phase was removed with a graduated syringe, and its volume and pH were measured.

Chemical Analyses. Total nitrogen (TN) and nonprotein nitrogen (NPN) in the silage were determined by the semimicro-Kjeldahl method [Method No. 46-12 of the AACC (1976)]. Samples for NPN determination were prepared by blending the samples with 3 volumes of 13.33% TCA (final TCA concentration 10%) and filtering through Whatman No. 3 paper under low suction. Filtrates were freed from any lipid material by centrifugation. TCA solubles were also analyzed for peptide nitrogen (Gornall et al., 1949) and α-amino nitrogen (Rosen, 1957). Egg albumin and glycine were used as standards for peptide and α-amino nitrogen (α-NH_2 N) determinations, respectively.

Gel Filtration. Aqueous phases from silages A–C were obtained as described under Assay of Proteinase, heated (85 °C for 20 min) to inactivate the enzymes, cooled, and centrifuged at 3640*g* for 30 min. The supernates were filtered through 0.45-μm pore size filters, and the absorbance (280 nm) and pH of the filtrates were measured. Aliquots of the filtrates (0.5 mL), suitably diluted to yield equivalent absorbances, were loaded on a Sephadex G-25 column. The following buffers whose pH values were within ±0.05 unit of the autolysate were chosen for elution to avoid sample precipitation on column due to pH changes. Citrate–phosphate buffers (0.11 M) at pH 3.2 and 2.8 were used for silages A and B, respectively, and equimolar glycine hydrochloride buffer at pH 2.3 was used for silage C.

RESULTS AND DISCUSSION

Proteinase Activities in Silages. The hydrolytic activities in silages A and B were assayed at two different pHs against various proteinase substrates: (a) at the optimum pH for a proteinase and (b) at the initial pH of ensilation. The activity against a substrate found to be stable at the optimum pH, but undetected at the ensilation pH, may still contribute appreciably to autolysis during the long periods of ensilation. As the viscera mainly consists of digestive organs, proteinase substrates with a bias toward digestive proteinases were mostly selected for screening. The activity observed against a particular substrate in this experiment is more indicative of the presence of a class/type of proteinase, rather than solely attributable to that proteinase, due to the crude nature of the silage.

Silage A (ensiled at pH 3.0) hydrolyzed a number of proteinase substrates (Table II). The activities against substrates of elastase and carboxypeptidases A and B appeared to have been totally inactivated by the acid pH conditions, which supports the earlier observations made in other fish (Clark et al., 1985; Cohen et al., 1981; Petra, 1970; Overnell, 1973). The hydrolysis of BTEE, a chymotrypsin substrate, was stable at pH 7.8 but could not be detected at pH 3.0. Chymotrypsins from most fish rapidly lose their activity when exposed to pH 3 or below (Croston, 1960; Jany, 1976; Prahl and Neurath, 1966), but the inactivation at pH 4 is reported to be slow (Cohen et al., 1981). Mammalian chymotrypsins are however highly stable at pH 3.0 (Wilcox, 1970).

Silage A actively hydrolyzed Azocoll at pH 1.7, indicating the presence of pepsin-type enzymes. Rainbow trout pepsin is reported to have an optimum pH of 3.0 but is also active at pH 1.7 (Twining et al., 1983). Hence, pepsin-type proteinases would be definitely involved in autolysis of silage A. The specific pepsin substrate *N*-acetyl-L-phenylalanyl-3,5-diiodo-L-tyrosine was tested but could not be used as the high concentrations of free amino acids in the silage interfered with the determination of small quantities of diiodo-L-tyrosine released. In addition, some fish pepsins are reported to be inactive against this substrate (Noda and Murakami, 1981).

Silage A hydrolyzed BAPNA, a common substrate for trypsin and acid thiol type proteinases, under the assay conditions for both enzymes. The substrate was hydrolyzed at the optimum pH for both enzymes as well as at the ensilation pH. Trypsins from some fish are stable, though not active under acid conditions (Hjelmeland and Raa, 1982; Croston, 1960) while others are unstable and are completely inactivated (Shin and Zall, 1986; Cohen et al., 1981). Under acid thiol proteinase assay conditions BAPNA was hydrolyzed by the silage more strongly at the ensilation pH than at the optimum pH of 6.4. This may be because the optimum pH of 6.4 used in the assay was established for acid thiol proteinases of mammalian origin (Barrett and Kirschke, 1981), while similar proteinase from a nonmammalian source (squid) has shown a much lower optimum pH of 3.0 (Sakai-Suzuki et al., 1986). The silage also hydrolyzed Gly-Phe–NA, a substrate for

Table II. Activity of Rainbow Trout Viscera Silage[a] Ensiled with Formic Acid (3% w/w), at an Initial pH of 3.0, toward Various Proteinase Substrates

substrate[b]	nominal enzyme assayed	units of act.[c]	activity opt pH	activity pH 3.0
Azocoll[d]	pepsin	pepsin, equiv/mg	0.10	
BTEE	chymotrypsin	sp act.	17.57	na[e]
BAP-NA	trypsin	sp act.	1.13	0.85
Suc(Ala)$_3$-NA	elastase	sp act.	na	na
Hip-Phe	carboxypeptidase A	sp act.	na	na
Hip-Arg	carboxypeptidase B	sp act.	na	na
Leu-NA	aminopeptidase	sp act.	2.31	0.77
BAP-NA	cathepsin B	sp act.	0.45	1.41
Gly-Phe-NA	cathepsin C	sp act.	0.45	0.21
Azocoll	general proteolytic act.[f]	A_{520}/mg per 50 min		0.06

[a] Enzyme samples were prepared for assay by filtration of aqueous phase of centrifuged 12-h-old silage. [b] See abbreviations for full names of substrates. [c] Pepsin equiv/mg = pepsin equivalents/milligram of silage protein. Specific activity = millimoles of product formed per minute per milligram of silage protein. A_{520}/mg per 50 min = change in absorbance at 520 nm per milligram of silage protein in 50 min. [d] Pepsin-like activity was not assayed at pH 3.0 as it would overlap with general proteolytic activity. [e] na = no activity. [f] General proteolytic activity was assayed at the initial pH of silage; optimum pH was not determined.

cathepsin C under the assay conditions for the enzyme, at both its optimum pH and the ensilation pH. Cathepsin C also has a fairly low optimum pH of 5.0 (McDonald et al., 1966; Hopsu-Havu et al., 1977). Leu-NA hydrolytic activity was observed in silage A both at the optimum pH for aminopeptidase and at the ensilation pH. However, aminopeptidases from rainbow trout and other fish are reported to be inactive below pH 4.0 (Bouck et al., 1975; VoVan et al., 1983); indicating the possible involvement of other exo-peptidases in the hydrolysis of this substrate.

Proteinase activity in the low-pH silage, silage B (ensiled at pH 2.0), was distinctly different from that of silage A. Proteolytic activities were observed only against Azocoll (0.01 pepsin equiv/mg of protein at pH 1.7) and Leu-NA (specific activity 0.49 at pH 7.0). The latter substrate was, however, not hydrolyzed at the ensilation pH. Thus, proteolytic activity against all other substrates appeared to have been inactivated, which was reflected in a much lower general proteolytic activity compared to silage A (0.01 absorbance unit/mg of protein per 50 min at pH 2.0). Hence, proteolytic activity in silage B would appear to be mostly due to pepsin-type enzymes.

The stable proteolytic activity in silage A toward several proteinase substrates is of great advantage when the proteinases responsible are sought to be recovered/isolated. The feasibility of such recovery has been demonstrated with cod, mackerel, and salmon silages (Reece, 1988). The low-pH silage B differed from silage A, principally in the lower proteolytic activity and the absence of any exo-peptidase activity at the ensilation pH. The effect of these differing proteinase complements was clearly demonstrated when autolysates from the two silages were analyzed.

Time Course of Autolysis in Silages. The liquefaction of silages as monitored by the increase in the volume of centrifugable aqueous phase (Figure 1) was fastest in silage A, which was also liquefied to a greater extent than silages B and C. The amounts of undigested fractions in the latter silages, correspondingly, were higher. Initial rates of liquefaction for all silages were comparable to cod viscera silages reported earlier (Raa and Gildberg, 1976; Gildberg and Raa, 1977), but the final extent of liquefaction was lower, which could be because of the inclusion of both the lipid and aqueous layers in calculating the liquefaction in earlier experiments.

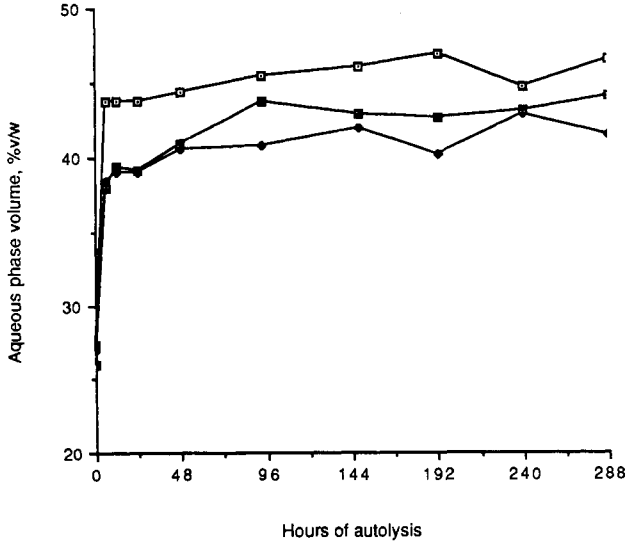

Figure 1. Course of liquefaction of different silages. Silages A (□), B (■), and C (●) were centrifuged and the volumes of aqueous layers expressed as a percent of the total weight of silage.

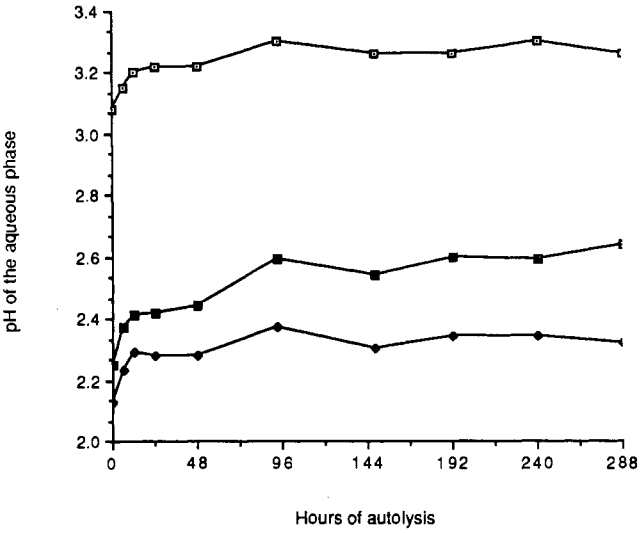

Figure 2. Changes in the pH of aqueous phases of silages during the course of autolysis. Aqueous phases obtained by the centrifugation of silages A (□), B (■), and C (●) were separated and their pH values measured.

The pH values of aqueous phases from all the silages increased during autolysis, but those of silages A and C, containing formic acid, increased by a lesser extent than silage B, ensiled with sulfuric acid only (Figure 2). The rise in pH during autolysis of fish silage has been commonly observed (Rattagol et al., 1979; Beddows et al., 1976), as also the buffering effect of formic acid against this increase (Reece, 1980).

The initial rate as well as the final extent of autolysis as measured by the degree of hydrolysis (DH = NPN/TN %) was highest in the case of silage A, followed by silages B and C, respectively (Figures 3–5). This confirmed the observations made using liquefaction as an autolysis index. After 48 h, autolysis was quite slow in all silages and additional proteolysis was limited, especially in silage C. The pattern of autolysis in silage A was similar to those of higher pH silages made with tem-

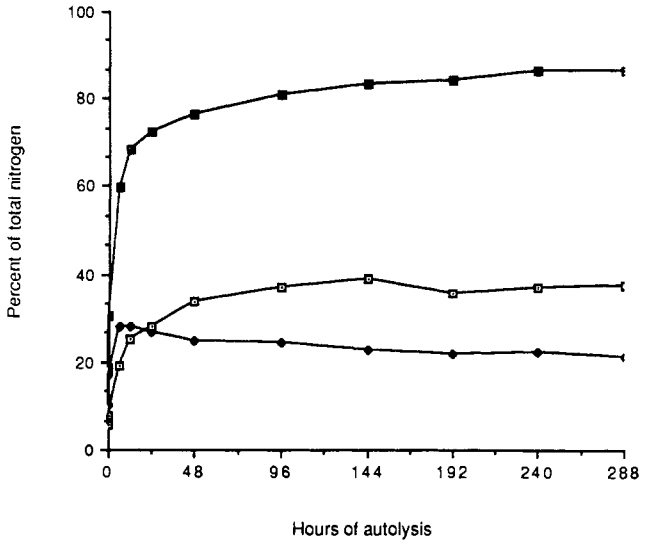

Figure 3. Changes in the nonprotein nitrogen (NPN) and its subfractions during the autolysis of silage A, ensiled with 3% formic acid at an initial pH of 3.0. Trichloroacetic acid solubles of the silage were analyzed for NPN (■), peptide nitrogen (●), and α-amino nitrogen (⊡) and expressed as percents of the total nitrogen in silage.

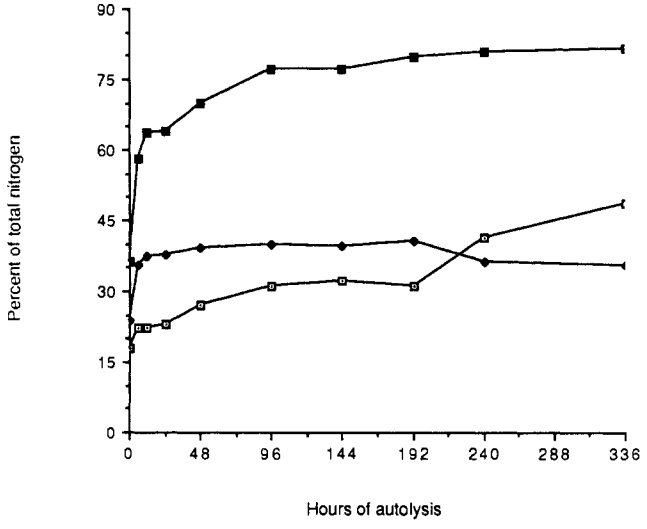

Figure 4. Changes in the nonprotein nitrogen (NPN) and its subfractions during the autolysis of silage B, ensiled with sulfuric acid at an initial pH of 2.0. Trichloroacetic acid solubles of the silage were analyzed for NPN (■), peptide nitrogen (●), and α-amino nitrogen (⊡) and expressed as percents of the total nitrogen in silage.

perate water fishes (Freeman and Hoogland, 1956; Tatterson and Windsor, 1974; Backhoff, 1976; Gildberg and Raa, 1977), except where autolysis was slowed by low ensilation temperatures (Backhoff, 1976; Raa and Gildberg, 1976) or by tropical fish resistant to autolysis (Hall et al., 1985).

The changes in the peptide and α-amino nitrogen (α-NH_2 N) fractions of silage A were highly substantive of the type of proteinase activity in this silage (Figure 3). An initial increase in peptide N was quickly followed by its continuous decline, while α-NH_2 N increased with the progress of autolysis. In light of the high activities against endo- and exo-peptidase substrates in silage A, these changes can be explained as an initial generation of oligopeptides by endo-peptidase action, which were further broken down to short peptides and free amino acids by the exo-peptidases. Similar increases in dialyzable or free amino nitrogen with concomitant decreases in oligopeptide or peptide fractions have been noticed in

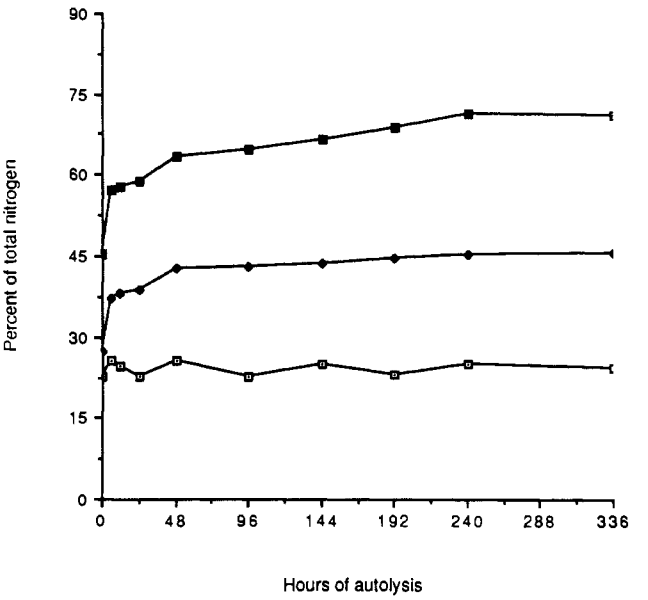

Figure 5. Changes in the nonprotein nitrogen (NPN) and its subfractions during the autolysis of silage C, ensiled with 3% formic acid and adjusted to pH 2.0 with sulfuric acid. Trichloroacetic acid solubles of the silage were analyzed for NPN (■), peptide nitrogen (●), and α-amino nitrogen (⊡) and expressed as percents of the total nitrogen in silage.

fish silages ensiled at pH 3–4 (Haard et al., 1985; Stone and Hardy, 1986). In contrast, peptide N in silage B reached higher levels despite its lower DH and declined after 192–240 h of ensilation (Figure 4). The increase in α-NH_2 N was gradual, exceeding the peptide N levels after 240 h. These changes can be explained in light of the hydrolytic activity observed in silage B against Leu-NA at the optimum pH for aminopeptidase activity (pH 7). Though the hydrolysis of this substrate was not detected at the ensilation pH (pH 2) within the relatively short time of the assay, the stable Leu-NA hydrolytic activity could be responsible for the gradual increase in α-NH_2 N over the autolysis period of 14 days.

Silage C was the least autolyzed among the three silages but had the highest peptide N levels even after 14 days (336 h) of ensilation (Figure 5). The α-NH_2 N levels remained virtually constant throughout the ensilation period, indicating complete suppression of exopeptidase activity. Since both silages B and C were initially ensiled at pH 2.0, maintenance of ensilation pH close to 2.0 in silage C would appear to be responsible for this suppression. In "acid-stabilized" silages of Pacific whiting, Stone and Hardy (1986) reported no increases in α-NH_2 N even after 42 days of storage but little proteolysis occurred in these silages (~45% DH in 42 days vs 71% DH in 14 days with silage C).

The size distribution of peptides in aqueous phases of the three silages showed that the peptides were roughly distributed in three size ranges (Figure 6): a high molecular weight (MW) fraction of 1300–>3500, an intermediate fraction of 500–1300, and a low-MW fraction of <500. Peptide fractions of high and intermediate MW were most abundant in silage C followed by silage B and silage A. The latter silage had considerable quantity of low-MW fractions unlike the other two silages. These results reconfirmed the near-absence of exo-peptidase activity in silages B and C, which would have produced low-MW fractions, as in the case of silage A. Greater amounts of low-MW peptides (<700) were found in pH 4.0 than in pH 2.0 silages by Reece (1980).

These experiments demonstrate the influence of the pH of ensilation on the proteinase complement in fish

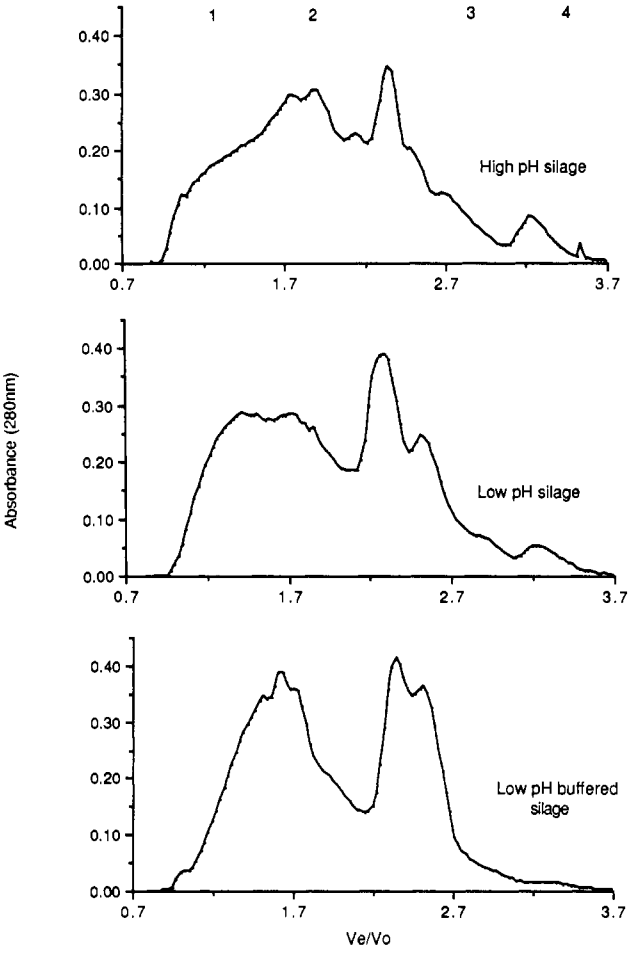

Figure 6. Gel filtration chromatography of the aqueous phases of silages A–C: sample size, 0.5 mL; fraction size, 2 mL; V_e, elution volume; V_0, void volume. Molecular weight standards: 1, glucagon (3550); 2, vitamin B_{12} (1570); 3, oxytetracycline hydrochloride (500); 4, tryptophan (200).

silage and the nature of autolytic products. At pH 3.0, both exo- and endo-peptidases, from digestive organs as well as tissues, were active in the autolysis of rainbow trout viscera silage. Lowering the pH of ensilation to 2.0 limited the proteolytic activity to pepsin-type proteinases and reduced the formation of short peptides and amino acids. This formation was completely suppressed when the rise of pH during autolysis was minimized by the presence of 3% formic acid in pH 2.0 silage.

ABBREVIATIONS USED

TCA, trichloroacetic acid; TN, total nitrogen; NPN, nonprotein nitrogen; α-NH_2 N, α-amino nitrogen; DH, degree of hydrolysis (NPN/TN %); peptide N, peptide nitrogen; BTEE, benzoyl-L-tyrosine ethyl ester; BAP-NA, *N*-benzoyl-DL-arginine *p*-nitroanilide; $Suc(Ala)_3$-NA, *N*-succinylalanylalanylalanine *p*-nitroanilide; Hip-Phe, hippuryl-L-phenylalanine; Hip-Arg, hippuryl-L-arginine; Leu–NA, leucine *p*-nitroanilide; Gly-Phe–NA, glycyl-L-phenylalanine *p*-nitroanilide.

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Registry No. Proteinase, 9001-92-7; pepsin, 9001-75-6; chymotrypsin, 9004-07-3; elastase, 9004-06-2; carboxypeptidase A, 11075-17-5; carboxypeptidase B, 9025-24-5; aminopeptidase, 9031-94-1; cathepsin B, 9047-22-7; cathepsin C, 9032-68-2; trypsin, 9002-07-7; formic acid, 64-18-6.

LITERATURE CITED

AACC. *Approved Methods of AACC*; American Association of Cereal Chemists: Washington, DC, 1976.

Backhoff, H. P. Some chemical changes in fish silage. *J. Food Technol.* **1976**, *11*, 353–363.

Barrett, A. J. Classification and nomenclature of proteinases. In *Proteinases in mammalian cells and tissues*; Barrett, A. J., Ed.; North-Holland: Amsterdam, 1977.

Barrett, A. J.; Kirschke, H. Cathepsin B, Cathepsin H and Cathepsin L. *Methods Enzymol.* **1981**, *80*.

Beddows, C. G.; Ismail, M.; Steinkraus, K. H. The use of bromelain in the hydrolysis of mackerel and the investigation of fermented fish aroma. *J. Food Technol.* **1976**, *11*, 379–388.

Bouck, G. R.; Schneider, P. W., Jr.; Jacobson, J.; Ball, R. C. Characterization and subcellular localization of leucine aminonaphthylamidase in rainbow trout. *J. Fish. Res. Board Can.* **1975**, *32*, 1289–1295.

Clark, J.; MacDonald, N. L.; Stark, J. R. Metabolism in marine flat fish-III Measurement of elastase activity in the digestive tract of dover sole (*Solea solea*). *Comp. Biochem. Physiol.* **1985**, *81B*, 695–700.

Cohen, T.; Gertler, A.; Birk, Y. Pancreatic proteolytic enzymes from carp Cyprinus carpio - I: Purification and physical properties of trypsin, chymotrypsin, elastase and carboxy- peptidase B. *Comp. Biochem. Physiol.* **1981**, *69B*, 639–646.

Croston, C. B. Tryptic enzymes of chinook salmon. *Arch. Biochem. Biophys.* **1960**, *89*, 202–206.

Dawson, R. M. C., Elliott, W. H., Jones, K. M., Eds. *Data for biochemical research*; Clarendon Press: Oxford, 1986.

Dean, D. D.; Domnas, A. J. The extracellular proteolytic enzymes of the mosquito-parasitizing fungus *Lagenidium giganteum*. *Exp. Mycol.* **1983**, *7*, 31–39.

Folk, J. E.; Schirmer, E. W. The porcine pancreatic carboxypeptidase A system - I. *J. Biol. Chem.* **1963**, *238*, 3884–3894.

Folk, J. E.; Piez, K. A.; Carroll, W. R.; Gladner, J. A. Carboxypeptidase B. IV. Purification and characterization of the porcine enzyme. *J. Biol. Chem.* **1960**, *235*, 2272–2277.

Freeman, H. C.; Hoogland, P. L. Processing of cod and haddock viscera, Laboratory experiments. *J. Fish. Res. Board Can.* **1956**, *13*, 869–877.

Gildberg, A.; Raa, J. Properties of a propionic/formic acid preserved silage of cod viscera. *J. Sci. Food Agric.* **1977**, *28*, 647–653.

Gornall, A. G.; Bardawill, C. J.; David, M. M. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **1949**, *177*, 751–766.

Haard, N. F.; Kariel, N.; Herzberg, G.; Feltham, L. A. W.; Winter, K. Stabilization of protein and oil in fish silage for use as a ruminant feed supplement. *J. Sci. Food Agric.* **1985**, *36*, 229–241.

Hall, G. M.; Keeble, D.; Ledward, D. A.; Lawrie, R. A. Silage from tropical fish I. Proteolysis. *J. Food Technol.* **1985**, *20*, 561–572.

Hjelmeland, K.; Raa, J. Characteristics of two trypsin type isozymes isolated from the artic fish capelin. *Comp. Biochem. Physiol.* **1982**, *71B*, 557–562.

Hopsu-Havu, V. K.; Fraki, I. E.; Jarvinen, M. Proteolytic enzymes of skin. In *Proteinases in mammalian cells and tissues*; Barrett, A. J., Ed.; North-Holland: Amsterdam, 1977.

Jany, K. D. Studies on the digestive enzymes of the stomachless bone fish *Carassius auratus* Gibelo. *Comp. Biochem. Physiol.* **1976**, *53B*, 31–38.

Kasafirek, E.; Fric, P.; Slaby, J.; Malis, F. p-Nitroanilides of 3-carboxyprionyl peptides. *Eur. J. Biochem.* **1976**, *69*, 1–13.

McDonald, J. K.; Reilly, T. J.; Zeitmann, B. B.; Ellis, S. Cathepsin C: A chloride requiring enzyme. *Biochem. Biophys. Res. Commun.* **1966**, *24*, 771–775.

Noda, M.; Murakami, K. Studies on proteinases from the digestive organs of sardine II. Purification of acid proteinases from the stomach. *Biochem. Biophys. Acta* **1981**, *658*, 27–34.

Otto, K.; RiesenKonig, H. Improved purification of cathepsin B 1 and cathepsin B 2. *Biochem. Biophys. Acta* **1975**, *379*, 462–475.

Overnell, J. Digestive enzymes of the pyloric caeca and of their associated mesentry in the cod. *Comp. Biochem. Physiol.* **1973**, *46B*, 519-531.
Petra, P. H. Bovine procarboxypeptidase and carboxypepti- dase A. *Methods Enzymol.* **1970**, *19*.
Pfleiderer, G. Particle-bound aminopeptidase from pig kidney. *Methods Enzymol.* **1970**, *19*.
Prahl, J. W.; Neurath, H. Pancreatic enzymes of the spiny dogfish I. Cationic chymotrypsinogen and chymotrypsin. *Biochemistry* **1966**, *5*, 2131-2145.
Raa, J.; Gildberg, A. Autolysis and proteolytic activity of cod viscera. *J. Food Technol.* **1976**, *11*, 619-628.
Raa, J.; Gildberg, A.; Strom, T. Silage production-Theory and practice. In *Upgrading wastes for feeds and food*; Ledward, D. A., Taylor, A. J., Lawrie, R. A., Eds.; Butterworths: London, 1983.
Rattagoal, P. P.; Wongchinda, N.; Swachathamwongratana, S. Studies on the nutritive value of fish silage for broiler chickens. In *Proceedings of the IPFC Workshop on fish silage production and its use*; FAO: Jakarta, Indonesia, 1979; pp 48-54.
Reece, P. Control and reduction of free fatty acid concentration in oil recovered from fish silage prepared from sprat. *J. Sci. Food Agric.* **1980**, *31*, 147-155.
Reece, P. Recovery of proteases from fish waste. *Process Biochem.* **1988**, *23*, 62-66.
Rosen, H. A modified ninhydrin colorimetric analysis for aminoacids. *Arch. Biochem. Biophys.* **1957**, *671*, 10-15.
Sakai-Suzuki, J.; Tobe, M.; Tsuchiya, T.; Matsumoto, J. J. Purification and characterization of acid cysteine proteinase from squid mantle muscle. *Comp. Biochem. Physiol.* **1986**, *85B*, 887-893.
Shin, D. H.; Zall, R. R. Purification and identification of a trypsin-like enzyme from the pyloric caeca of cod. *Process Biochem.* **1986**, *21*, 11-15.
Stone, F. S.; Hardy, R. W. Nutritional value of acid stabilized silage and liquefied fish protein. *J. Sci. Food Agric.* **1986**, *37*, 797-803.
Stone, F. S.; Hardy, R. W.; Spineldin, J. Autolysis of phytic acid and protein in Canola meal, wheat bran and fish silage blends. *J. Sci. Food Agric.* **1984**, *35*, 513-519.
Tarky, W.; Agarwala, G. P.; Pigot, G. M. Protein hydrolyzate from fish waste. *J. Food Sci.* **1973**, *38*, 917-918.
Tatterson, I. N.; Windsor, M. L. Fish silage. *J. Sci. Food Agric.* **1974**, *25*, 369-379.
Twining, S. S.; Alexander, P. A.; Huibregtse, K.; Glick, D. A pepsinogen from rainbow trout. *Comp. Biochem. Physiol.* **1983**, *75B*, 109-112.
VoVan, T.; Kusakabe, I.; Murakami, K. Properties of two aminopeptidases from sardines. *Agric. Biol. Chem.* **1983**, *47*, 2453-2454.
Walsh, K. A.; Wilcox, P. E. Serine proteases. *Methods Enzymol.* **1970**, *19*.
Wilcox, P. E. Chymotrypsinogens-Chymotrypsins. *Methods Enzymol.* **1970**, *19*.

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Interaction of β-Lactoglobulin with κ-Casein in Micelles As Assessed by Chymosin Hydrolysis: Effect of Temperature, Heating Time, β-Lactoglobulin Concentration, and pH

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Heat-induced interaction of β-lactoglobulin (β-Lg) with κ-casein (κ-C) on intact micelles was studied by following the kinetics of chymosin hydrolysis. The rate of enzymic reaction was determined on the basis of the release of glycomacropeptide (GMP) soluble in 8% TCA. Heating of β-Lg with κ-C and with casein micelles in Jenness-Koops buffer at pH 6.8 inhibited chymosin hydrolysis, resulting in decreased initial velocity (V_i) and GMP release. Heating casein micelles alone did not affect chymosin hydrolysis. V_i and the release of GMP decreased steadily with increasing temperature of heating from 60 °C and reached a maximum at 85 °C. The decreases in V_i and GMP release at 85 °C were 37 and 26%, respectively. Inhibition reached a maximum after heating for 10 min at 85 °C, with 38% decrease in V_i and 28% decrease in GMP. A heating time of 1 min at 85 °C caused a 9% decrease in V_i and 15% decrease in GMP release. Decrease in V_i and the release of GMP was proportional to the concentration of β-Lg (0.05–0.50%) added to casein micelles. Heating of casein micelles and β-Lg at pH 6.0 or 7.5 did not improve chymosin hydrolysis, whereas lowering to pH 5.8 and readjusting to pH 6.8 before chymosin addition improved chymosin hydrolysis.

The heat-induced interaction of β-lactoglobulin (β-Lg) and κ-casein (κ-C) has been extensively studied (Sawyer, 1969; Smits and van Brouwershaven, 1980; Euber and Brunner, 1982; Doi et al., 1981, 1983; Haque et al., 1987; Haque and Kinsella, 1988). Using ^{3}H-labeled β-Lg, Smits and van Brouwershaven (1980) presented evidence of association of casein micelles and β-Lg. The existence of a β-Lg and κ-C complex in heated milk has been reported (Snoeren and van der Spek, 1977; Elfagm and Whee-

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