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# Heat-Induced Changes in Sulfhydryl Groups and Disulfide Bonds in Fish Protein and Their Effect on Protein and Amino Acid Digestibility in Rainbow Trout (*Salmo gairdneri*)

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Ground fillets of frozen Alaska pollock (*Theragra chalcogramma*), a low-fat fish, and Pacific mackerel (*Pneumataphorus japanicus*), a high-fat fish, heated for 20 min at temperature intervals ranging from 40 to 115 °C showed a linear decrease in the content of –SH (sulfhydryl) groups and a concomitant increase in the content of S–S (disulfide) bonds from 50 to 115 °C. At 95 °C, the reaction was rapid and had reached an equilibrium after 20 min. These experiments indicate that temperatures higher than 50 °C are required for oxidative transformation of –SH groups to S–S bonds. Heating at 115 °C caused a loss in cystine plus cysteine. Heating to 95 °C and drum-drying caused the formation of S–S bonds from –SH groups and reduced protein and amino acid digestibility when fed to rainbow trout (*Salmo gairdneri*) as compared with the raw fish protein. Freeze-drying did not affect digestibility and no formation of S–S bonds was found. It is postulated that heat-induced S–S cross-linking from –SH oxidation causes a reduction in protein and amino acid digestibility in drum-dried samples. The effect of heating on digestibility was greater in the low-fat pollock than in the high-fat mackerel.

Sulfhydryl groups and disulfide bonds are important in maintaining structure and functions of native proteins (Saxena and Wetlaufer, 1970) and play important roles in functional properties of proteinaceous foods (Connell, 1964; Wolf, 1970). Disulfide cross-linking in proteins may occur from oxidation of sulfhydryl groups and sulfhydryl–disulfide interchange (Cecil and McPhee, 1959; Friedman, 1973). This has been demonstrated by heating fish (Itoh et al., 1979a,b; Itoh et al., 1980), meat (Hamm and Hofmann, 1965), soybean (Wolf, 1970), and lactalbumin (Schnack and Klostermeyer, 1980) and in frozen storage of fish (Buttkus, 1970; Tsuchiya et al., 1979). Heating that affected the status of cysteine/cystine residues was found to reduce protein utilization by animals (Rios Iriarte and Barnes, 1966; Dvorschak, 1970; Waibel et al., 1977), and

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it has been assumed (Belikov et al., 1981; Friedman et al., 1982) that disulfide cross-linking hampers the attack by proteolytic enzymes, which decreases protein digestibility. This theory is consistent with the finding that cleavage of disulfide bonds increases in vitro protein digestibility (Boonvisut and Whitaker, 1976), but there has been no substantiation in vivo.

The importance of sulfhydryl groups and disulfide bonds on the nutritional quality of proteins is generally acknowledged, and fish protein is known to be easily damaged during handling and processing. In spite of these facts, studies on changes of sulfhydryl groups and disulfide bonds in fish protein and their impact on its quality are limited. This paper presents data on the effect of heating and drying on the status of the sulfhydryl groups and disulfide bonds in fish protein and correlates these findings with in vivo digestibility of the protein. Because hydroperoxides from oxidizing polylenoic fatty acids may cause oxidation of sulfur-containing amino acids (El-Zeany et al., 1975; Finley and Lundin, 1979), fish with a low content of fat (pollock, <1.0% fat) was compared with fish with a high content of fat (mackerel, >5% fat).

## MATERIALS AND METHODS

**Fish and Sample Preparation.** Gutted and headed Alaska pollock (*Theragra chalcogramma*) and whole Pacific mackerel (*Pneumataphorus japanicus*), frozen and stored at -30 °C, were thawed overnight at 3 °C, filleted, skinned, and ground twice through a plate with 5-mm holes before being divided into portions for further treatment. Freeze-dried and drum-dried samples of pollock used in the digestibility trial were from a separate lot of fish, gutted and headed before freezing, and thus were not directly comparable with the other pollock samples.

**Sample Treatment.** Samples of 100 g of ground fish in polyethylene bags with free access to air were heated in a water bath for 20 min at 40, 50, 60, 70, and 80 °C for 0, 5, 10, 20, 40, and 60 min at 95 °C. Further samples were heated in a retort at 115 °C in sealed $^1/_2$-lb (307 × 200) C-enameled cans for 20 min after the internal temperature of 115 °C was reached.

Samples used in the digestibility trial were heated in open polyethylene bags containing 1000 g of ground fish and placed in a water bath with a 95 °C temperature for 20 min after the internal temperature in the fish had reached 95 °C. Other samples used in the digestibility trial were freeze-dried over 48 h with a plate temperature of 40 °C or drum-dried on a double-drum dryer using steam pressure of 4.92 kg/cm$^2$ (145 °C) with a revolution rate of 1 per minute. After drying, the samples were ground in a ball mill. Before freeze-drying and after drum-drying, 250 mg of TBHQ (*tert*-butylhydroquinone, Eastman Chemical Products, Kingsport, TN) and 100 mg of citric acid were added per 1000 g of solids.

**Chemical Analysis.** Protein (N × 6.25) in fish was determined by the conventional Kjeldahl method and in feeds and excrements in the digestibility study by a micro-Kjeldahl method as described by AOAC (1980).

Sulfhydryl (-SH) groups were determined by a modification of the method described by Sedlak and Lindsay (1968). A sample containing about 30 mg of protein was dissolved in 8.0 mL of 0.2 M Tris buffer (pH 8.2)-0.02 M disodium EDTA solution (wet samples) or in 8.0 mL of 0.2 M Tris buffer (pH 8.2)-0.02 M $Na_2$EDTA -2% SDS (sodium dodecyl sulfate) solution (dry samples). After standing for 2 h, 0.5 mL of 0.016 M DTNB [5,5′-dithiobis(2-nitrobenzoic acid)] and 31.5 mL of absolute methanol were added with mixing. The solution was allowed to stand in covered tubes at room temperature for 15 min, centrifuged at 3000*g* for 15 min, and filtered through a Whatman filter paper No. 4, and the absorbency was read at 415 nm.

Disulfide (S-S) bonds reduced with sodium borohydride ($NaBH_4$) were determined as -SH groups along with the native -SH groups. The previously determined native -SH groups were then subtracted to give the number of disulfide bonds. A sample containing about 35 mg of protein was added to 4.0 mL of 0.6 M $NaBH_4$ in 8 M urea with 2 drops of octyl alcohol (to avoid foaming) and allowed to stand for 2 h (dry samples 3 h). Remaining $NaBH_4$ was destroyed by the addition of 1.1 mL of 2 N HCl and the pH of the solution brought to 8.2 by the addition of 0.9 mL of 2 N NaOH. To an aliquot of 0.5 mL 1.5 mL of 0.2 M Tris buffer (pH 8.2)-0.02 M $Na_2$EDTA solution, 0.2 mL of 0.005 M DTNB, and 7.8 mL of absolute methanol were added. The solution was mixed and allowed to stand in covered tubes at room temperature for 15 min, centrifuged at 3000*g* for 15 min, and filtered through a Whatman No. 4 filter paper before reading the absorbency at 412 nm. DBC (dye-binding capacity) was determined as described by Jacobsen et al. (1972).

**Chromium Determination.** The determination of chromium used in feeds and excrements was modified from the method of Furakawa and Tsukahara (1966) in the following manner. Samples containing 1-5 mg of $Cr_2O_3$ were added to Erlenmeyer flasks containing 5 mL of sulfuric acid and 5 mL of nitric acid. These solutions were boiled gently until all of the organic material was digested. The flasks were cooled to room temperature, and 0.5 mL of 2% sodium molybdate ($Na_2MoO_4$) and 3 mL of perchloric acid ($HClO_4$) were added. The flasks were reboiled until the solutions changed to yellow or orange. Boiling was continued for 3 min to stabilize the color. The solutions were cooled and diluted to an appropriate chromium concentration (1-20 ppm) in a volumetric flask with deionized distilled water. The chromium concentration was measured directly with an inductively coupled argon emission spectrophotometer (Model 975, Plasma AtomComp, Jarrell-Ash Division, Fisher Scientific Co.).

**Amino Acids.** Amino acid analyses were done on a Beckman 118CL amino acid analyzer. The dried samples of diet and feces were extracted overnight on a goldfisch extractor with hexane (bp 68-69 °C). Extracted samples were ground to a powder, and an aliquot was weighed into an ignition tube for hydrolysis. The samples were hydrolyzed in 6 N HCl for 24 h in a Kontes heating block at 110 °C according to the method of Spackman et al. (1958). The oxidation of cystine/cysteine to cysteic acid was done by adding 50 μL of dimethyl sulfoxide ($Me_2SO$) according to the method of Spencer and Wold (1969) and Williams et al. (1979).

**Digestibility Study.** The composition of the diets used in the digestibility study is shown in Table I. The different fish proteins were the sole source of protein in the diets and were included to provide about 29 g of protein/100 g of DM (dry matter). Herring oil was added to the diets made from pollock in order to make the diets similar in lipid content. Water was added to the diets made from dry fish protein to faciltate pelleting. Chromium oxide was added to the diets at a level of about 1%. After mixing, the diets were ground through a plate with 4-mm holes, placed in thin layers on plates, frozen at -30 °C, and stored frozen until fed. Rainbow trout (average weight 278 g) were randomly placed into eight tanks of 20 fish each. One diet was fed to each tank for 3 weeks at a level of 1.7 g of DM in feed per 100 g of fish body weight. Collection of excrements was done the last 2 days of each week from

**Table I. Composition of Diets in Percent of Dry Matter (DM)**[a]

| | |
|---|---|
| fish (pollock and mackerel) | 30–40 |
| fish oil | 0–8.5 |
| dextrin | 30 |
| α-cellulose | 12 |
| guar gum | 4.5 |
| pregelatinized wheat starch | 4.5 |
| carboxymethylcellulose | 2.2 |
| mineral mixture[b] | 2.2 |
| vitamin mixture[c] | 4.5 |
| chromic oxide | 1 |

[a]DM of diet 1 (raw mackerel) was 51.0%, diet 2 (mackerel heated to 95 °C) 53.9%, diet 3 (raw pollock) 43.2%, diet 4 (pollock heated to 95 °C) 43.2%, diet 5 (freeze-dried mackerel) 52.5%, diet 6 (drum-dried mackerel) 52.4%, diet 7 (freeze-dried pollock) 52.4%, and diet 8 (drum-dried pollock) 52.4%. [b]Mineral mixture provided in grams per kilogram of DM in diets: Ca, 5.1; P, 7.6; Mg, 1.1; Na, 2.5; K, 8.6; Cl, 2.1; S, 1.4; Fe, 0.4. [c]Vitamin mixture provided per kilogram of DM in the diets: α-tocopheryl acetate, 1500 IU; vitamin $B_{12}$, 0.18 mg; riboflavin, 159 mg; niacin, 565 mg; *d*-pantothenic acid, 317 mg; menadione bisulfite complex, 54 mg; folic acid, 38 mg; pyroxidine hydrochloride, 64 mg; thiamin mononitrate, 77 mg; biotin, 2 mg; inositol, 794 mg; ascorbic acid, 2.7 g.

anesthetized (Tricaine, Argent Chemical Laboratories, Redmond, WA 98052) fish by stripping from the dorsal fins to the anus. Excrements from each tank were pooled for each week and stored frozen (−10 °C) pending analysis. Determinations on protein and chromium oxide were done on samples dried in a draft oven overnight at 105 °C. Lyophilized samples were used for amino acid analysis.

The study was conducted according to a factorial design with type of fish (pollock and mackerel) as one factor and treatment of fish protein (raw, heated, freeze-dried, and drum-dried) as the second factor. Analysis of variance was conducted on the digestibility values and orthogonal comparisons were made to determine the significance of main effects and interactive effects (Snedecor and Cochran, 1974).

## RESULTS

**Contents of Sulfhydryl Groups and Disulfide Bonds.** The standard curve for the determination of –SH groups was done with GSH (reduced glutathione). The color of the solution was relatively stable over 4 h (OD decreasing by 0.02 unit/h). The accuracy of the determination given as the deviations between duplicates of 90 determinations was 3.2 ± 2.6%. Adding 2% SDS to the Tris buffer increased the measurable –SH groups in unheated samples by 8–10% but had negligible effects on samples heated to 95 °C. The addition of 2% SDS also increased the content of –SH groups in dry samples by about 60% compared to Tris buffer alone. Recovery of –SH groups from cystine and GSH, alone and together with fish, was nearly 100%.

The physical nature of the samples had an effect on the accuracy of the determination of the disulfide bonds (S–S) since all of them had to be reduced by the borate to –SH groups for the chemical analysis. One hour was sufficient for wet pollock samples while wet mackerel samples needed 2 h and dry samples 4 h. The accuracy of the determination of –SH groups plus S–S bonds judged by the deviations between duplicates of 90 determinations, was 3.5 ± 2.7%. Recovery of cysteine alone and in combination with fish was 100% (±7.9%). Analyses of bovine plasma albumin gave 55.9 mM –SH + S–S/2 per 16 g of N, which is close to the reported value of 52.3 mM/16 g of N (Tristram and Smith, 1963).

The effect of heating on the content of –SH groups and S–S bonds is shown in Table II and Figures 1 and 2. There were no significant differences between pollock and mackerel in the content of –SH groups and S–S bonds, and both fish responded to heat treatment in a similar fashion when heated for 20 min. No increase in S–S bonds occurred at temperatures up to 50 °C. However, from 50 to 115 °C for mackerel and from 50 to 95 °C for pollock, the content of S–S bonds increased linearly with temperature. Pollock showed no further increase in S–S bonds above 95 °C.

The content of –SH groups increased slightly up to 50 °C and decreased thereafter (for mackerel from 60 °C) in a linear fashion with increasing temperatures up to 115 °C.

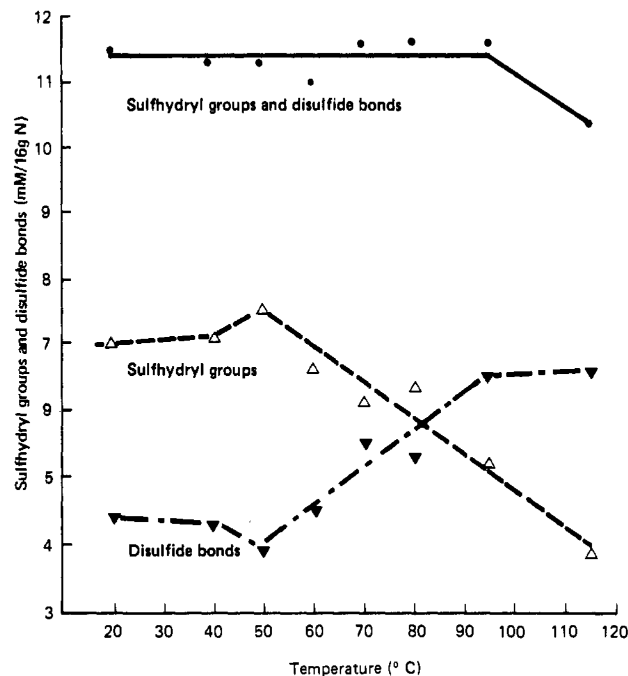


**Figure 1.** Content of sulfhydryl groups plus disulfide bonds/2 (●), sulfhydryl groups (△), and disulfide bonds/2 (▼) in pollock protein heated for 20 min to various temperatures.

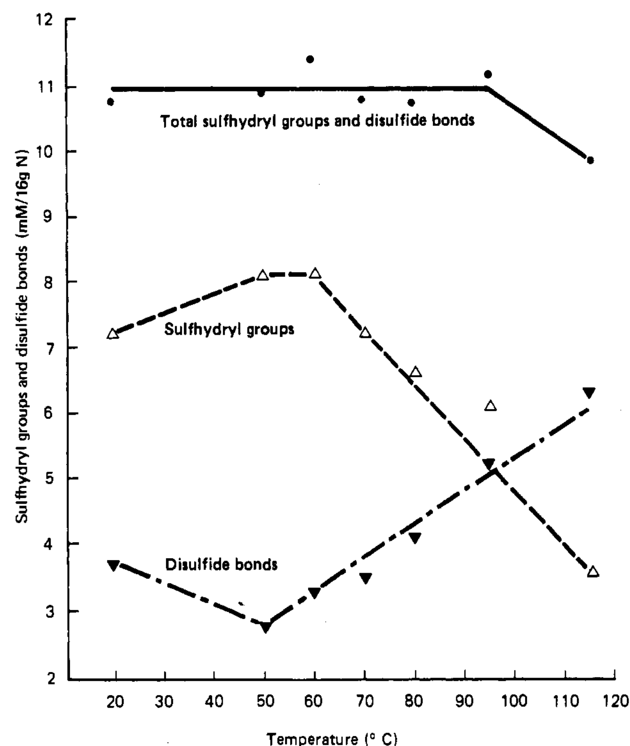


**Figure 2.** Content of sulfhydryl groups plus disulfide bonds/2 (●) sulfhydryl groups (△), and disulfide bonds/2 (▼) in mackerel protein heated for 20 min to various temperatures.

Table II. Effect of Heating on the Content[a] of -SH Groups and S-S Bonds in Pollock and Mackerel Proteins

| | temp, °C | pollock | | | | mackerel | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | $N^b$ | -SH + S-S/2 | -SH | S-S/2 | $N^b$ | -SH + S-S/2 | -SH | S-S/2 |
| untreated | | 12 | 11.5 ± 0.4 | 7.0 ± 0.5 | 4.4 ± 0.3 | 6 | 10.8 ± 0.3 | 7.2 ± 0.3 | 3.7 ± 0.4 |
| heated for 20 min | 40 | 3 | 11.3 ± 0.4 | 7.1 ± 0.3 | 4.3 ± 0.4 | | | | |
| | 50 | 3 | 11.3 ± 0.7 | 7.5 ± 0.1 | 3.9 ± 0.6 | 3 | 10.9 ± 0.4 | 8.1 ± 0.3 | 2.8 ± 0.2 |
| | 60 | 3 | 11.0 ± 0.2 | 6.6 ± 0.4 | 4.5 ± 0.5 | 3 | 11.4 ± 0.4 | 8.1 ± 0.1 | 3.3 ± 0.4 |
| | 70 | 3 | 11.6 ± 0.5 | 6.1 ± 0.2 | 5.5 ± 0.4 | 3 | 10.8 ± 0.5 | 7.2 ± 0.3 | 3.5 ± 0.7 |
| | 80 | 3 | 11.6 ± 0.5 | 6.3 ± 0.1 | 5.3 ± 0.5 | 3 | 10.8 ± 0.2 | 6.6 ± 0.3 | 4.1 ± 0.3 |
| | 95 | 3 | 11.6 ± 0.6 | 5.2 ± 0.5 | 6.5 ± 0.3 | 4 | 11.2 ± 0.4 | 6.1 ± 0.5 | 5.2 ± 0.4 |
| | 115 | 4 | 10.4 ± 0.3 | 3.9 ± 0.5 | 6.6 ± 0.2 | 2 | 9.9 | 3.6 | 6.3 |

[a] SH/16 g of N; average ± standard deviation. [b] $N$ = number of determinations.

Table III. Proximate and Amino Acid Composition and Content of Sulfhydryl Groups and Disulfide Bonds in Samples Used in the Digestibility Trial

| | mackerel | | | | pollock | | | |
|---|---|---|---|---|---|---|---|---|
| | raw | cooked (95 °C) | freeze-dried | drum-dried | raw | cooked (95 °C) | freeze-dried | drum-dried |
| moisture, % | 69.8 | 65.8 | 3.5 | 11.4 | 80.2 | 79.9 | 5.5 | 10.9 |
| in DM[a] | | | | | | | | |
| protein | 73.8 | 72.7 | 72.3 | 74.6 | 91.9 | 91.5 | 93.9 | 93.3 |
| lipids[b] | | | 25.0 | 22.6 | | | 4.2 | 4.0 |
| ash | | | 5.8 | 4.5 | | | 5.8 | 5.9 |
| water-soluble protein, g/16 g of N | | | 19.7 | 17.5 | | | 26.4 | 22.9 |
| amino acids,[c] g/16 g of N | | | | | | | | |
| Ala | 5.2 | 6.2 | 5.2 | 5.4 | 6.0 | 6.2 | 5.3 | 3.9 |
| Arg | 5.2 | 5.0 | 4.6 | 4.9 | 5.4 | 6.0 | 6.0 | 5.2 |
| Asp | 8.0 | 10.0 | 8.4 | 8.7 | 10.2 | 10.6 | 9.2 | 11.6 |
| Glu | 12.9 | 14.7 | 12.3 | 12.8 | 15.6 | 16.5 | 14.4 | 18.0 |
| Gly | 4.4 | 4.6 | 4.0 | 4.1 | 4.4 | 4.5 | 3.7 | 4.8 |
| His | 5.9 | 4.8 | 5.0 | 4.8 | 1.9 | 2.0 | 2.0 | 1.8 |
| Ile | 3.7 | 5.1 | 4.4 | 4.5 | 4.7 | 5.1 | 4.4 | 5.5 |
| Leu | 7.1 | 8.5 | 7.2 | 7.5 | 8.5 | 8.6 | 7.5 | 9.3 |
| Lys | 6.2 | 7.9 | 5.6 | 6.5 | 6.9 | 8.2 | 6.0 | 7.7 |
| Met | 1.8 | 2.8 | 1.8 | 2.0 | 2.3 | 2.3 | 2.0 | 2.5 |
| Cyst | 1.7 | 1.9 | 2.8 | 2.0 | 1.8 | 2.6 | 1.7 | 2.0 |
| Phe | 2.9 | 3.5 | 2.9 | 3.1 | 3.4 | 3.2 | 2.9 | 3.5 |
| Ser | 3.5 | 4.0 | 3.3 | 3.4 | 4.2 | 4.1 | 3.4 | 4.2 |
| Thr | 3.9 | 4.8 | 3.9 | 4.0 | 4.3 | 4.5 | 3.7 | 4.7 |
| Tyr | 3.2 | 3.1 | 2.8 | 3.0 | 3.2 | 3.1 | 3.4 | 3.1 |
| Val | 4.2 | 5.3 | 4.5 | 5.3 | 4.0 | 5.2 | 4.7 | 6.3 |
| DBC,[d] mM/16 g of N | | | 107 | 109 | | | 98 | 109 |
| -SH, mM/16 g of N | 6.6 | 5.7 | 6.5 | 3.5 | 6.2 | 5.2 | 8.0 | 6.1 |
| S-S/2, mM/16 g of N | 3.4 | 4.4 | 3.1 | 4.9 | 4.8 | 5.6 | 2.8 | 4.9 |
| (-SH + S-S)/2, mM/16 g of N | 10.0 | 10.1 | 9.6 | 8.4 | 11.0 | 10.8 | 10.8 | 11.0 |

[a] DM = dry matter. [b] Total lipids determined according to Bligh and Dyer (1959). [c] Determined after oxidation with $Me_2SO$ except for arginine, histidine, and tyrosine. [d] DBC = dye-binding capacity.

Temperature had no significant effect on the total content of -SH groups plus S-S bonds (cystine + cysteine/2) up to 95 °C, but from 95 to 115 °C there was a significant reduction ($P < 0.05$) in the content of -SH groups plus S-S bonds.

The effect of heating time at 95 °C on the content of -SH groups and S-S bonds is shown in Figure 3. Temperature readings within the samples of ground fish showed the core temperature increased linearly with time and reached 95 °C after 4 min. The reaction proceeded at a rapid rate and reached an equilibrium after 20 min.

**Apparent Digestibility of Protein and Amino Acids.** The proximate and amino acid composition of the samples used in the digestibility trial are shown in Table III together with the contents of -SH groups and S-S bonds. For mackerel, freeze-drying had no significant effect on the content of -SH groups and S-S bonds as compared with the raw sample, while drum-drying reduced the content of -SH groups by 3.1 mM/16 g of N, increased the content of S-S bonds/2 by 1.4 mM/16 g of N, and reduced the content of -SH groups plus S-S bonds/2 by 1.6 mM/16 g of N. Drum-dried pollock had 1.9 mM/16 g of N lower content of -SH groups, 2.1 mM higher content of S-S bonds/2, and about an equal content of -SH groups plus S-S bonds as freeze-dried. Apparent protein and amino acid digestibilities are shown in Table IV. Table V shows the orthogonal comparisons of treatment effects.

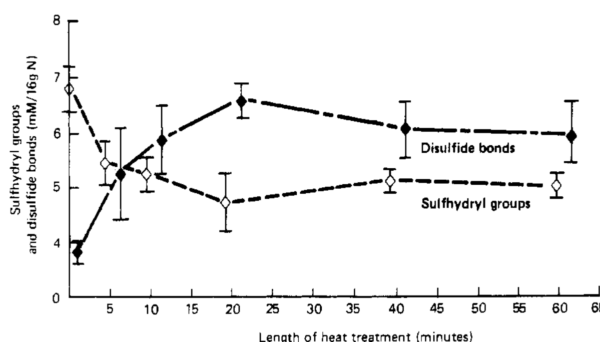


Figure 3. Effect of time of heat treatment at 95 °C of pollock protein on the content of sulfhydryl groups (◇) and disulfide bonds (◆). Bars show the standard deviation of three replicate determinations.

Table IV. Apparent Digestibility (Percent) of Protein and Amino Acids: Average and SEM[a] of Three Replicate Determinations

| | mackerel | | | | pollock | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | raw | cooked (95 °C) | freeze-dried | drum-dried | raw | cooked (95 °C) | freeze-dried | drum-dried | SEM |
| protein | 86.5 | 87.4 | 89.2 | 84.0 | 84.2 | 81.2 | 87.5 | 83.0 | 1.18 |
| Ala | 93.7 | 95.4 | 94.8 | 91.3 | 91.5 | 89.5 | 94.4 | 86.2 | 0.90 |
| Arg | | | 95.2 | 93.2 | | | 96.3 | 94.3 | |
| Asp | 86.0 | 83.2 | 89.9 | 85.9 | 83.0 | 78.6 | 88.8 | 86.3 | 1.11 |
| Glu | 89.8 | 92.5 | 93.8 | 89.4 | 88.9 | 87.0 | 92.4 | 89.9 | 0.96 |
| Gly | 87.6 | 85.9 | 88.3 | 84.9 | 82.2 | 81.0 | 87.2 | 85.2 | 1.09 |
| His[b] | | | 96.3 | 89.6 | | | 90.7 | 86.8 | |
| Ile[c] | 91.7 | 92.0 | 92.4 | 88.3 | 89.1 | 86.4 | 91.7 | 90.8 | 1.09 |
| Leu | 93.2 | 94.1 | 95.3 | 91.5 | 92.4 | 90.2 | 94.5 | 92.3 | 0.70 |
| Lys | 94.6 | 95.4 | 95.6 | 91.7 | 91.8 | 91.1 | 95.2 | 92.1 | 0.82 |
| Met[c] | 88.8 | 93.8 | 91.5 | 85.4 | 89.5 | 86.0 | 91.3 | 88.0 | 1.61 |
| Cys[c] | 63.1 | 56.5 | 76.7 | 60.4 | 57.7 | 60.6 | 72.2 | 67.2 | 3.70 |
| Met | | | | | | | | | |
| +Cys[c] | 76.6 | 79.9 | 84.6 | 73.0 | 75.5 | 72.6 | 83.4 | 72.7 | 3.50 |
| +Phe[b] | 91.6 | 92.1 | 95.9 | 88.0 | 92.5 | 87.2 | 93.4 | 91.3 | |
| +Ser | 86.6 | 87.8 | 88.7 | 86.3 | 86.1 | 83.8 | 89.5 | 86.5 | 0.93 |
| +Thr | 86.4 | 88.9 | 90.1 | 84.6 | 84.6 | 82.4 | 88.8 | 85.2 | 1.12 |
| +Tyr[b] | | | 92.1 | 86.3 | | | 92.4 | 88.4 | |
| +Val | 89.4 | 92.8 | 93.4 | 87.6 | 84.9 | 85.7 | 92.4 | 90.1 | 1.75 |

[a] Standard error of treatment means. [b] One determination per treatment. [c] Two replicate determinations per treatment.

Table V. Orthogonal Comparisons of Treatment Effects on Apparent Protein and Amino Acid Digestibility

| | main effects | | | | | | | | interactions | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | mackerel/pollock | | wet/dry samples | | raw/cooked (95 °C) samples | | freeze-dried/drum-dried samples | | wet/dry samples | | raw/cooked (95 °C) samples | | freeze-dried/drum-dried samples | |
| | diff | $P$[a] | diff | $P$ | diff | $P$ | diff | $P$ | diff | $P$ | diff | $P$ | diff | $P$ |
| protein | 2.8 | <0.01 | −1.1 | >0.2 | 1.1 | >0.2 | 4.9 | <0.001 | 1.5 | <0.2 | −1.9 | >0.2 | 0.3 | >0.2 |
| Ala | 3.4 | <0.001 | 0.8 | >0.2 | 0.1 | >0.2 | 5.9 | <0.001 | 0.6 | >0.2 | −0.2 | >0.2 | 2.4 | <0.05 |
| Arg[b] | −1.1 | | | | | | 2.0 | | | | | | | |
| Asp | 2.1 | <0.05 | −5.0 | <0.1 | 3.6 | <0.01 | 3.3 | <0.05 | 1.7 | <0.05 | −0.8 | >0.2 | 0.8 | >0.2 |
| Glu | 1.8 | <0.05 | −1.8 | <0.05 | 0.4 | >0.2 | 3.5 | <0.01 | 1.4 | <0.2 | −2.3 | <0.05 | 1.0 | >0.2 |
| Gly | 2.8 | <0.01 | −2.2 | <0.01 | 1.5 | >0.2 | 2.7 | <0.05 | 2.4 | <0.01 | 0.3 | >0.2 | 0.7 | >0.2 |
| His[b] | 4.2 | | | | | | 5.3 | | | | | | | |
| Ile | 1.6 | <0.2 | −1.0 | >0.2 | 1.2 | >0.2 | 2.6 | <0.2 | 1.7 | <0.2 | −1.0 | >0.2 | 1.1 | >0.2 |
| Leu | 1.2 | <0.2 | −0.9 | >0.2 | 0.7 | >0.2 | 3.1 | <0.001 | 1.2 | <0.05 | −1.6 | <0.05 | 0.8 | >0.2 |
| Phe[b] | 0.8 | | −1.3 | | 2.4 | | 5.0 | | | | | | | |
| Lys | 1.8 | <0.01 | −0.4 | >0.2 | 0.0 | | 3.5 | <0.01 | 1.8 | <0.01 | −0.8 | >0.2 | 0.4 | >0.2 |
| Met | 1.2 | >0.2 | 0.5 | >0.2 | −0.7 | >0.2 | 4.7 | <0.05 | 1.2 | <0.2 | −4.3 | >0.2 | 1.4 | >0.2 |
| Cys | −0.2 | >0.2 | −9.7 | <0.2 | 0.2 | >0.2 | 10.7 | <0.05 | 0.9 | >0.2 | 4.8 | >0.2 | 5.7 | <0.2 |
| Met + Cys | 2.4 | >0.2 | −2.3 | >0.2 | −0.2 | >0.2 | 11.1 | <0.05 | 1.7 | >0.2 | −3.1 | >0.2 | 0.5 | >0.2 |
| Phe[b] | 0.8 | | −1.3 | | 2.4 | | 5.0 | | | | | | | |
| Ser | 0.9 | >0.2 | −1.7 | <0.05 | 0.5 | <0.2 | 2.7 | <0.01 | 1.4 | <0.2 | −2.6 | <0.2 | −0.5 | >0.2 |
| Thr | 2.3 | <0.05 | −1.6 | <0.2 | −0.2 | >0.2 | 4.5 | <0.01 | 1.9 | <0.05 | −3.5 | <0.2 | 1.0 | >0.2 |
| Tyr[b] | −1.2 | | | | | | 4.9 | | | | | | | |
| Val | 2.5 | <0.2 | −2.7 | <0.2 | −1.5 | <0.2 | 4.1 | <0.2 | 3.2 | <0.05 | −1.3 | >0.2 | 1.8 | >0.2 |

[a] $P$ = probability of statistical significance. [b] One sample per treatment only.

Arginine, histidine, and tyrosine were determined (without the addition of DMSO) in fecal samples from fish fed the dried samples in week 3 only. The average digestibilities for protein and amino acids were from 1% to 4% higher in mackerel than in pollock, except for arginine, cystine, and tyrosine where it was higher in pollock than in mackerel. Dried samples had in general higher digestibilities than wet samples, due to the high digestibility of the freeze-dried samples.

Compared with the raw samples, samples heated to 95 °C had an average of 1.1% lower digestibility for protein and 0.1–2.4% lower digestibility for individual amino acids with the exception of methionine, threonine, and valine, which had the highest digestibility in the heated samples. Only the difference for aspartic acid was statistically significant ($P < 0.05$) between raw and cooked samples. Although interactions between type of fish (i.e., mackerel vs. pollock) and heating were statistically significant ($P < 0.05$) for glutamic acid and leucine only, heating apparently had different effects on the two types of fish. Thus, heating reduced the digestibility of protein and amino acids in pollock but had negligible effects in mackerel.

Drum-dried samples had 4.9% lower digestibility of protein and from 2.0% to 11.1% lower digestibility for the individual amino acids compared with freeze-dried samples. All differences except those for arginine, histidine, isoleucine, phenylalanine, tyrosine, and valine were found to be statistically significantly ($P < 0.05$). The effects of drum-drying were consistent for mackerel and pollock except for alanine, where a significant interaction was found due to a much greater effect in pollock than in mackerel.

## DISCUSSION

The average content of –SH groups plus S–S bonds/2 in unheated pollock and mackerel fillets corresponded to a cysteine/2 content of 1.4 and 1.3 g/16 g of N, which is

in agreement with reported figures for mackerel (Krause and Mahan, 1979) but somewhat higher than that previously reported for pollock (Meinke et al., 1982). Our determination of –SH groups was dependent on their being reactive with DTNB. From our work, it was found that in undenaturated fish protein only 90% of the –SH groups were reactive with DTNB without the presence of SDS. The portion of the –SH groups being reactive with DTNB in the unheated fish in our study is thus considerably higher than that (70%) found for meat by Tinbergen (1970) but is in agreement with the results of Sedlak and Lindsay (1968) testing different protein denaturating solvents. The unreactivity of some –SH groups in unheated fish was evident from the low content of –SH groups and high content of S–S bonds. However, this effect was significant only in dried fish that had not been dissolved in SDS. On the basis of values obtained from fish heated to 50 °C, 34% of the total cystine plus cysteine/2 in pollock and 25% in mackerel was present as cysteine/2. To our knowledge, no comparative figures for fish are available, but for beef myofibrils Hamm and Hofmann (1965) found that of the total content of cystine plus cysteine/2 (1.6 g/16 g of N), 43% was present as cysteine/2.

In our study, heating for 20 min at temperatures higher than 50 °C caused oxidative transformation of sulfhydryl groups to disulfide bonds, which is in agreement with the previous findings for fish (Itoh et al., 1979a,b, 1980), for meat (Hamm and Hofmann, 1965), and for milk protein (Kisza and Rotkiewics, 1974). Disulfide cross-linking from sulfhydryl oxidation has also been found to occur during frozen storage of fish protein (Buttkus, 1970; Tsuchiya et al., 1979; Mathews et al., 1980), which would suggest that the content of S–S bonds in our untreated frozen fish could be higher than in unfrozen fish.

When the samples in our study were subjected to 115 °C, there was a loss of about 10% in the –SH groups plus S–S bonds/2. This loss of cystine plus cysteine units due to elevated temperature is in agreement with previous observations on cod muscle protein (Miller et al., 1965), soy proteins (Rios Iriarte and Barnes, 1966), pork, beef, and chicken meat protein (Dvorschak, 1970), blood proteins (Waibel et al., 1977), $\alpha$-lactalbumin (Schnack and Klostermeyer, 1980), bovine plasma albumin (Bjarnarson and Carpenter, 1970), and beef myofibrils (Hamm and Hofmann, 1965). The latter author found that the loss in cysteine plus cystine was accompanied by the formation of $H_2S$, which is in line with early descriptions of the reaction mechanism (Schoberl and Eck, 1936).

The digestibility study was designed to study the effect of heating on the digestibility of protein and individual amino acids with and without drying (cooking at 95 °C vs. drum-drying) and to separate the effect of heating and drying (freeze-drying vs. drum-drying) and to correlate these effects with the formation of disulfide bonds. Within-diet variations in the digestibilities of protein and individual amino acids were relatively small with a CV (coefficient of variation) of about 1%, with the exception of methionine and cysteine/cystine, for which the within-diet variations were relatively large (CV about 2% and 5%, respectively). When the fish samples were subjected to higher temperatures, disulfide bonds were formed from –SH groups. No disulfide bond formation occurred during freeze-drying. The formation of disulfide bonds was associated with a significant reduction in protein and individual amino acid digestibility in drum-dried samples compared to freeze-dried samples. Although disulfide bonds were formed in cooked samples, protein and amino acid digestibilities were not significantly reduced in these samples, with the exception of aspartic acid. The effect of cooking on digestibility was variable in the mackerel samples, but in the pollock samples, the digestibility of protein and most amino acids was lower compared to that of the raw samples. The cooked pollock samples had a higher –SH content than did the cooked mackerel sample, which could account for the difference between fish in digestibility. The observation that depression in digestibility by heating generally coincided with the formation of S–S bonds from –SH groups does not necessarily mean that the reduced digestibility was caused by disulfide cross-linking. Lysine also is sensitive to heat-induced cross-linkages in proteins (Bjarnarson and Carpenter, 1970). However, the finding that drum-dried mackerel had about an equal value for DBC (a criterion for the presence of free and reactive lysine) as freeze-dried would indicate that cross-linkages via lysine residues were not involved. On the other hand, drum-drying reduced the content of –SH groups and protein and amino acid digestibility. If secondary disulfide cross-linking due to heating was the cause of the reduction in protein digestibility in the drum-dried samples, the digestibility of cysteine/cystine would be expected to be more significantly affected than that of the other amino acids, which was, in fact, the case, confirming previous observations by Skrede and Opstvedt (1979).

Numerous experiments have studied the effect of heating on protein digestibility, but only those combining nutritional studies with measurements of the status of the –SH groups and S–S bonds are directly relevant to this work. Early studies (Evans and Butts, 1949a,b) indicated that overheating of soybean meal caused a reduction in in vitro cystine availability. Dvorschak (1970) found that roasting of meat and fish (110–140 °C) had no effect on the total content of amino acids or on FDNB-reactive lysine as compared with the raw materials but reduced the content of –SH groups and in vitro peptic and pancreatic protein digestibility. Several workers (Rios Iriarte and Barnes, 1966; Waibel et al., 1977; Evans and Butts, 1949a,b) have observed that excess heating caused a loss in total cysteine/cystine and a reduction in the digestibility of protein and amino acids, in particular of cystine. Although disulfide cross-linkages were not measured in these experiments, it may be assumed that temperatures that caused cysteine/cystine loss also would have led to a substantial degree of disulfide cross-linking. Noting that the effect of heating on in vitro protein digestibility of various materials was correlated to the content of cystine in their proteins, Belikov et al. (1981) postulated that the deteriorative effect of heat was due to the formation of disulfide cross–linkages. The hypothesis that disulfide cross-linkages formed by the oxidation of –SH groups reduces protein digestibility is also substantiated by the fact that cleavage of disulfide bonds has increased in vitro pepsin digestibility (Boonvisut and Whitaker, 1976). Contrary to our results, Gabaudan et al. (1980) did not find differences in protein digestibilities for trout between freeze-dried and drum-dried krill and brine shrimp.

The observation that freeze-drying gave consistently higher protein digestibility compared with raw fish may indicate that denaturation without the formation of secondary cross-links promotes protein and amino acid digestibility. Thus, it may be assumed that the net effect of heat denaturation on protein digestibility appears as the difference between the positive effect of denaturation on one side and the negative effect of secondary cross-linking on the other.

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# Stability of Pyrrolizidine Alkaloids in Hay and Silage

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The stability of pyrrolizidine alkaloids (PAs) in hay and silage samples with various amounts of *Senecio alpinus* L. was studied. While the PA content in hay remains constant over months, the PAs in silage were found to be destroyed to a great extent, but the degradation of PAs was much less complete in the lower concentration range. A quantitatively important PA-degradation product in silage was identified as retronecine. A second PA-derived compound was tentatively identified by GC–MS as a dehydratation product of retronecine with a $M^+ = 137$. Silage with a *S. alpinus* percentage of 3.5–23 still contained macrocyclic PAs in a concentration of ca. 20 mg/kg wet weight. Such a silage cannot be recommended as a safe fodder for cattle.

Plants containing pyrrolizidine alkaloids (PAs) are responsible for increasing animal health problems in various countries all over the world (Bull et al., 1968; Johnson, 1979; Lüthy et al., 1981). The fate of PAs in silage is a controversial point in literature: Vardiman (1952) reported that the toxicity of silage made from *Senecio ridellii* is greatly reduced or even nontoxic to calves. On the other hand, Donald and Shanks (1956) described a massive outbreak of ragwort poisoning in England with cattle fed on silage containing ragwort. However, in both cases no chemical analysis on PA content was performed.

According to a recent analysis *Senecio alpinus* L. contains nine different PAs with seneciphylline (see Chart I) as the major component and with a total PA content in

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