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Changes in protein fractions of rainbow trout (*Oncorhynchus mykiss*) gravads during production and storage

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Abstract

Gravad fish products belong to a group of low-processed products obtained from fresh fish by sprinkling fillets with a mixture of sugar and salt and then placing them in cold storage. Little is known about changes in the tissue during the production and storage of gravads. The purpose of the present study was to investigate the type and scope of changes in rainbow trout (*Oncorhynchus mykiss*) gravad proteins during processing and vacuum storage at 3 °C and −30 °C. The results showed that the solubility of protein was barely affected by the gravading process, but did change during the cold storage and freezing of the gravad. Catheptic activity decreased with gravading and further storage. A SDS-PAGE analysis revealed that the intensity of the M-protein and troponin bands was reduced, while the bands of α-tropomyosin and the myosin heavy chain increased when the trout muscle was subjected to the gravading process. Two bands with molecular weights of 255 and 135 kDa disappeared in the gravad, and new bands with molecular weights of 163 and 117 kDa appeared. An HPLC analysis revealed that gravading had a limited effect on the relative areas of peaks corresponding to low molecular weight substances of protein origin, while more significant changes were observed during gravad storage. Moreover, the gravading process caused a lowering of the actin and myosin denaturation temperature, which was proved using the DSC method. In conclusion, changes in rainbow trout muscle protein appeared to have a crucial effect on product quality.
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Keywords: Gravad; Rainbow trout; Fish protein; Storage stability; Cathepsin activity

1. Introduction

Minimally-processed products play an important role in the range of food obtained from fish. This group consists of well-known and popular European herring products, e.g., salted and marinated herring, as well as the less popular Maatjes, Tidbits, Schnell-maaties and Surstromming (Knochel, 1993). However, other fish are also a good raw material for delicatessen, minimally-processed products. Gravads, traditional Scandinavian products belonging to this group, are obtained mainly from salmon, greenland halibut and trout, though occasionally also from mackerel and herring. Their processing involves filleting and sprinkling the fillets with a mixture of salt and sugar, frequently also with pepper and dill, followed by 1–4 days of cold storage. The final product is either eaten directly after processing or vacuum packed and cold stored, the storage period depending on the salt and sugar content of the gravad and the storage temperature (Danfors, 1994; Paarup, Ruiz-Capillas, Morales, Lopez, & Moral, 1996).

Until now, the problem of the microbiological safety of such products, especially with respect to *Listeria monocytogenes* occurrence, has attracted a lot of attention (Loncarevic, Tham, & Danielsson-Tham, 1996; Paarup et al., 1996). As a matter of fact, little is known about the transformations taking place in protein fractions during the gravading process and subsequent cold storage. It is a crucial problem, as protein is the main organic component of the muscle tissue determining its nutritional and sensory quality, as well as its technological properties (Kołczak, 1983). Moreover, the *post-mortem* proteolysis products not only

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contribute to nutritional value, but can also have physiological properties, such as immunostimulating or antihypertensive activity (Bauchart et al., 2007). In this context, the object of the present study was to determine the type and range of changes in rainbow trout (*Oncorhynchus mykiss*) proteins during the gravading process and gravad storage. This, in turn, would lead to a better understanding of both the nature of the gravading process and the effects of storage on gravad quality.

2. Materials and methods

2.1. Gravad preparation and storage

Rainbow trout (*Oncorhynchus mykiss*) of live weight approx. 300 ± 30 g (28–32 cm in length) were collected in the winter before sexual maturity, killed by a blow on the head, eviscerated and immediately chilled in ice. Within 4 h, they were filleted with the skin left on. The gravading process involved sprinkling both sides of each fillet with a mixture of salt and sugar (1:2) at a rate of 350 g of mixture/kg of fillets, matching them in pairs, meatside to meatside, and placing them in layers in enamelled containers under light pressure for 48 h at 3 °C (Michalczyk & Surówka, 2003).

The gravads thus obtained were then removed from the brine, dried with paper cloth, vacuum packed in polyethylene bags and stored at 3 °C for 12 weeks and at −30 °C for 24 weeks. Immediately before analysis, samples were taken from the white dorsal muscle and minced by passing them twice through a mincer with 3 mm diameter holes.

2.2. Protein solubility and enzyme extract preparation

Protein solubility was determined by the extraction method described by Dyer, French, and Snow (1950). Briefly, 5 g of minced sample was homogenised for 30 s with 50 ml cold (0 °C) 0.5 M KCl at maximum speed in a Diax 900 homogenizer (Heidolph, Schwabach, Germany), equipped with a G6 blade. The homogenate was kept for 20 min at 2 °C and then stirred in an ice/water bath (0 °C) with a mechanical stirrer (500 rpm, 15 min), rested again for 10 min and centrifuged (5000*g*, 15 min) in a MLW K-70D cooled (4 °C) centrifuge (Engelsdorf, Germany). Soluble protein in the supernatant was assayed by the Kjeldahl method, using K-435 and B-324 combustion and distillation units (Büchi, Flawil, Switzerland). The supernatant was also used as an enzyme extract to determine cathepsin activity.

2.3. Cathepsin activity

Cathepsin activity was determined by the Anson method (Bergmeyer, 1965), using as a substrate a 2% bovine hemoglobin (Merck, Darmstadt, Germany) in 8 M urea solution brought to pH 4. A mixture of 5 ml of substrate and 1 ml of enzyme extract was incubated at 40 °C for 60 min. The hydrolysis was stopped by adding 10 ml 5% trichloroacetic acid (TCA) in 2.4 M urea. Then the mixture was kept for 30 min at room temperature, filtered, and the TCA soluble proteolysis products were analysed in the filtrate, using the Folin–Ciocalteau reagent according to Lowry, Rosebrough, Farr, and Randall (1951). A blank was prepared as described above, except that TCA was added before the enzyme extract and incubation was omitted. The activity unit was taken to be the amount of enzyme which, during 1 min under the conditions of the experiment, released the amount of Folin-positive material which had the same absorbance as 1 μM tyrosine at 750 nm.

2.4. Differential scanning calorimetry (DSC)

A Shimadzu (Kyoto, Japan) DSC-60 differential scanning calorimeter was used to investigate the effect of the gravading process and freezing on the temperature and enthalpy of the denaturation of trout proteins. A heating rate of 5 °C/min was applied to ca. 15 mg samples of raw fish meat and gravad in hermetically sealed aluminium pans. Indium metal was used for calibration.

2.5. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Freshly minced samples (1.0 g) were homogenized in 20 ml distilled water. The aliquots of homogenate as well as brine released during gravading process were diluted (1:1) with a denaturing buffer (0.125 M Tris, 4% SDS, 20% glycerol, 2% 2-mercaptoethanol, pH 6.8) and heated for 90 s in a boiling water bath. The extracts were centrifuged at 5000*g* for 15 min and the clear supernatant was collected. SDS-PAGE was carried out according to the Laemmli (1970) method using a 12% w/v gel concentration. A Hoefer Mighty Small SE 260 unit was used in conjunction with an EPS 301 power supply unit (Amersham Pharmacia Biotech, Uppsala, Sweden). Electrophoregrams were scanned and analysed using Image Master TotalLab (Amersham Pharmacia Biotech, Uppsala, Sweden) software. The molecular weights of the bands were estimated using MW-SDS-200 and MW-SDS-70L marker kits (Sigma Chemical Co., St. Louis, MO, USA).

2.6. High performance liquid chromatography (HPLC) analysis

Minced samples of mass 5 g were homogenized with 30 ml of buffer (0.45 M KCl, 3.38 mM KH_2PO_4, 15.5 mM Na_2HPO_4, 10 mM $Na_4P_2O_7$ and 5 mM $MgCl_2$,, pH 7.5). The homogenate was left for 15 min and 35 ml of 0.1% trifluoroacetic acid (TFA) was added. Additionally, to calibrate chromatograms, samples were prepared in which 0.1% TFA was replaced with 10% trichloroacetic acid (TCA), which is able to precipitate poly- and macropeptides. The whole was mixed for 15 min and centrifuged (3000*g*, 10 min) in a MLW K70D centrifuge (Engelsdorf,

Germany). The supernatant was filtered through a MilleX-LCR_{13} (Millipore) 0.5 μm membrane filter and 50 ml of the filtrate was injected onto a chromatography column.

HPLC analyses were performed with a Merck–Hitachi LaChrom HPLC System (Darmstadt, Germany), consisting of an interface (Model D-7000), a gradient pump (Model L-7000), a column oven (Model L-7350), an auto sampler (Model L-7250) and a diode array detector (Model L-7450). HSM System Manager software was employed for the components control and data acquisition. Separations were carried out on a WP 300 RP-18 LiChroCART 250–4 column (Merck, Darmstadt, Germany) at 30 °C. Eluent A was 0.1% TFA in water, and eluent B 0.07% TFA in water and acetonitrile (1:9 v/v). A flow rate of 1 ml/min with eluent A for 10 min and a linear gradient from 0% to 66% of eluent B for 100 min were applied. Detection was monitored at 220 nm and after each run the integration area of peaks was determined, after which the chromatograms obtained were separated into peaks corresponding to specific groups of proteinaceous substances. This was based on the results of a calibration procedure carried out with free amino acids and extracts of TCA solution. Most free amino acids and other low-molecular substances absorbing at 220 nm eluted in 2.7 to 8.0 min. Peptides non-precipitable by 10% TCA eluted within 8–85 min, whereas polypeptides and macropeptides precipitable by 10% TCA eluted in 85 to 110 min. Retention times for tyrosine, phenylalanine and tryptophan were ca. 10.5, 17.5, and 27.5 min, respectively.

2.7. Statistical analysis

Analyses were run in triplicate for each characteristic. The results were analysed statistically with the CSS Statistica package (Stat Soft, Tulsa, OK, USA). The significance of differences between means was determined by one-way ANOVA at a confidence level of 5%.

3. Results and discussion

3.1. Protein solubility

The basic parameter affecting the functional characteristics of proteins is their solubility, which is closely associated with secondary and tertiary structure change. Merely sprinkling the trout fillets with the gravading mixture makes them release brine, which contains, among other things, non-protein nitrogen substances and proteins. Table 1 presents protein solubility in 0.5 M KCl of the raw material and the cold-stored gravaded product. The gravading process was not found to affect the solubility of proteinaceous substances noticeably. During further storage of the samples, the solubility increased significantly. Conversely, the freezing of gravads caused a decrease in solubility, which, after storage at −30 °C, increased and remained at a higher level than that in the fresh product until the end of the 24-week storage period (Table 2). The decrease in protein solubility due to freezing can be explained by freeze denaturation (Benjakul & Bauer, 2000; Sikorski, 1980), but its further increase after two-month storage is surprising. Of possible significance here was the presence of sucrose acting as a cryoprotectant. According to Herrera and Mackie (2004), cryoprotectants are able to preserve the structural stability of the protein and slow down aggregation rates, which also maintains solubility.

3.2. Cathepsin activity

Protein fraction in fish muscle is strongly related to cathepsin activity. As a result of gravading and later cold storage, there was a consistent tendency for it to decrease (Table 1). A lowering of proteolytic enzyme activity in fish caused by salting is known in the literature (Reddi, Constantinides, & Dymsza, 1972). In the product analysed here, it might have been additionally caused by the partial oxidation of thiol groups in the active enzyme centres (Fik, Surówka, & Leszczyńska-Fik, 1988) or their interactions with sugar (Golovkin & Meluzova, 1975). In a study by Fik et al. (1988) concerning unprocessed trout stored at −2 °C, no such lowering of cathepsin activity in the 30-day storage period was noted. Despite the fact that cathepsin activity decreased in cold-stored gravads, their activity was probably responsible for the steady increase in protein solubility observed in the present study (see Table 1). The

Table 1
Solubility of muscle proteins and specific cathepsin activity in cold-stored gravad

Storage time	Protein solubility[A] (%)	Specific cathepsin activity[A] (μmol Tyr/min mg)
Raw fish	35.1 ± 0.66[aB]	6.64 ± 0.56
Gravad immediately after production	36.2 ± 0.83[a]	4.92 ± 0.59[a]
2 weeks	43.7 ± 0.66	4.14 ±0.59[ab]
4 weeks	48.8 ± 0.78	3.23 ± 0.41[b]
6 weeks	57.4 ± 0.32	3.06 ± 0.45[bd]
8 weeks	86.4 ± 4.91	2.47 ± 0.20[d]

[A] Mean values ± standard deviation.
[B] The mean values in columns marked with the same letter do not differ significantly ($p > 0.05$).

Table 2
Solubility of proteins and specific cathepsin activity in freeze stored gravad

Storage time (weeks)	Protein solubility[A] (%)	Specific cathepsin activity[A] (μmol Tyr/min mg)
Raw fish	35.1 ± 0.7[aB]	6.64 ± 0.56[a]
Gravad immediately after production	36.2 ± 1.8[a]	4.92 ± 0.59
Gravad after freezing	30.8 ± 0.6	6.58 ± 0.49[a]
8 weeks	61.6 ± 0.4	3.59 ± 0.14[b]
16 weeks	57.7 ± 0.8[b]	3.15 ± 0.34[b]
24 weeks	55.7 ± 2.5[b]	3.54 ± 0.66[b]

[A] Mean values ± standard deviation.
[B] The mean values in columns marked with the same letter do not differ significantly ($p > 0.05$).

freezing of gravad caused the activity of the enzymes tested to increase to the normal level for raw fish. However, after only 8-weeks' storage a distinct decrease could be observed, the level then remaining more or less constant until the investigations were completed (Table 2). Maintaining of cathepsin activity during freezing and frozen storage has also been observed by Ming, Gen, and Shann (2000) who stored mackrel surimi at −20 °C for eight weeks.

3.3. Differential scanning calorimetry (DSC)

The effect of treating trout muscle tissue with salt and sugar is reduced hydration and the loss of some proteins to the brine, as well as changes in conformation affecting the structural stability of proteins. The thermal stability of proteins is a good indicator of such changes. The denaturation temperature and the enthalpy of denaturation can be observed by the DSC method (Hastings, Rodger, Park, Matthews, & Anderson, 1985; Thorarinsdottir, Arason, Geirsdottir, Bogason, & Kristbergsson, 2002). In fish muscle proteins most commonly identified are two endothermic peaks of maximum temperatures lower than in mammalian muscle proteins, which is caused by a generally lower thermostability of fish proteins (Hastings et al., 1985).

The first peak on the thermogram refers to myosin and the second to actin denaturation. What should, however, be taken into account is that the remaining myofibril proteins (e.g., tropomyosin, troponin and actinin), as well as sarcoplasmic proteins and stromas have an effect, albeit small, on the shape of the DSC curves (Beas, Wagner, Crupkin, & Añon, 1990). The results obtained by the DSC method for raw trout meat, a freshly produced gravad and a gravad just after freezing are shown in Table 3. The data show that gravading caused a shift in denaturation temperatures for both fractions (myosin and actin) towards the lower range of values, as well as a substantial loss of myosin transition enthalpy. However, the actin transition enthalpy doubled. It is generally considered that NaCl lowers the denaturation temperature of both actin and myosin as well as their transition enthalpies (Hastings et al., 1985; Park & Lanier, 1989). On the other hand, according to Thorarinsdottir et al. (2002), a lowering of the water content causes a slight shift in transition temperatures towards the higher range of values. Hagerdal and Martens (1976) explained that proteins then become more thermostable due to the formation of inter- and intramolecular electrostatic interactions and the formation of hydrogen bonds in moieties previously occupied by water. Changes in the denaturation temperature and enthalpy of denaturation caused by the freezing of gravad were small and statistically insignificant. Furthermore, according to Hastings et al. (1985), freezing and immediate thawing had little effect on the thermal values of cod muscle.

Summarising, the results obtained by the DSC method prove that the process of gravading leads to the modification of structural stability in trout proteins, while subsequent gravad freezing does not affect this stability.

Table 3
The results of DSC analysis of raw fish, gravad, and gravad immediately after freezing

Specification		Raw fish	Gravad immediately after production	Gravad immediately after freezing
Myosin	Peak I, T_{max} (°C)	45.2 ± 1.6^{A}	42.3 ± 0.5^{aB}	41.2 ± 0.7^{a}
	Peak I, T_{onset} (°C)	39.4 ± 0.6	37.9 ± 0.3^{a}	37.8 ± 0.9^{a}
	Peak I, ΔH (J/g dry matter)	6.38 ± 1.16	0.75 ± 0.13^{a}	0.60 ± 0.07^{a}
Actin	Peak II, T_{max} (°C)	75.9 ± 0.7	69.9 ± 0.6^{a}	68.8 ± 0.5^{a}
	Peak II, T_{onset} (°C)	72.7 ± 0.5	63.6 ± 0.7^{a}	62.4 ± 1.7^{a}
	Peak II, ΔH (J/g dry matter)	1.10 ± 0.12	2.20 ± 0.39^{a}	2.39 ± 0.40^{a}

[A] Mean values ± standard deviation.
[B] The mean values in rows marked with the same letter do not differ significantly ($p > 0.05$).

3.4. Electrophoretic (SDS-PAGE) analysis

The quantitative results of electrophoretic separation of proteins in raw trout meat, gravad and brine obtained during its production, as well as in cold-stored and frozen products, are shown in Table 4. Based on data from the literature (Jasra, Jasra, & Talesara, 2001; Lin & Park, 1996; Ünlüsayin, Kaleli, & Gulyavuz, 2001) and calibration performed, some bands were assigned to respective proteins: a band of molecular weight 215 kDa corresponded to myosin heavy chain (MHC); 180 kDa to M protein; 148 kDa to C protein; 102 and 107 kDa to α-actinin; 42.7 kDa to actin; 39.5 kDa to β-tropomyosin; 37.9 kDa to troponin T; 35.6 kDa to α-tropomyosin; and the set of bands from 35 to 17.6 kDa corresponded to myosin light chain. The band which appeared at molecular weight 14.1 kDa was assigned to non-separated low-molecular proteinaceous substances. Gravading resulted in an increase in the intensity of the MHC band. This was accompanied by a drop in the level of M-protein, which is responsible for maintaining an ordered structure within the thick filaments (Yuming & Hultin, 1997). There was also found to be a relatively low quantity of troponin and some of the proteins assigned here to the myosin light chain (17.6–35 kDa), while the intensity of the band representing α-tropomyosin increased. The result of the process described was also the disappearance of fractions with a molecular weight of 255 and 135 kDa and the appearance of 163 and 117 kDa bands. The number of bands representing the fractions passing to the brine was ten fewer than those identified in the muscle tissue of raw and processed trout. No presence of, among others, M-protein, C-protein and troponin was found in the brine, but two bands appeared, of molecular

Table 4
Changes of relative content of protein fractions during gravading and storage of products

Protein type	Molecular weight (kDa)	Raw trout		Fresh gravad		Brine		Cold-stored gravad (8 weeks)		Fresh frozen gravad		Freeze stored gravad (8 weeks)	
		Band (no)	Intensity (%)	Band (no)	Intensity (%)	Band (no)	Intensity (%)	Band (no)	Intensity (%)	Band (no)	Intensity (%)	Band (no)	Intensity (%)
	280.0	1	2.14	1	4.31	1	2.78	1	1.93	1	6.14	1	1.69
	274.0	2	4.70	2	5.59			2	12.9	2	7.31	2	10.2
	255.0	3	2.07			2	1.67						
MHC[A]	215.0	4	23.7	3	25.6	3	3.31	3	20.0	3	27.1	3	23.0
M protein	180.0	5	1.40	4	0.18			4	0.315	4	0.381	4	0.271
	163.0			5	1.63			5	2.62	5	1.96	5	2.01
C protein	148.0	6	0.865	6	1.22			6	3.53	6	1.80	6	1.40
	135.0	7	0.219										
	117.0			7	0.349			7	0.269	7	0.280	7	0.554
α-Actinin	107.0	8	1.61	8	1.82	4	0.299	8	1.54	8	1.67	8	1.53
α-Actinin	102.0	9	1.60	9	2.15	5	1.57	9	1.909	9	2.091	9	2.34
	96.0	10	0.173	10	0.308			10	0.494	10	0.341	10	0.546
	86.0	11	0.388	11	0.558			11	0.853	11	0.523	11	0.681
	63.0	12	2.87	12	2.30	6	1.27	12	2.37	12	1.99	12	2.47
	61.0					7	3.81						
	51.0	13	4.61	13	1.93	8	8.63	13	2.23	13	1.88	13	2.73
	50.0	14	0.618	14	0.863	9	3.00	14	0.903	14	0.497	14	0.494
Actin	42.7	15	23.0	15	23.4	10	16.3	15	20.2	15	22.3	15	20.2
β-Tropomyosin	39.5	16	5.13	16	4.73	11	15.5	16	4.52	16	3.94	16	4.52
Troponin T	37.9	17	0.674	17	0.276			17	0.913	17	0.375	17	1.21
α-Tropomyosin	35.6	18	8.48	18	9.73	12	6.11	18	10.7	18	8.98	18	10.9
	35.0	19	0.725	19	1.82			19	0.935	19	1.12	19	1.09
	32.0	20	0.668	20	0.039			20	0.087	20	0.036	20	0.070
	31.4	21	1.91	21	1.40	13	2.15	21	0.762	21	0.987	21	1.00
	29.2					14	0.453						
	28.5	22	1.14	22	0.690	15	2.90	22	0.702	22	0.331	22	0.652
	24.3	23	2.46	23	1.48	16	4.94	23	1.64	23	1.01	23	1.66
	23.4	24	0.463	24	0.224			24	0.170	24	0.008	24	0.337
	23.1	25	0.163	25	0.061	17	0.529	25	0.025	25	0.018	25	0.116
	21.3	26	1.37	26	0.202			26	0.263	26	0.813	26	0.661
	20.8	27	1.78	27	2.40	18	0.436	27	2.51	27	1.45	27	2.52
	18.3	28	0.097	28	0.453			28	0.250	28	0.251	28	0.734
	17.6	29	1.05	29	0.592	19	2.37	29	0.663	29	0.454	29	0.754
NSB[B]	14.1	30	3.94	30	3.72	20	22.0	30	3.72	30	3.95	30	3.63

[A] MHC: myosin heavy chain.
[B] NSB: non-separated low molecular protein substances.

weight of 61.0 and 29.2 kDa. The relative intensity of bands representing proteinaceous substances of molecular weight below that typical for α-tropomyosin 35.6 kDa was 35.8% in brine, whereas in raw fish and gravad only 15.8% and 13.0%, respectively. This difference is mainly caused by the high level of non-separated low-molecular substances in brine (band no. 20). According to Mackie et al. (2000) when describing collaborative eletrophoretic studies of fish proteins in some of the laboratories, slight changes in the SDS-PAGE patterns of raw and gravaded trout were noted, but many institutions did not report any differences.

In the present study, it was shown that when gravads were cold stored for 8 weeks, the intensity of the 274 and 163 kDa bands and the C-protein increased compared to the fresh gravad. This was accompanied by a lowering of the relative intensity of bands representing the actin and myosin heavy chain. It is therefore possible that during cold storage, partial aggregation of these two myofibrillar proteins occurs. On the other hand, the lowering of the actin and MHC bands could be due to their degradation caused by cathepsins (Ashie & Simpson, 1997). Hultmann, Rørå, Steinsland, Skåra, and Rustad (2004) suggest that the conformation of the myofibrillar proteins may change as a result of increased salt content in the muscle, making the proteins more susceptible to attack by endogenous proteases. The freezing process of gravad resulted in an increase in the intensity of the six heaviest protein bands (1–6) (see Table 4), while there was a tendency for most of the remaining bands to decrease compared with the fresh gravad. Frozen storage for 8 weeks caused a decrease in the relative intensity of the MHC and actin bands compared with freshly frozen gravad. Owusu-Ansah and Hultin (1986) also observed a tendency for the content of MHC to decrease with the length of frozen storage in cod. The data from the literature, however, tends rather to report no changes in actin (Kim, Loveridge, & Taub, 1984; LeBlanc & LeBlanc, 1989). Despite the quantitative differences observed, all the bands present in fresh gravad were also found in cold-stored and frozen products.

3.5. High performance liquid chromatography (HPLC) analysis

In order to arrive at a more complete description of low-molecular fractions not separated by electrophoresis, HPLC analysis was employed. Table 5 summarises the results of the separations of fractions extracted with 0.1% TFA from raw trout, fresh, frozen and stored gravad and brine. Gravading did not seem to cause substantial changes in the relative intensity of the peaks recorded. Compared with the raw fish, only a limited increase in the relative area of peaks assigned to amino acids, and a decrease in the intensity of peaks representing peptides, poly- and macropeptides were recorded. There was, however, a marked change in the number of registered peaks, particularly in the cases of poly- and macropeptides, which doubled. Further storage of the product caused much larger changes than the gravading process itself. After 4 weeks of cold storage a considerable increase in the relative area of peaks representing amino acids was observed. A slight increase was also noted in the area of peptide peaks, and the relative content of poly- and macropeptides decreased markedly. These changes were accompanied by increase in the number of peaks in the peptides, and reduction in poly- and macropeptides. According to Özogul, Ahmad, Hole, Özogul, and Deguara (2006), an increase in biogenic amines can also be observed during the cold storage of trout.

In general, the freezing process and further storage for 24 weeks moderately elevated the relative area of peaks assigned to amino acids and peptides, and reduced the area for poly- and macropeptides compared to the fresh gravad. In addition, a reduction in the number of recorded peaks on chromatograms was observed. This could be due to freeze denaturation, although the extent of it could not have been great as it would have been limited by the sugar

Table 5
Analysis of HPLC chromatograms of protein fractions obtained from trout muscle tissue, a gravad, and brine

	Amino acids relative area (%)	Tyrosine relative area (%)	Phenylalanine relative area (%)	Tryptophan relative area (%)	Peptides		Polypeptides and macropeptides		Total area under peaks (arbitrary units)
					Number of peaks	Relative area (%)	Number of peaks	Relative area (%)	
Raw trout	31.1	0.46	0.03	0.6	2	9.1	7	58.8	3345
Fresh gravad	32.4	1.2	0.4	1.0	3	7.8	15	57.2	2823
Four weeks cold-stored gravad	49.9	4.0	1.1	3.1	5	9.7	11	32.2	1778
Fresh frozen gravad	37.1	1.4	0.3	1.1	2	9.8	11	50.2	2522
24-Weeks freeze stored gravad	45.1	1.5	0.4	1.2	1	11.2	9	40.6	2083
Brine	17.1	0.7	0.8	2.7	7	39.5	13	39.2	1906

present in the gravad, which can act as a cryoprotectant (Herrera & Mackie, 2004; Sikorski, 1980). The tendency observed in the present investigation is in accordance with the results reported by LeBlanc and LeBlanc (1989), who examined proteins in cod fillets during frozen storage. The brine released during the gravading process had the lowest relative content of amino acids among the samples examined. Nearly 80% of the area under peaks recorded for brine was assigned to peaks representing peptides and poly- and macropeptides. It should be emphasized that in the peptide group there were even more peaks found than in the gravad, and their relative area was approx. five times higher. Also worth noticing is the relatively large diversity of poly- and macropeptides recorded in the brine (13 peaks).

Generally, despite the continuous increase of protein solubility in 0.5 M KCl (see Table 1) due to both gravading and further storage, investigations by HPLC showed a reduction in the total area under the chromatograms, corresponding to a decline in the amount of proteinaceous substances soluble in 0.1% TFA. Due to the substantial sensory activity of such compounds, the changes observed may have a significant effect on the flavour characteristics of gravad.

4. Conclusions

As a result of the gravading process, rainbow trout muscle loses part of its proteinaceous substances, which enter the brine. This leads to changes in the relative levels of individual proteins in the muscle tissue, a process which continues when the gravad is frozen and is particularly pronounced during both cold and frozen storage. To a large degree this applies to the low molecular weight proteinaceous substances that were analysed in this study by the HPLC method. Thus, due to the potentially high sensory activity of these substances, the changes observed may have a significant effect on the taste of the gravad. The above changes in the fractions of gravad protein are probably, to some degree, caused by cathepsin activity, which is, however, lower in gravad than in a raw fish.

The gravading process also weakens the structural stability of trout muscle protein, which, in turn, may lead to a decrease in actin and myosin denaturation temperatures. However, the process does not affect changes in protein solubility, which increases only during product storage.

References

Ashie, I. N. A., & Simpson, B. K. (1997). Proteolysis in food myosystems – a review. *Journal of Food Biochemistry, 21*(2), 91–123.

Bauchart, C., Chambon, C., Mirand, P. P., Savary-Auzeloux, I., Rémond, D., & Morzel, M. (2007). Peptides in rainbow trout (*Oncorhynchus mykiss*) muscle subjected to ice storage and cooking. *Food Chemistry, 100*, 1566–1572.

Beas, V. E., Wagner, J. R., Crupkin, M., & Añon, M. C. (1990). Thermal denaturation of hake (*Merluccius hubbsi*) myofibrillar proteins. A differential scanning calorimetric and electrophoretic study. *Journal of Food Science, 55*(3), 683–687.

Benjakul, S., & Bauer, F. (2000). Physicochemical and enzymatic changes of cod muscle proteins subjected to different freeze–thaw cycles. *Journal of the Science of Food and Agriculture, 80*(8), 1143–1150.

Bergmeyer, H. U. (1965). *Methods of enzymatic analysis*. New York and London: Academic Press.

Danfors, S. (1994). Easter and spring specialities: egg, gravlax and spinach. *Var-Foeda, 46*(2), 106–111 (in Swedish).

Dyer, W. J., French, H. W., & Snow, J. M. (1950). Proteins in fish muscle. I. Extraction of protein fractions in fresh fish. *Journal of the Fisheries Research Board of Canada, 7*, 585–593.

Fik, M., Surówka, K., & Leszczyńska-Fik, A. (1988). Application of superchilling to prolong the keeping time of rainbow trout. *Nahrung, 32*(3), 291–300.

Golovkin, N. A., & Meluzova, L. A. (1975). Progress in refrigeration science and technology. In: *The fourteenth international congress of refrigeration*, Moscow, p. 753.

Hagerdal, B., & Martens, H. (1976). The influence of water content on the stability of myoglobin to heat treatment. *Journal of Food Science, 41*(4), 933–937.

Hastings, R. J., Rodger, G. W., Park, R., Matthews, A. D., & Anderson, E. M. (1985). Differential scanning calorimetry of fish muscle: the effect of processing and species variation. *Journal of Food Science, 50*(2), 503–506.

Herrera, J. J. R., & Mackie, I. M. (2004). Cryoprotection of frozen stored actomyosin of farmed rainbow trout (*Oncorhynchus mykiss)* by some sugars and polyols. *Food Chemistry, 84*(1), 91–97.

Hultmann, L., Rørå, A. M. B., Steinsland, I., Skåra, T., & Rustad, T. (2004). Proteolytic activity and properties of proteins in smoked salmon (*Salmo salar*) – effect of smoking temperature. *Food Chemistry, 85*, 377–387.

Jasra, S. K., Jasra, P. K., & Talesara, C. L. (2001). Myofibrillar protein degradation of carp (*Labeo rohita* (Hamilton)) muscle after post-mortem unfrozen and frozen storage. *Journal of the Science of Food and Agriculture, 81*(5), 519–524.

Kim, H. J., Loveridge, V. A., & Taub, I. A. (1984). Myosin cross-linking in freeze-dried meat. *Journal of Food Science, 49*(3), 699–703.

Knochel, S. (1993). *Fish fermentation technology*. The United Nations University, pp. 53–70.

Kołczak, T. (1983). *Biological principles of meat technology*. Kraków: Publishers AR (in Polish).

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head bacteriophage T4. *Nature, 227*, 680–685.

LeBlanc, E., & LeBlanc, R. (1989). Separation of cod (*Gadus morhua*) fillet proteins by electrophoresis and HPLC after various frozen storage treatments. *Journal of Food Science, 54*(4), 827–834.

Lin, T. M., & Park, J. W. (1996). Extraction of proteins from pacific whiting mince at various washing conditions. *Journal of Food Science, 61*(2), 432–438.

Loncarevic, S., Tham, W., & Danielsson-Tham, M. L. (1996). Prevalence of *Listeria monocytogenes* and other *Listeria spp.* in smoked and gravad fish. *Acta Veterinaria Scandinavica, 37*(1), 13–18.

Lowry, O. H., Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951). Protein measurements with the Folin phenol reagent. *Journal of Biological Chemistry, 193*, 265–275.

Mackie, I. M., Craig, A., Etienne, M., Jerome, M., Fleurence, J., Jessen, F., et al. (2000). Species identification of smoked and gravad fish products by sodium dodecylsulphate polyacrylamide gel electrophoresis, urea isoelectric focusing and native isoelectric focusing: a collaborative study. *Food Chemistry, 71*(1), 1–7.

Michalczyk, M., & Surówka, K. (2003). A method of manufacturing gravad rainbow trout preserves (*Oncorhynchus mykiss*) and their acceptability by consumers (in Polish). *Żywność, 36*(3), 131–140, Supl.

Ming, L. H., Gen, H. C., & Shann, T. J. (2000). Effect of mackrel cathepsins L and L-like, and calpain on the degradation of macrel surimi. *Fisheries Science, 66*(3), 558–568.

Owusu-Ansah, Y. I., & Hultin, H. O. (1986). Chemical and physical changes in red hake fillets during frozen storage. *Journal of Food Science, 51*(6), 1402–1406.

Özogul, Y., Ahmad, J. I., Hole, M., Özogul, F., & Deguara, S. (2006). The effects of partial replacement of fish meal by vegetable protein sources in the diet of rainbow trout (*Onchorynchus mykiss*) on post mortem spoilage of fillets. *Food Chemistry, 96*(4), 549–561.

Paarup, T., Ruiz-Capillas, C., Morales, J., Lopez, E., & Moral, A. (1996). "Gravad"-type rainbow trout, packed in vacuum and modified atmosphere: microbiological, biochemical and sensory aspects. Refrigeration and aquaculture. In: *Proceedings IIR conference-commission C2*, Bordeaux (France), pp. 333–337.

Park, J. W., & Lanier, T. C. (1989). Scanning calorimetric behavior of tilapia myosin and actin due to processing of muscle and protein purification. *Journal of Food Science, 54*(1), 49–51.

Reddi, P. K., Constantinides, S. M., & Dymsza, H. A. (1972). Catheptic activity of fish muscle. *Journal of Food Science, 37*(5), 643–648.

Sikorski, Z. E. (1980). *Technology of seafoods*. Warszawa: Wydawnictwa Naukowo-Techniczne (in Polish).

Thorarinsdottir, K. A., Arason, S., Geirsdottir, M., Bogason, S. G., & Kristbergsson, K. (2002). Changes in myofibrillar proteins during processing of salted cod (*Gadus morhua*) as determined by electrophoresis and differential scanning calorimetry. *Food Chemistry, 77*(3), 377–385.

Ünlüsayin, M., Kaleli, S., & Gulyavuz, H. (2001). The determination of flesh productivity and protein components of some fish species after hot smoking. *Journal of Science of Food and Agriculture, 81*(7), 661–664.

Yuming, F., & Hultin, H. O. (1997). Solubility of the proteins of mackerel light muscle at low ionic strength. *Journal of Food Biochemistry, 21*(6), 479–496.