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Modification of the properties of gelatin from skins of Baltic cod (*Gadus morhua*) with transglutaminase

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Abstract

The aim of the investigations was to optimize the procedure of gelatin extraction from the skins of Baltic cod (*Gadus morhua*) and to modify the properties of fish gelatin, using transglutaminase, to form gels at room temperature. A temperature of 45 °C and 30 min of extraction was established as optimal conditions for preparing gelatin from cod skins. Fish gelatin in 5% solutions formed gels at room temperature in the presence of transglutaminase (0.15–0.7 mg of enzyme protein/ml), depending on the reaction time. Transglutaminase was also active in crosslinking of fish gelatin at 4–5 °C. The deformation of gels obtained from 5% gelatin solutions after a 24-h incubation with transglutaminase, at concentration 0.25 mg/ml, measured at 6–8 °C, was the same as or slightly lower than in control samples (without enzyme). Fish gelatin gels, enzymatically crosslinked, did not melt after 30 min of heating in a boiling water bath.

Keywords: Baltic cod; Fish skins; Fish gelatin; Transglutaminase

1. Introduction

Gelatin, the product of partial hydrolysis of collagen, finds a variety of applications in the food, photographic and pharmaceutical industries. Viscosity of gelatin solutions, gel strength, gelling and melting temperatures govern where gelatin is used. The properties of gelatin gels depend on the source and pretreatment of the raw material and parameters of the process. They are also affected by concentration of the gelatin, pH, presence of interacting compounds, gel maturation time and temperature.

There are two methods of obtaining gelatin from bones and skins of mammals – an acid process, using pigskins as raw material (gelatin A with isoelectric points at pH 6–9) and an alkaline process, using hides and bones of cattle (gelatin B with isoelectric points at pH about 5) (Stainsby, 1987). The type of chemical pretreatment and parameters of extraction can influence the length of polypeptide chains and the functional properties of the gelatin. The gelling strength of commercial gelatins, expressed as Bloom value, ranges from 100 to 300, but gelatins with Bloom values of 250–260 are most desired (Holzer, 1996). In the case of fish gelatins, such high gel strength characterizes only those extracted from skins of warm-water fish (Grossman & Bergman, 1992). However, fish skins may serve as a valuable source of gelatin for religious or health reasons.

The gelling properties of gelatin are greatly influenced by the origin of raw material used in the process. This results from differences in contents of proline and hydroxyproline in collagens of different species and is connected with the temperature of the habitat of the animals. The thermal shrinkage, denaturation temperature of collagens and melting temperature of gelatins derived from the skins of cold-water fish are significantly lower than those of collagens and gelatins from skins of warm-blooded animals and fish living in warm waters, due to the lower imino acid content and decreased proline hydroxylation degree (Gilsenan & Ross-Murphy, 2000; Gómez-Guillén et al., 2002; Norland, 1990; Piez & Gross, 1960; Yamaguchi, Lavéty, & Love, 1976).

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Furthermore, Burjanadze (2000) found that the dependence of denaturation temperature on 4-hydroxyproline content is hyperbolic and the total Gly–Pro–Hyp sequence content is one of the main factors influencing collagen thermostability. The properties of gelatin aslo depend on the molecular weight distribution of collagenous components and on the α_1/α_2 ratio (Gómez-Guillén et al., 2002). According to Normand, Muller, Ravey, and Parker (2000), only α, β and γ components participate in elastic properties of gelatin.

The gelatin produced from skins of fish living in cold waters does not gel at room temperature – its gelling temperature is below 8–10 °C (Norland, 1990). This feature offers fresh applications of fish gelatin. For example, such gelatin is the base for light-sensitive coating for the electronics trade and is a good medium for precipitating silver halide emulsions because this process can be carried out at lower temperature than with warm-blooded animal gelatin (Norland, 1990). On the other hand, the same feature limits the use of such gelatin as a gelling component in food production. Therefore, for wide applications, studies on the modification of gelling properties of fish gelatin are conducted.

One possibility for improving of gelling strength, viscoelastic properties and melting temperature of gelatin is the addition of $MgSO_4$ and glycerol (Fernández-Díaz, Montero, & Gómez-Guillén, 2001; Sarabia, Gómez-Guillén, & Montero, 2000). Limited, available data showed that such an effect could be achieved, also, by enzymatic modification of gelatin with transglutaminase (Fernández-Díaz et al., 2001). The enzyme catalyses the formation of crosslinking bonds between γ-amide groups of glutamine and ϵ-amino groups of lysine. This reaction leads to substantial changes in the functional properties of plant and animal proteins and gives possibilities for formation of products with improved rheological and sensory properties (Nielsen, 1995).

The direct procedures used for preparing fish gelatin involve mild chemical pretreatment of raw material and mild temperature conditions of the extraction process. However, prolonged time of extraction is usually used (Fernández-Díaz et al., 2001; Gómez-Guillén et al., 2002; Gudmundsson & Hafsteinsson, 1997; Sarabia et al., 2000).

The objective of the investigations was to search for a possibility of shortening of the time of extraction of gelatin from the skins of Baltic cod and to determine optimum conditions of enzymatic crosslinking of gelatin so that the gel could be obtained at room temperature.

2. Materials and methods

The source of transglutaminase was a commercial product (Ajinomoto Co's Transglutaminase Activa®-WM, Tokyo, Japan). The preparation contained 99% of maltodextrin and 1% of transglutaminase. The dry preparation was stored at −20 °C. Before use, 6 g of preparation was mixed with 20 ml water at 0 °C for 15 min and centrifuged at 10,000*g* for 15 min at 0 °C.

The skins were mechanically separated from fresh Baltic cod (*Gadus morhua*). The residue of adherent tissues was removed manually. The cleaned skins were frozen at −20 °C and minced in a meat grinder using mesh diameter $\phi = 3$ mm, mixed thoroughly and stored at −20 °C before use.

In order to determine optimum conditions for gelatin extraction, the minced, raw material was gently stirred with water (1:6) at a temperature range of 30–60 °C during 15–120 min. The samples were centrifuged at 10,000*g* for 30 min at 15 °C and hydroxyproline was determined in the supernatant. On the basis of the obtained results, the procedure of gelatin extraction was established.

The freeze-dried fish gelatin was dissolved in distilled water at room temperature and transglutaminase solution was added. The 2% and 5% gelatin solutions were incubated at room temperature or at 4–5 °C up to 24 h with 0.05–0.7 mg of enzyme protein/ml of reaction mixture. After an appropriate time, the formation of gel at room temperature was checked visually. Samples kept at 4–5 °C were after 24 h transferred to room temperature and, during 2 h, the behaviour of the gels was observed. Further experiments were conducted at concentrations of enzyme of 0–0.25 mg/ml. The samples were incubated at 4–5 °C and, after 20 h, the deformation of the gels was determined. In some cases the measurements were made at room temperature.

The dry weight and total nitrogen of the skins were determined according to AOAC (1990) methods. Hydroxyproline was determined after hydrolysis of the material in 6 M HCl for 6 h at 105 °C, using the colorimetric method recommended by ISO (Anonymous, 1978). The protein content in the transglutaminase solution was determined according to Lowry, Rosebrough, Farr, and Randall (1951).

The gel-forming ability of gelatin was determined in a test based upon the least protein concentration as the endpoint (LCE) according to Trautman (1966), but without previous heating of the dissolved sample of fish gelatin. The commercial pork gelatin, used for comparison, was dissolved in water at 60 °C. The tubes, containing gelatins at different concentrations, were kept for 1 h at 0 °C and 20 h at 4–5 °C. The endpoint was the least concentration of gelatin at which all the content of the sample remained inside when the tube was inverted.

The deformation of gels under stress (expressed as the depth of penetration) was measured in the penetrometer (Veb Feinmess Dresden) with flat end cylindrical plungers, 2 cm in diameter weighting from 115 to 690 g.

3. Results and discussion

3.1. Effect of temperature and extraction time of cod skins with water on the yield of gelatin

The process for obtaining of gelatin from skins of fish, including cold-water species, includes chemical treatment of the raw material. In the method applied by Gudmundsson and Hafsteinsson (1997) for preparing odourless fish gelatin, rinsed and cleaned cod skins were treated with diluted solutions of sodium hydroxide, followed by sulphuric acid, and finally citric acid. In another procedure the raw material, cleaned with water and 0.8 M NaCl, was treated with diluted sodium hydroxide and acetic acid (Gómez-Guillén et al., 2002; Montero & Gómez-Guillén, 2000; Sarabia et al., 2000). The viscoelastic properties and the gel strength of gelatin obtained in this way were much better than those of gelatin prepared according to Gudmundsson and Hafsteinsson (Montero & Gómez-Guillén, 2000). Gudmundsson and Hafsteinsson (1997) showed that treating of skins with solutions of sodium hydroxide and sulphuric acid (higher than 0.2%) and citric acid (above 1%) decreased the yield of gelatin and reduced the gel strength. In both these procedures, each step of chemical treatment was repeated three times and gelatin was extracted with water overnight at 45 °C. Presumably, gelatin can also be degraded during prolonged extraction, conducted even at relatively low temperature. Cod and hake gelatins, prepared by Gómez-Guillén et al. (2002), were characterized by lower α_1/α_2 ratios than those of megrim and sole skins. According to these authors, this was caused by damage or partial loss of α_1-chains during the extraction procedure. In the present method of preparation of gelatin for enzymatic modifications, the chemical treatment was omitted and the possibility of shortening the time of extraction was checked. However, minced raw material was used for easier extraction of gelatin.

The thermal solubility of skin collagen in water depended on the extraction temperature and time. It increased significantly above 30 °C. At 40 °C, about 50% of collagen was dissolved after 30 min of gentle stirring of the skins in water. At 45–60 °C, after 15–30 min about 85–90% of collagen was converted to soluble gelatin. The extractability of gelatin did not change significantly during further heating up to 120 min (Fig. 1). Based on these results, a temperature of 45 °C and time of extraction of 30 min was established as optimal conditions for preparing gelatin from cod skins.

Baltic cod skins used in the experiments contained, on a wet weight basis, about 24% crude protein (N × 6.25) and about 80% of that was collagen (Table 1). Using the procedure shown in Fig. 2, for optimal conditions of extraction of cod skins, the yield of gelatin amounted to 12.3% of the weight of raw material (64% of collagen content in skins). Gudmundsson and Hafsteinsson (1997), using prolonged extraction of whole cod skins, achieved a yield of gelatin between 11% and 14%, depending on the concentrations of sodium hydroxide, sulphuric and citric acid solutions used in the preliminary treatment of raw material, i.e., 64–82% of collagen content in skins. A lower yield of gelatin from cod skin, about 7%, was obtained by Gómez-Guillén et al. (2002).

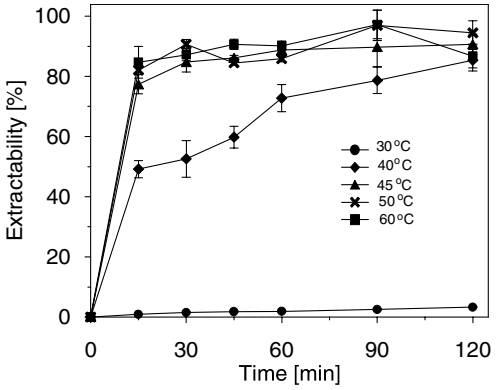

Fig. 1. Effect of temperature and time on the yield of gelatin extracted from minced skins of cod with water (1:6)[a]. [a]Mean values ± SD from three separate samples.

Table 1
Chemical composition of Baltic cod skins[a]

Component	(%)
Dry weight	27.6 ± 0.62
Protein (N × 6.25)	24.3 ± 0.58
Collagen[b]	19.1 ± 0.95

[a] Mean values ± SD of three separate samples.
[b] The conversion factor for calculating the content of cod skin collagen from hydroxyproline was 14.7.

Our results show that it is possible to shorten the time of extraction of skins of cold-water fish from about 12 h down to 30 min, but minced raw material must be used. Because of the collagen characteristics, fish skins are difficult to mince in a meat grinder. However, they can be comminuted easily after previous treatment with diluted acetic acid (1: 6) at a temperature below 15 °C for 2 h (Sadowska, Kołodziejska, & Niecikowska, 2003). The gelatin obtained in our experiments is soluble in water at room temperature; 5% solution is slightly yellow but the fishy odour is not eliminated. Therefore, chemical pretreatment will also be essential for obtaining a odourless product.

3.2. Modifications of gelatin with transglutaminase

In the case of gelatin from skins of fish of cold-water species, it is necessary to modify this product to make it

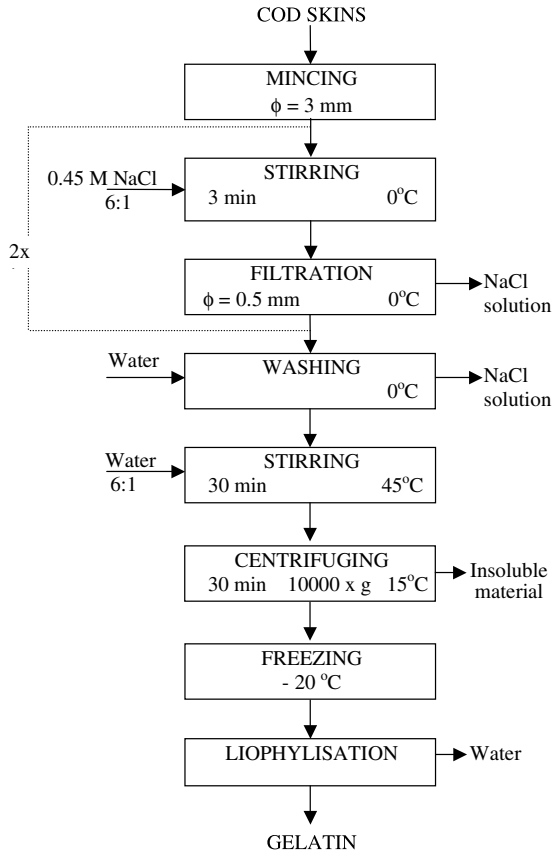

Fig. 2. Flow sheet of the procedure used for extraction of gelatin from minced skins of cod.

able to form a gel at room temperature. Sarabia et al. (2000) and Fernández-Díaz et al. (2001) showed that the properties of fish gelatin can be improved, including increasing of melting and gelling temperature, in the presence of glycerol and $MgSO_4$, $(NH_4)_2SO_4$, or NaH_2PO_4. However, these compounds must be added in relatively high concentrations, for example, 15% of glycerol and 0.1–0.5 M $MgSO_4$. For this reason, such a method of modification of gelatin may be limited for use by the food industry.

The LCE of pork and fish gelatin did not differ significantly after 1 h incubation at 0 °C; after maturation for 20 h at 4–5 °C, the LCE values of the two gelatins were the same – 0.6% (Table 2). However, as expected, the deformation of gel formed at 4–5 °C from fish gelatin in 2% solution was much higher than that of pork gelatin. In preliminary experiments, the effect of transglutaminase at different concentrations on fish gelatin in 2% and 5% solutions was checked visually. In the case of 2% solutions of gelatin, incubated at room temperature for 24 h, gels were not formed in the presence of transglutaminase of concentrations lower than 0.35 mg/ml. They were obtained during 1–8 h at higher concentrations of enzyme protein (Table 3). The gels were not transparent as an effect of excessive crosslinking and exuded water after their mechanical breaking. A lower concentration of transglutaminase for gelation at room temperature was needed in the case of 5% fish gelatin solutions and the properties of more concentrated gelatin gels were better than those of 2% gels. The leaking of water after mechanical breaking appeared only at concentrations of enzyme of 0.55–0.7 mg/ml (Table 3). These data show that not only is the concentration of enzyme important for enzymatic modification of fish gelatin but also the concentration of substrate.

The transglutaminase was also active in crosslinking fish gelatin at 4–5 °C. The results were somewhat different from those obtained at room temperature because gels formed at this temperature did not melt at room temperature, even at concentrations of 0.1 mg enzyme/ml in 2% or 5% gelatin solutions (Table 4). However, these gels were very weak and, after mixing, resembled rather viscous sols. The gels formed at 4–5 °C were transparent over a broader range of enzyme concentration than gels formed at room temperature. Gelation of unmodified gelatin occurs by physical crosslinking, which generally leads to the formation of "junction zones" and finally a three-dimensional branched network (Gilsenan & Ross-Murphy, 2000). The gel strength of such gelatin depends on time and temperature of maturation and increases when temperature decreases (Choi & Regenstein, 2000). In enzymatically modified gelatin gels, wherein covalent bonds also participate in the three-dimensional branched network formation, those parameter are also very important because they affect the activity of the enzyme. We also found that the structures of enzymatically crossl-linked fish gelatin gels were not destroyed during 30 min of heating in a boiling water bath.

Fernández-Díaz et al. (2001) found that, at a concentration of transglutaminase preparation of 10 mg/ml,

Table 2
Properties of fish and pork gelatin

Source of gelatin	Least concentration endpoint (LCE) (% of protein)		Penetration depth for 2% gelatin gels[a] (mm)
	A	B	
Pork skin	0.9	0.6	2.9 ± 0.3
Baltic cod skin	1.1	0.6	12.4 ± 1.1

A, LCE after 1 h at 0 °C; B, LCE after 20 h at 4–5 °C.
[a] Mean values ± SD of five determinations. The deformation of gel was measured after 20 h at 4–5 °C. The weight of the plunger was 115 g.

Table 3
Effect of transglutaminase concentration on the gel-forming ability of fish gelatin at room temperature

Enzyme concentration (mg/ml)	Gelatin (%)	Time of reaction (h)						
		0.5	1	2	4	6	8	24
0.15	2	Sol	Sol	Sol	Sol	Sol	Sol	Sol
	5	Sol	Sol	Sol	Sol	Sol	Sol	Gel[a]
0.20	2	Sol	Sol	Sol	Sol	Sol	Sol	Sol
	5	Sol	Sol	Sol	Sol	Sol	Gel[a]	Gel
0.25	2	Sol	Sol	Sol	Sol	Sol	Sol	Sol
	5	Sol	Sol	Sol	Sol	Gel[a]	Gel	Gel
0.30	2	Sol	Sol	Sol	Sol	Sol	Sol	Sol
	5	Sol	Sol	Sol	Gel[a]	Gel	Gel	Gel
0.35	2	Sol	Sol	Sol	Sol	Sol	Gel[a]	Gel[a,b]
	5	Sol	Sol	Gel[a]	Gel	Gel	Gel	Gel
0.40	2	Sol	Sol	Sol	Sol	Gel[a]	Gel	Gel[b]
	5	Gel[a]	Gel[a]	Gel	Gel	Gel	Gel	Gel
0.55	2	Sol	Gel[a]	Gel	Gel	Gel	Gel	Gel[b]
	5	Gel	Gel	Gel	Gel	Gel	Gel	Gel[b]
0.70	2	Sol	Gel	Gel	Gel	Gel	Gel	Gel[b]
	5	Gel	Gel	Gel	Gel	Gel	Gel	Gel[b]

[a] Weak gel.
[b] Liquid is produced.

Table 4
Effect of transglutaminase concentration on the melting of fish gelatin gels at room temperature[a]

Enzyme concentration (mg/ml)	Gelatin (%)	Time (min)		
		30	60	90
0	2	Sol	Sol	Sol
	5	Sol	Sol	Sol
0.05	2	Sol	Sol	Sol
	5	Sol	Sol	Sol
0.1 – 0.7	2	Gel	Gel	Gel
	5	Gel	Gel	Gel

[a] Samples were incubated at 4–5 °C for 20 h.

Table 5
The deformation of gels formed from pork and fish gelatin in 2% solution incubated with 0.25 mg transglutaminase/ml at 4–5 °C for 20 h

Sample	Penetration depth[a] (mm)	
	A	B
Fish gelatin	10.1 ± 0.3	–
Control (without enzyme)	12.4 ± 1.1	–
Pork gelatin	3.1 ± 0.5	8.2 ± 0.7
Control (without enzyme)	2.9 ± 0.3	14.1 ± 0.9

The deformation of gels was measured at 6–8 °C, weight of the plug: A, 115 g; B, 690 g.
[a] Mean values ± SD of five determinations.

Table 6
The effect of concentration of transglutaminase on the deformation of gels formed from pork gelatin in 2% solutions incubated at 4–5 °C for 20 h

Enzyme concentration (mg/ml)	Penetration depth[a] (mm)
0	5.9 ± 0.4
0.05	5.4 ± 0.4
0.10	5.0 ± 0.4
0.15	4.4 ± 0.1
0.25	4.0 ± 0.1

The deformation of gels was measured at 6–8 °C, the plunger weight was 250 g.
[a] Mean values ± SD of five determinations.

gel strengths of gelatins from cod and hake skins after 16–18 h at 7 °C were, respectively, about 2 and 2.5 times higher than gelatins not modified enzymatically. According to these authors, transglutaminase preparations at concentrations higher than 15 mg/ml increased the gelling and melting temperature and produced a very elastic gel, but with lower gel strength than that obtained at lower concentration. Our experiments, first conducted with pork gelatin, showed that the deformation of 2% gels of pork gelatin, modified with transglutaminase at the highest concentration, 0.25 mg of enzyme protein/ml, was similar to the control sample without enzyme when a plunger of 115 g weight was used (Table 5). However, with a plunger of 250 g weight the deformation of 2% gels gradually decreased with increase of the concentration of enzyme protein in the studied range of 0–0.25 mg/ml (Table 6). This range of concentrations of enzyme protein, determined by the method of Lowry et al. (1951), corresponded to a 0–35 mg preparation of transglutaminase/ml. The difference in the gel strength of pork gelatin in the presence of 0.25 mg transglutaminase and that without enzyme was even more clear when deformation of the samples was determined with a plunger of 690 g weight (Table 5) or when the measurements were done at room temperature (Table 7). Similar results were obtained for 5% pork gelatin gels.

Modification of 2% fish gelatin with transglutaminase, at a concentration of 0.25 mg/ml, resulted in a decrease in deformation of gel as compared with that formed without enzyme (Table 5). However, the gels were not transparent, but milky and formed a liquid exudate. These unfavourable features increased during standing at room temperature which suggested that transglutaminase was still active in crosslinking of gelatin. In the case of 5% fish gelatin, differences in the gelling properties between modified and unmodified gels were seen when the deformation was measured under higher stress (Table 8). The data obtained with pork gelatin showed that the gel strengths of enzymatically modified gels differed at 4–5 °C and room temperature much less than those formed without enzyme (Table 7). The deformation of 5% fish gelatin gels, formed in the presence of 0.25 mg of enzyme protein/ml, was considerably lower at room temperature than at 4–5 °C (Table 8).

In all experiments, transglutaminase, at 0.25 mg/ml, slightly decreased the gel deformation. According to Fernández-Díaz et al. (2001), such a concentration can worsen the gel strength of fish gelatin. However, it is possible that the preparations of transglutaminase differed in enzyme activity. In some of our experiments, performed earlier with another batch of transglutaminase preparations, no melting fish gelatin gels were formed at lower concentrations of enzymes than those here. Moreover, it would also be interesting to check whether maltodextrin, contained in the transglutaminase preparation, influences the rheological properties of gelatins.

Table 7
The deformation of gels formed from pork gelatin in 2% and 5% solutions incubated with 0.25 mg transglutaminase/ml at 4–5 °C for 20 h measured at 6–8 °C (A) and at room temperature (B)

Sample	Penetration depth[a] (mm)	
	A	B
2% Pork gelatin [b]	4.0 ± 0.1	5.3 ± 0.2
Control (without enzyme)[b]	5.9 ± 0.4	Samples thoroughly punctured
5% Pork gelatin [c]	2.6 ± 0.1	3.5 ± 0.1
Control (without enzyme)[c]	3.0 ± 0.2	6.4 ± 0.3

[a] Mean values ± SD of five determinations.
[b] Weight of the plunger was 250 g.
[c] Weight of the plunger was 690 g.

Table 8
The effect of the concentration of transglutaminase on the deformation of gels formed from fish gelatin in 5% solutions incubated at 4–5 °C for 20 h measured at 6–8 °C (A) and at room temperature (B)

Concentration of the enzyme protein (mg/ml)	Penetration depth[a] (mm)	
	A	B
0[b]	3.8 ± 0.4	Sol
0.10[b]	3.2 ± 0.2	Weak gel
0.25[b]	3.2 ± 0.3	8.2 ± 0.5
0[c]	8.4 ± 0.3	Sol
0.25[c]	7.0 ± 0.3	12.6 ± 1.3

[a] Mean values ± SD of five determinations.
[b] The plunger weight was 250 g.
[c] The plunger weight was 690 g.

4. Conclusion

The skins of cod are a suitable raw material for extracting gelatin. Using minced skin significantly shortens the time of extraction in comparison with extraction methods where whole skins are used. About 85% of collagen from minced cod skins is converted to soluble gelatin after 30 min at 45 °C. Using these parameters, the yield of gelatin on a large laboratory scale was lower, amounting to 12.3% of the weight of the raw material (64% of collagen content in skins). However, it is still a satisfactory recovery of the collagenous material from the skins.

Modification of fish gelatin with transglutaminase allows the use of such a product as a gelling component in food production. The possibility of conducting the enzymatic reaction below room temperature is a great advantage. However, it is important to use enzyme and substrate in proper concentrations. Excessive enzymatic crosslinking of the material may not improve but may even lead to worsening of the product properties. On the other hand, fish gelatin gels cannot be produced at all if the concentration of enzyme is too low. Therefore, even in practical use of transglutaminase preparations for modification of fish gelatin, it would be important to determine the enzyme activity in order to perform enzymatic reaction at concentrations of enzyme which ensure desired properties of the product.

The enzymatically crosslinked gels did not melt during heating in the boiling water bath. This property may be useful when the activity of the enzyme must be inhibited. If the gel structure is not destroyed at higher temperatures, fish gelatin can find application as a gelling component of sterilized products.

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