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Optimization of condition for demineralization Baltic cod (*Gadus morhua*) backbone

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Abstract

Fish offal, among others, backbones, separated during mechanical processing of raw material can serve as an additional source of proteins, especially of collagen. To obtain native collagen previous deprotenization and demineralization of backbones is necessary. Therefore, the aim of this work was to determine optimal parameters for demineralization of Baltic cod backbones with HCl solutions, as well as by complexing metal ions with ethylenediamineteraacetic acid (EDTA). The best demineralization effect was achieved in 1.0 M HCl solution. After 24 h of extraction repeated three times, 99% of mineral substances were solubilized. Simultaneously, the smallest loss of collagen, up to 3%, was observed in these conditions. The process conducted in EDTA solution was less effective than that with HCl solution. After four times repeated 24 h extraction in 0.1 M EDTA solution only about 58% of mineral compounds were removed. Somewhat better efficiency was reached with 0.5 M EDTA solution. The yield of the process amounted to about 72%. Demineralization with EDTA did not cause loss of collagen.

Keywords: Cod; Backbone; Demineralization; Collagen

1. Introduction

Fish offal such as backbones, skins and heads which are separated during mechanical processing can serve an additional source of proteins, especially of collagen. The backbones are one of the major by-products yielding about 15% of the fish weight. They contain collagen, the proteins of connective tissue, as well as mineral salts, mainly calcium phosphate and carbonate. Content of individual components in osseous elements depends on the animal species. The collagen content in the backbones of bigeye snapper (*Priacanthus tayenus*), Atlantic cod (*Gadus morhua*), and Nile perch (*Lates niloticus*) amount to about 37, 30 and 25% of the dry weight, respectively (Gildberg, Arnesen, & Carlehög, 2002; Kittiphattanabawon, Benjakul, Visessanguan, Nagai, & Tanaka, 2005; Muyonga, Cole, & Duodu, 2004). Similar quantities of collagen are contained in the bones of warm-blooded animals like: cattle (33%) and pigs (29%) (Palka, Sikorski, & Sadowska, 1981), which are industrial sources of gelatin.

Bones, including fish backbones, are mineralized material with highly complex hierarchical structure. The basic building block of the bone is the mineralized collagen fibril, which is composed of very hard material, the mineral and much softer collagen fibrils. Mineralized collagen fibrils are always present in bundles or arrays aligned along their length. These fibril arrays organize into four common patterns: arrays of parallel fibrils, woven fiber structure, plywood-like structure, and radial fibril arrays. At a higher level of organization, the initially deposited primary bone undergoes internal remodeling and form the secondary bone with a central canal for blood vessels and nerves, which is called the "haversian system" (Wang, Cui, Ge, & Wang, 2004).

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It is possible to obtain native collagen or gelatin from bones after previous demineralization. Such collagen is called ossein. To dissolve mineral salts from osseous elements HCl is used. EDTA can also be used for this purpose since it forms soluble salts with many metals and minerals. According to the literature data, EDTA has been applied for demineralization of fish backbones, but the effectiveness of this process was not determined (Ikoma, Kobayashi, Tanaka, Walsh, & Mann, 2003; Nagai, Izumi, & Ishii, 2004; Nagai & Suzuki, 2000). Fish collagen, particularly from species living in cold waters, has different physicochemical proprieties, (e.g. solubility in acid medium) as compared with that from warm-blooded animals (Yamaguchi, Lavety, & Love, 1976). Therefore, the methods used for demineralization of cattle and pig bones cannot be applied for fish backbones. Moreover, at the high concentration of HCl usually used in this process the fish collagen is dissolved.

To date, fish backbones have been demineralized in HCl solutions at concentrations of 0.6–0.8 M in a process conducted from 2 to 12 days depending on the origin of backbones (Morimura et al., 2002; Muyonga et al., 2004). Demineralization conditions must be adapted to the properties of the raw material. Fish are cartilage-skeletal or bone-skeletal with different degrees of calcification. Cartilages contain on a dry weight only 10% of mineral substances while human bones contain about 58% (Zawistowski, 1973). Therefore, the aim of this work was to determine the best conditions for demineralization of Baltic cod backbones in HCl and EDTA solutions, simultaneously, without loss of collagen content.

2. Materials and methods

2.1. Raw material

Fish backbones were mechanically separated from fresh Baltic cod (*Gadus morhua*). Samples were minced in a meat grinder, using a mesh diameter of $\phi = 3$ mm. After thorough mixing of the minced backbones, approximately 500 g samples were prepared, and were stored at −20 °C in polyethylene bags. The dry weight, total nitrogen, ash and hydroxyproline contents in the raw material were determined.

2.2. Removal of non-collagenous protein

Non-collagenous protein were removed from minced backbones according to procedures described by Skierka, Sadowska, and Majewska (2006). The samples were mixed with 0.1 M NaOH solution (1:2, w/v), and kept at 4 °C. After 24 h the mixture was centrifuged at 10,000 g for 20 min and the supernatant discarded. This procedure was repeated twice. The residue, after alkaline extraction, was washed thoroughly with cold tap water to remove remaining muscle proteins and NaOH until the wash water reached neutral pH. The dry weight, ash, total nitrogen, and hydroxyproline contents in the deproteinised backbone were determined.

2.3. Demineralization process

The backbones were decalcified using 0.1, 0.5 M EDTA solutions (pH 7.5), or 0.1, 0.5, 1.0 M HCl solutions (1:5, w/v). The samples were homogenized at 4000 rpm for 2 min at 4 °C and next kept for 1–4 days at 4 °C with changing of the solution once a day. The demineralized backbones were then filtered through cotton-cloth. The dry weigh and ash content in the residue and the hydroxyproline content in all supernatants were determined. The yield of the demineralization was calculated using the following formula:

$$W = (A - B)/A \times 100$$

where: W – the yield of demineralization of backbones (%); A – concentration of ash in the raw material (%); B – concentration of ash in the demineralized sample (%).

Collagen loss (%) was determinated indirect method as the ratio of hydroxyproline extracted with HCl or EDTA solutions to their initial concentration in the raw material.

2.4. Dry weight, ash and total nitrogen content

The dry weight, ash and total nitrogen were determined according to AOAC methods (1990). The established conversion factor of nitrogen to protein was 6.25 (Sadowska, Kołodziejska, & Niecikowska, 2003).

2.5. Hydroxyproline

The hydroxyproline content 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 established conversion factor used for calculating of the collagen in the cod backbone from hydroxyproline content was 14.7 (Sadowska et al., 2003).

2.6. Statistical analysis

All experiments were replicated five times. Mean values with standard deviations (SD) were reported. Duncan's multiple-range test was used to evaluate significant differences ($P < 0.05$) between the means for each sample.

3. Result and discussion

3.1. Chemical composition of cod backbone

The collagen content in raw backbone was 24% of the dry weight. Taking into consideration the proportion of collagen in total protein, it can be evaluated that the raw backbones contain 52% of non-collagenous proteins, peptides and free amino-acid, in dry weight (Table 1). The rest

Table 1
Chemical composition of cod backbones on the basis of dry weight[a]

Component	Concentration (%)	
	Raw	After deproteinization
Crude protein (N × 6.25)	76 ± 4.4	63 ± 4.8
Ash	28 ± 1.3	41 ± 3.7
Collagen (hydroxyproline × 14.7)	24 ± 1.1	55 ± 2.6

[a] Mean values ± SD from five separate samples.

of the dry weight consisted of 28% of ash. These results are quite similar to the chemical composition of Atlantic cod backbone (Gildberg et al., 2002). Non-collagenous protein in cod backbone can be completely recovered by alkali extraction. After deproteinisation, cod backbones contained 41% of ash, and 55% of collagen in the dry weight.

3.2. Demineralization of cod backbone by HCl solution

The yield of demineralization depends both on the concentration of HCl solution and time of the process (Fig. 1). The amount of dissolved minerals increased with the increase of HCl concentration within a studied range. The largest percentage of the total content of minerals (about 100%) was removed by treating backbones with 1.0 M and 0.5 M HCl solutions for 3 days with changing the solution once a day. Using the same procedure with 0.1 M HCl solution, 44% of minerals were extracted. The amount of dissolved minerals was diminished when the process was conducted without changing of the HCl solution. The solubility of minerals after 24 and 48 h of treatment was similar, and amounted to about 83 and 87% in 1.0 M HCl, 74 and 72% in 0.5 M HCl, 10 and 15% in 0.1 M HCl, respectively.

The results presented in Fig. 1 characterize relatively large standard deviations, especially after demineralization with 1.0 M HCl solution. This is caused by very small content of minerals in the dry mass of osein and in consequence problems with the precise determination of ash residue in samples.

During the demineralization in HCl, the part of collagen contained in backbones was solubilized. The solubility of collagen depended on the concentration of acid. Loss of collagen from backbones after triplicate demineralization for 24 h in 0.1 M and 0.5 M HCl solutions was from 8.3–13.9 and 2.3–9.1%, respectively. In such acidic medium (Table 2) collagen swells and this facilitates its solubility. The decrease of the dry mass of backbones confirmed this phenomenon (Table 3). Swelling of the material during demineralization in 0.1 M and 0.5 M HCl solutions also caused difficulties during sample filtration. The collagen was considerably less soluble in 1.0 M HCl solution. The loss of collagen amounted to only from 0.2 to 3.2%. The hydration of collagen is at a maximum in the pH range 3–4. At low pH, like in the case of backbones decalcified in 1.0 M HCl solutions (Table 2), the water uptake of collagen is significantly lower. The structure of collagenous fibres is tightened and solubility of collagen is reduced, because there is less space for water between macromolecules.

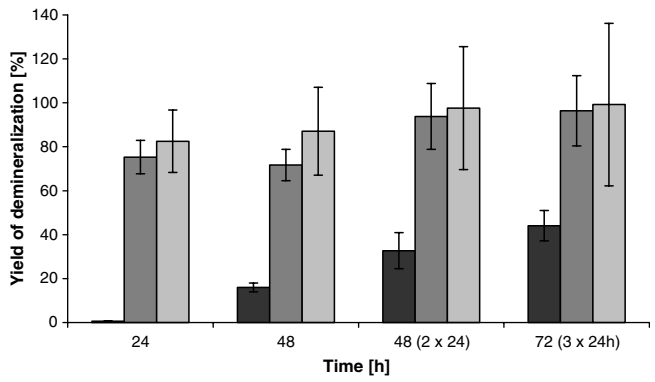

Fig. 1. Effect of time and HCl concentration on the yield of demineralization. Concentration of HCl: 0.1 M (■), 0.5 M, (■), 1.0 M (■).

3.3. Demineralization of cod backbone with EDTA solution

The 0.1 M EDTA solution was less efficient as an extractant of mineral salt than HCl solution. During 72 h of demineralization about 31% of mineral salts was removed and about 58% after 96 h (Fig. 2). Larger efficiency of removing of mineral salts was achieved in 0.5 M solution of EDTA (about 72% after 72 h). The extension of the time of demineralization in 0.5 M EDTA solution to 96 h brought only small changes in the content of ash in backbones. The three-stage process of demineralization was more efficient in comparison with the continu-

Table 2
pH of the mixture backbones with HCl or EDTA solution before and after 24 h demineralization

Concentration of solution	pH of the mixture	
	Before the process	After the process
HCl		
0.1 M	1.0	4.3
0.5 M	0.3	1.6
1.0 M	0.0	1.1
EDTA		
0.1 M	7.5	7.8
0.5 M	7.5	7.5

Table 3
Concentration of dry weight in the cod backbone after demineralization in HCl[a]

HCl concentration	Dry weight (%)			
	24 h	48 h	48 (2 × 24) h	72 (3 × 24) h
0.1 M	15 ± 2.9a	18 ± 5.6a	12 ± 2.8a	10 ± 2.5a
0.5 M	18 ± 3.7a	17 ± 2.6a	14 ± 1.5ab	11 ± 1.3a
1.0 M	25 ± 7.1a	26 ± 5.6a	28 ± 2.8b	23 ± 1.3b

[a] Mean values ± SD from five separate samples. Means in the same columns with different letters are significant difference ($P < 0.05$).

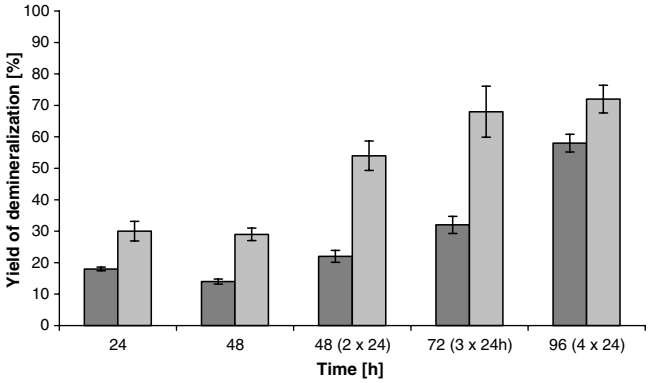

Fig. 2. Effect of time and EDTA concentration on the yield of demineralization. Concentration of EDTA: 0.1 M (■), 0.5 M (□).

Table 4
Concentration of dry weight in the cod backbone after demineralization in EDTA[a]

EDTA concentration	Dry weight (%)				
	24 h	48 h	48 (2 × 24) h	72 (3 × 24) h	96 (4 × 24) h
0.1 M	27 ± 1.0a	27 ± 1.1a	25 ± 1.4a	25 ± 2.4a	26 ± 1.2a
0.5 M	31 ± 1.1a	30 ± 1.0a	30 ± 1.0a	32 ± 0.7a	31 ± 0.9a

[a] Mean values ± SD from five separate samples. Means in the same columns with different letters are significant difference ($P < 0.05$).

ous process (Fig. 2). The material in the presence of EDTA did not undergo swelling and therefore the water content in demineralized backbones was not dependent on the concentration of this substance (Table 4). For these reasons, the difficulties with filtration, which were observed during using of 0.1 M and 0.5 M HCl solutions, did not occur in the case of EDTA. Moreover, demineralization of backbones with EDTA solution did not cause the loss of collagen. The insolubility of collagen results from slightly alkaline extracting medium (Table 2), the pH of the solution corresponds to isoelectric point of collagen.

4. Conclusion

The best effect of demineralization of cod backbones, almost 100%, was achieved with 1 M HCl solution during 72 h of extraction with changing the solution once a day. The loss of collagen amounted to only about 0.2–3.2%. In the same conditions, but with using 0.5 M EDTA solution, it was possible to remove 65% of mineral salts from backbones, and additionally about 7% after prolongation of demineralization by a further 24 h. In contrast to HCl, EDTA does not cause any loss of collagen, but the efficiency of the process is lower.

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