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Food Chemistry 105 (2007) 1302–1306
Food Chemistry
www.elsevier.com/locate/foodchem

Analytical, Nutritional and Clinical Methods

The influence of different acids and pepsin on the extractability of collagen from the skin of Baltic cod (*Gadus morhua*)

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Received 26 January 2007; received in revised form 22 February 2007; accepted 17 April 2007

Abstract

Solutions of (0.5 M) citric, lactic and acetic acids and 0.15 M HCl were used for the extraction of collagen from the whole skins of Baltic cod (*Gadus morhua*). The extractions were performed at a temperature of 4 °C for 24, 48 and 72 h using a solid/solution ratio of 1:6 (w/v). Of the acids used, HCl was the least effective solvent for collagen. The maximal yield of collagen extracted with citric acid was 60%. Collagen extraction with acetic or lactic acid give a maximal yield of about 90% with HCl yielding of only 18%. After enzymatic treatment of cod skin the yield of protein extracted with HCl and citric acids increased to 40% and 20%, respectively. Collagen was completely solubilized under the same conditions in acetic and lactic acids. Electrophoretic analysis of collagens extracted in HCl and citric acids with enzymatic treatment proved that the isolated protein was denaturated. The solutions of acetic and lactic acids are solvents for native collagen.

Keywords: Cod; Collagen; Fish offal

1. Introduction

Fish offal, such as bones, scales, fins, as well as the skins and collagenous membranes of squid separated during mechanical processing, can serve as an alternative source of connective tissue, but they have not been rationally utilized up till now. Fibres or fibrils of collagen, depolymerized to the basic structure – tropocollagen, can be reconstituted to native structures or other forms of fibrils. This raises many possibilities for increasing the number of products from collagen with different functional properties and uses. Collagen from total tissue can be isolated by direct extraction with organic acids (acetic, chloracetic, citric, lactic) or inorganic acid (hydrochloric). The yield of extracted collagen depends on the animal species used and the age and parameters of extraction. A number of studies have referred to collagen from different marine animals extracted by acetic acid. The solubility of skin collagen in 0.5 M acetic acid solution varies between 2% and 90%. (Ciarlo, Paredi, & Fraga, 1997; Muyonga, Coleb, & Duodub, 2004; Nagai & Suzuki, 2002b; Nagai, Yamashita, Taniguchi, Kanamori, & Suzuki, 2001; Sadowska, Kołłodziejska, & Niecikowska, 2003). The literature on the extraction of collagen by different acids and their influence on protein yield is limited.

It is possible to increase the yield of collagen extracted by mechanical, chemical or enzymatic pretreatments. In the case of fibrils and fibres reconstitution, the essence of the process is to select an adequate pretreatment of raw material to extract the ripe, insoluble collagen without modification of the macromolecule (Reich, 1970). From a chemical point of view, it means the necessity of removing numerous intra- and intermolecular covalent cross-links, mainly involving lysine and hydroxylysine residues, ester bonds and bonds with saccharides. One of the methods to increase the yield of collagen extraction is to apply the enzymatic pretreatment of connective tissue by proteolytic

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doi:10.1016/j.foodchem.2007.04.030

enzymes non-specific for collagen such as: pepsin, trypsin, pancreatin (Higheberger, 1961; Nishihara, 1962) ficin, bromelain or papain (Hochstadt, Park, & Lieberman, 1960). These enzymes remove only the non-helical ends (telopeptides) of the collagen. Because of the cutting of the telopeptides region they remove intermolecular cross-links, even the most stable in an acid medium (Bailey & Light, 1989; Hickman et al., 2000). During enzymatic treatment of connective tissue, not only the physico-chemical properties of collagen are changed, but also non-collagen proteins are hydrolyzed. The increase in solubility of collagen after enzymatic treatment depends on fish species. Collagen from skins of some fish species is completely soluble in acetic acid by enzymatic digestion (Nagai, Araki, & Suzuki, 2002a; Nagai & Suzuki, 2002b; Nagai et al., 2001; Senaratne, Park, & Kim, 2006), but for other species, the solubility of collagen after enzymatic treatment increases by only about 5% (Jongjareonrak, Benjakul, Visessanguan, Nagai, & Tanaka, 2005). Large differences in collagen solubility after enzymatic treatment can be caused by different location and type of intermolecular cross-links, especially those with saccharide participation. Collagen from the mantle of squid *Loligo pealei* was almost completely soluble in 0.2 M acetic acid solution after treatment with α-amylase (Hunt, Grant, & Lebovitch, 1970).

In the literature, there is no information about the influence of different acids and pepsin concentration on the yield of native collagen extracted from Baltic cod (*Gadus morhua*). Therefore, the aim of this work was to compare the yields of collagen extracted from fish skin with different acids: hydrochloric, acetic, citric and lactic. The objective of the investigation was also to check, by the electrophoresis method, that collagen was not denatured during the acid extraction, and hydrolyzed by pepsin.

2. Materials and methods

2.1. Raw material

The skins of fresh Baltic cod (*G. morhua*) were mechanically separated with the residue of adhering tissues removed manually. After thorough mixing of the skins, samples (approximately 500 g), were prepared and stored at −20 °C in polyethylene bags. For chemical analysis, the frozen samples were minced in a meat grinder, using a mesh diameter of $\varnothing = 3$ mm. The dry weight and hydroxyproline content in the raw material were determined and amounted to 26.5% and 1.3%, respectively. Pepsin (300 U/mg) powder for enzymatic treatment was purchased from Fluka.

2.2. Isolation of collagen from skins

Collagen was extracted from the whole skin according to Fig. 1. Extractions were performed at 4 °C for 24, 48 and 72 h, with solutions of 0.15 M HCl and 0.5 M of citric, acetic or lactic acid. The samples were mixed using a solid/solution ratio of 1:6 (w/v). The final pHs of the mixtures with HCl, citric, lactic and acetic acids were: 2.0, 2.2, 2.8, 2.9, respectively. Extraction of collagen with enzymatic treatment was achieved by the same method. Concentration of enzyme in the samples amounted to 3.3, 6.6, 10 and 20 mg/g raw material. Hydroxyproline was also determined in the supernatants. The yield of the extraction (%) was calculated as the ratio of extracted hydroxyproline to initial concentration in fish skin. The experiments for each acid, with and without pepsin digestion, were replicated four times. All solutions of collagen were analyzed using the electrophoresis method.

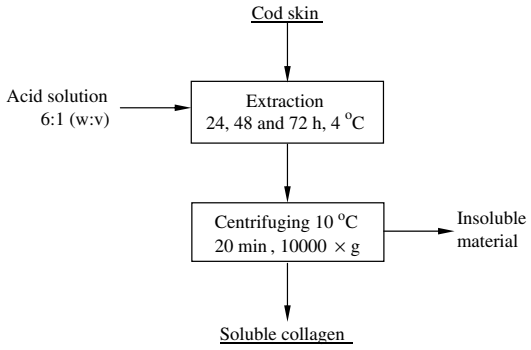

Fig. 1. Flow sheet of the procedure used for isolation of collagen from cod skin with different acids.

2.3. Dry weight and hydroxyproline content

The dry weight was determined according to AOAC methods (AOAC, 1990). 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).

2.4. SDS–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed according to the method of Laemmli (1970) using 8% separation gel and 5% stacking gel. Collagen samples were dissolved in sample buffer (Tris–HCl, pH 6.8 containing 2-mercaptoethanol, 2% SDS). Samples containing approximately 3 μg of collagen/μl were heated at 50 °C for 5 min. A 10 μl of sample was loaded per well. Protein bands were stained with Coomassine Brilliant Blue R250 and dissolved in a mixture of water, methanol, and acetic acid (4:5:1, v/v/v) and destained using a solution containing methanol, water and acetic acid (1:8:1, v/v/v). High-molecular-weight markers (Sigma Chemical Co., St. Louis, Mo, USA) were used to estimate the molecular weights of the proteins.

2.5. Statistical analysis

All experiments were replicated four times. Mean values with standard deviations (SD) were reported.

3. Results and discussion

3.1. The effect of acid and time on the extraction of collagen

Among the acids tested (Fig. 2), HCl was the least effective solvent for collagen extraction from the skins. During 72 h of extraction only about 18% of collagen was dissolved. The amount of dissolved collagen was increased when the skins were incubated in citric acid. In this acid 60% of collagen was dissolved. The solubility of the collagen in acetic and lactic acid was similar with an average of 90%. In lactic acid, collagen was completely solubilized after 24 h of extraction. Prolongation of extraction time from 24 to 72 h increased collagen solubility in the residual acid. The largest increase in solubility of collagen in citric and acetic acid was observed over 48 h. The incomplete solubility in the chosen acids, suggests that inter-molecular cross-links are still present in collagen. The first stage of solubilization of this protein is hydration of fibrous collagen which proceeds by exposure to acids or bases. Cattle skin collagens have a maximum degree of swelling near pH 3 (Gustavson, 1956). A very low pH value, as in the case of 0.15 M HCl (pH 0.87), reduces water absorption of collagen. This is probably due to the positively charged amine groups of protein bonding with anions (Cl^-), reducing electrostatic repulsive forces between one-nominal charged groups. In consequence, the structure of collagenous fibres is tightening, the ability of bonding water decreases, and the solubility of collagen is therefore reduced. Below pH 2 proteins are denaturated. Collagenous fibres are shrinking making protein hydration impossible because there is less space for water among the macromolecules. Digestion with strong acid for a longer time could be a reason for partial chemical hydrolysis of protein. The pH of citric acid was low, about 1.8, while pH's of acetic and lactic acid were 2.3 and 2.2, respectively.

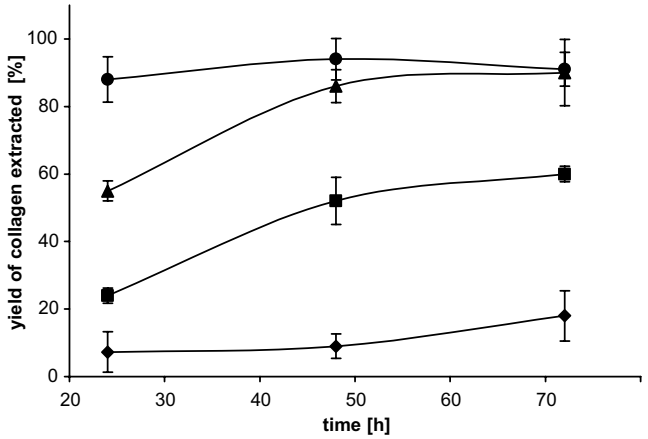

Fig. 2. The effect of acid and time on the yield of extracted collagen: ♦, HCl; ■, citric acid; ▲; acetic acid; ●, lactic acid.

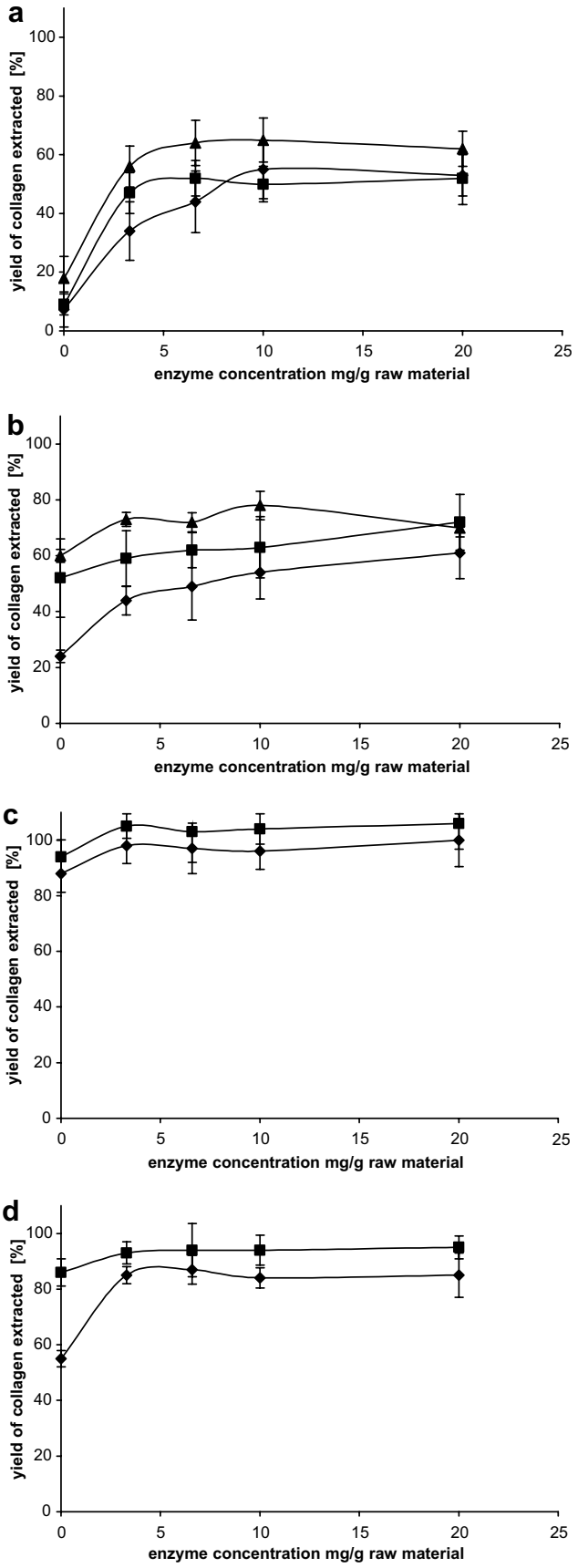

Fig. 3. Effect of pepsin concentration and time on the yield of extracted collagen: ♦, 24 h; ■, 48 h; ▲, 72 h (a, HCl; b, citric acid; c, lactic acid; d, acetic acid).

3.2. Effect of concentration of enzyme and time on extraction of collagen

The solubility of collagen in HCl (Fig. 3a) and citric acids (Fig. 3b) depended upon the enzyme concentration. The maximum solubility of collagen was obtained at an enzyme concentration of 10 mg/g raw material. The larger concentration of enzyme did not increase the solubility of collagen, whereas in the case of lactic acid (Fig. 3c) and acetic acid (Fig. 3d) the lowest concentration of enzyme – 3.3 mg/g raw material, was sufficient to solubilize skin collagen completely. The largest influence of enzyme concentration on solubility of collagen in citric acid and HCl was observed during 24 h of extraction (Fig. 3a and b). After enzymatic treatment of fish skin in citric acid, a maximum of 75% of collagen was extracted, about 10% more than in HCl. Enzymatic treatment in citric acid, for longer than 24 h, caused an increase in solubility of collagen by about 20%, but in the case of HCl by about 40%. Enzymatic treatment of skin in acetic acid shortened the extraction time to 24 h. Solubility of collagen, after pepsin digestion increased from 55% to 90%. Collagen from cod skin was solubilized completely after enzymatic treatment in lactic acid, after 24 h.

The yield of collagen extraction in HCl and citric acid was dependant upon the reaction time. During 24 h of protein extraction in HCl with pepsin treatment (3.3 mg/g raw material), about 33% of collagen was solubilized, and after 72 h about twice more. Whereas in citric acid with the same concentration of enzyme the yield of collagen extracted after 72 h increased by about 65% in comparison with the 24 h digestion.

3.3. Electrophoretic characterization of collagen

The SDS–PAGE analysis showed that collagen from Baltic cod skin contained two different chains α_1 and α_2 (Fig. 4). The β-component present in the electrophoretogram confirms that collagen contains inter molecular cross-links. This kind of dimer was also observed in collagen of skate (*Raja kenojei*) (Mizuta, Hwang, & Yoshinaka, 2002), rabbit, carp (Sato, 1999), brown backed toadfish (*Lagocephalus gloveri*), (Senaratne et al., 2006), Dover sole (*Solea vulgaris*) (Giménez, Turnay, Lizarbe, Montero, & Gómez-Guillén, 2005), Brownstripe red snapper (*Lutjanus vitta*) (Jongjareonrak et al., 2005) and Nile perch (*Lates niloticus*) (Muyonga et al., 2004).

The collagen extracted in HCl and citric acid with pepsin treatment contained products of enzymatic hydrolysis which were below 116 kDa in the electrophoretogram. Collagen extracted in citric acid was further degradated. Dissolved collagen was degradated by pepsin during 24 h of extraction. Within the next two days, no new products of degradation of protein appeared. This excludes HCl and citric acid as solvents of native collagen. Electrophoretic analysis of collagen extracted in acetic and lactic acid with enzymatic treatment has proved that isolated protein was native. Only α- and β-components, which did not undergo enzymatic digestion, were observed in the electrophoretogram.

Gustavson (1956) suggests that the weak organic acids cause hydrotropic swelling. This means that hydrogen bridges existing in collagen are replaced by hydrogen bonds with a hydrotropic substance. In consequence, hydrophobic bondings are removed causing denaturation of dissolved collagen. The undissociated molecules of organic acid are responsible for the hydrotropic swelling. This phenomenon explains the existence of enzymatic degradation products with molecular mass below 116 kDa observed in the electrophoretogram.

4. Conclusions

The best solvents for collagen were acetic acid and lactic acid. Collagen from cod skin extracted by these acids with enzymatic treatment, was completely dissolved, while under the same conditions HCl and citric acids extracted only 65% and 75% of collagen, respectively. The solvents

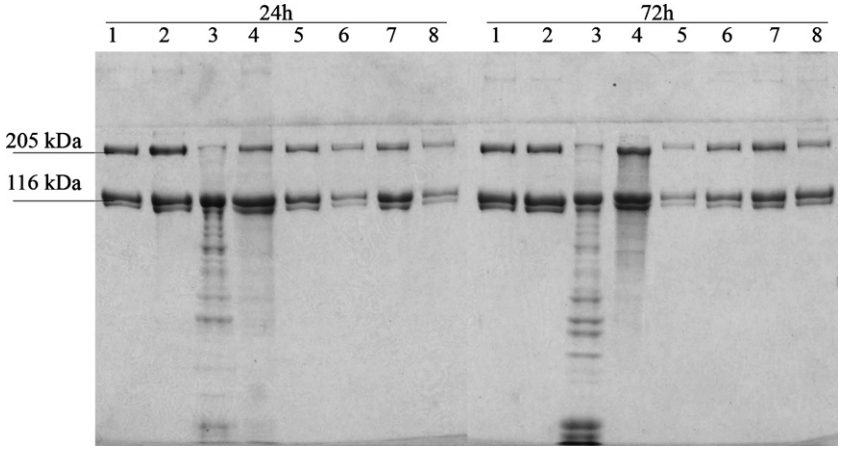

Fig. 4. SDS–PAGE pattern of collagen from cod skin extracted with different acids: 1, acetic acid with pepsin treatment; 2, lactic acid with pepsin treatment; 3, citric acid with pepsin treatment; 4, HCl with pepsin treatment; 5, acetic acid; 6, lactic acid; 7, citric acid; 8, HCl.

for native collagen are 0.5 M acetic and 0.5 M lactic acid. Collagen fibrils solubilized in acetic acid and lactic acid did not undergo enzymatic degradation. Only α_1, α_2 and β-chains were observed in the electrophoretogram.

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