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Isolation and characterization of a thermally stable collagen preparation from the outer skin of the silver carp *Hypophthalmichthys molitrix*

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ABSTRACT

Currently the main sources of collagen are still the skins of cows and pigs. However, the recent outbreaks of bovine spongiform encephalopathy (BSE) have sparked the development of new, alternative sources of safe collagen for industrial uses. In this work we studied the possibilities of using silver carp *Hypophthalmichthys molitrix* skin waste as a potential rich source of collagen. The collagen-containing preparation extracted from silver carp skins was a homogeneous, viscous gel, free of mechanical impurities. It was transparent and had a pH of 3.64. The water content varied from 95.0 to 97.5%. Total nitrogen was 14.3%, and the hydroxyproline content was 7.2%. After hydrolysis, glycine was the major amino acid in the collagen from the silver carp skins. There were also relatively high contents of proline, hydroxyproline, alanine and glutamic acid, in decreasing order of magnitude. The isolation procedure did not eliminate non-collagenous proteins extracted under the acidic conditions that probably thermally stabilize the isolated protein. Collagen is the main protein in the preparation, with an average total protein content of 75%. The protein consisted of two α chains (α1 and α2) classified as type I collagen. The remaining 25% of the protein fraction comprises smaller fragments that appear under the α bands corresponding to molecular masses ranging from 70–150 kDa, including a probable elastin band (8%); the remainder are unknown proteins and peptides (17%). The denaturation temperature (revealing thermal stability) of collagen from silver carp skin investigated using circular dichroism spectroscopy and differential scanning calorimetry was 34.5–34.8 °C.

1. Introduction

Collagen is the main protein of connective tissue in animals and the most abundant protein in mammals, making up about 25% of the total protein content (Sikorski et al., 1990). It is a unique protein, able to form insoluble fibers with a high tensile strength and containing a right-handed triple superhelical rod consisting of three polypeptide chains (Gelse et al., 2003). Isolated from natural sources, it has a wide applicability in various branches of industry. In the non-denatured form, it has pharmaceutical and biomedical uses, such as tissue engineering for implants in humans, inhibition of angiogenic diseases, treatment of hypertension, urinary incontinence and osteoarthritis (Lee et al., 2001). It is also a very attractive substrate for many fragrance and cosmetic applications (Tzaphlidou, 2004). In the denatured form (gelatin) it is widely used in the food industry (Slade and Levine, 1987). The main sources of collagen nowadays are still the skins of cows and pigs. However, the recent outbreaks of bovine spongiform encephalopathy (BSE) have sparked the development of new, alternative sources of safe collagen for industrial uses (Gareis, 2002).

The focus has therefore shifted to fish skin, bones and scales, obtained from fish processing waste, as an alternative source of collagen. Several papers have reported on the characteristics and possible isolation procedures of skin and bone collagen from various fish species (Yata et al., 2001; Nagai and Suzuki, 2002; Sadowska et al., 2003; Muyonga et al., 2004; Jongjareonrak et al., 2005; Zhang et al., 2007). Especially if waste material is used, the technology aims not only make a value-added product, but also to dispose of malodorous waste. The low thermal stability of fish proteins, when compared to mammalian collagen, limits some of its various uses.

In this work we studied the possibilities of using silver carp *Hypophthalmichthys molitrix* skin waste as a potential rich source of collagen. The isolated protein was characterized for its content and thermal stability. The silver carp belongs to the order *Cypriniformes* and is a member of the *Cyprinidae* family. This benthopelagic fish, naturally inhabiting freshwaters in China and Eastern Siberia, has been introduced around the world for both aquaculture and the control of algal blooms. The world production of this species in aquaculture exceeds 1 million tons per year. The waste from silver carp processing is therefore a potentially vast source of collagen.

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2. Materials and methods

2.1. Fish

All fish (silver carp *H. molitrix*) used in this study originate from the farm located in the Balaton Lake (Hungary). Except for natural phytoplankton no additional feeding was applied. They were caught in the age from 3 to 5 years old (weight of 2 to 8 kg). Silver carp skins (both sides and the bottom) — by-products of filleting, prior to extraction, were washed with chilled distilled water for 10 min and freed from adhering tissues.

2.2. Preparation of collagen

All the preparative procedures were carried out at 5 °C. The cleansed skins (0.3 kg) were extracted with 4 l of 0.5% glycolic acid (Sigma Aldrich) for 18 h. The extract (4 l) was then passed through a set of polyester filters (Amafilter B.V.) with decreasing pore sizes (from 10 to 5 μm). Filtration was repeated several times until a transparent and odorless gel preparation was obtained (3 l).

2.3. Amino acid analysis

The amino acid composition of the silver carp skin collagen (Inventia Polish Technologies Ltd.) was analyzed at BioCentrum Ltd. (Kraków, Poland) (Biocentrum, 2008). The protein samples were hydrolyzed in the gaseous phase with 6 M HCl at 115 °C for 24 h. The unhydrolyzed residue was also analyzed for the presence of free amino acids. The liberated amino acids were converted into phenylthiocarbamyl (PTC) derivatives and analyzed by high-pressure liquid chromatography (HPLC) on a PicoTag 3.9×150 mm column (Waters, Milford, MA, USA).

2.4. Hydroxyproline and total nitrogen

The hydroxyproline content was determined photometrically after hydrolysis of the extract samples in 3 M sulfuric acid for 16 h at 105 °C, oxidation of the hydroxyproline using chloramines T, and finally reaction with *p*-dimethylaminobenzoic aldehyde to obtain a reddish complex. The hydroxyproline content was determined according to a method recommended by the ISO (Anonymous, 1978).

The total nitrogen content in the dry weight of extract was determined on an EA 1108 elemental analyzer (Carlo Erba Instruments).

2.5. SDS-polyacrylamide gel electrophoresis

Sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis was run according to the method of Laemmli (1970). Samples (10 μg of the purified isoenzymes) or reference proteins were incubated for 5 min at 100 °C in 35 mM Tris–HCl buffer (pH 7.8) containing 5% SDS, 10% 2-mercaptoethanol, 20% glycerol and 0.03% bromophenol. The electrophoresis was carried out at room temperature on 5% and 10% polyacrylamide gels using a vertical slab gel apparatus (MINIPOL 2 Unit — P.P.H. BIOTEST, Poland). The electrode buffer was 0.19 M Glycine–Tris+0.01% SDS, pH 8.3. A constant current of 10 mA per gel was applied for 2 h. The proteins were stained with Coomassie Brilliant Blue R-250. The protein bands were scanned and the band intensities quantified using the Quantity one program (Biorad). Molecular weight markers were from Fermentas International Inc. (Canada). SDS-polyacrylamide gel electrophoresis was done for collagen stored at 4 °C and 25 °C.

2.6. Differential scanning calorimetry (DSC)

Denaturation temperatures were evaluated on a VP-DSC (MicroCal) differential scanning microcalorimeter. 0.5 g samples were immersed in 100 ml of 0.5% glycolic acid (Sigma Aldrich). The temperature was raised at a constant rate of 90 °C/h from 10 to 60 °C. The standard deviation was 0.06 kcal/(mol×°K). The denaturation temperature was determined using software provided by the instrument's manufacturer (Origin 7.0).

2.7. Circular dichroism measurement

CD spectra were measured using J-20 and J-815 spectrometers (JASCO, Easton, MD). Lyophilized collagen (0.05 g) was diluted with 5 ml 0.1 M glycolic acid and the solution placed in a quartz cell with a path length of 0.1 or 1 cm. CD spectra measurements were performed at 26 °C, 28 °C, 30 °C, 31 °C, 32 °C and 34 °C for wavelengths of 193–260 nm at a scan speed of 2 cm/min. The mean molar ellipticity (θ) was calculated using the mean residue molecular weight and expressed in deg×cm^2/dmol. The data were cumulated three times. In order to determine collagen denaturation temperatures, the rotatory angle at a fixed wavelength of 221 nm, $[\theta]_{221}$, was measured as a function of temperature. The denaturation temperature, T_m, was determined as the temperature at which the change in ellipticity (θ) was half complete.

2.8. Determination of viscosity

Collagen samples (0.04 to 0.4 g/l) were prepared in the same manner as the CD sample. A DV-II Viscometer (Brookfield Engineering Labs) was used to measure viscosity. 0.5 ml of the sample solution was incubated at 25 °C for 15 min. The viscosity (cP) was calculated using the Herschel–Bullkley equation (De Larrard et al., 1998).

3. Results and discussion

3.1. Proximate analysis of collagen and amino acid composition

The collagen-containing preparation extracted from silver carp skins was a homogeneous, viscous gel, free of mechanical impurities (viscosity=2,178,079±4357 cP). It was transparent (pigment-free) and had a pH of 3.64. The water content varied from 95.0 to 97.5%. Total nitrogen was 14.3%, and the hydroxyproline content was 7.2%. The dry weight protein content was estimated at 89.4%. The well-known conversion factor of nitrogen to collagen is 6.25 (Sadowska et al., 2003), the same as is used to calculate non-collagenous proteins. Taking into consideration the proportion of collagen in the total protein, calculated on the basis of the hydroxyproline content in the samples, the skins contained 14.4% of non-collagen free amino acids, peptides and proteins on a dry weight basis. The collagen content in the skin amounts 75% in the dry weight (89.4%–14.4%). Apart from the protein/nitrogen fraction there were small amounts of lipids, mucopolysaccharides and ash. In cod skins, the collagen and non-collagenous protein contents depend upon the fishing period. When food is lacking, albumins and globulins are degraded and the amount of collagen in the skins increases (Love et al., 1976; Sikorski et al., 1984). The differences between fish species and the isolation method of collagen could also results in the different quantity of the collagen. This fact could be responsible for the difference between our results and those of others. For example Sadowska et al. (2003) reported 71% of collagen content in the Baltic cod skin, Muyonga et al. (2004) found 61% of the collagen content in the Nile perch skin whereas Nagai and Suzuki (2000b) reported 51.4% of the collagen content in the Japanese sea-bass, 49.8% in the chub mackerel and 50.1% in the bullhead shark, respectively.

The amino acid composition, expressed as residues per 1000 total residues, is shown in Table 1. The amino acid content in a sample before hydrolysis was glycine, arginine, tyrosine, proline and alanine. The amount of hydroxyproline, the amino acid characteristic of collagen, was lower. Hydroxyproline is an endogenous amino acid and its free presence in the collagen extract could have a considerable