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Parameters affecting the isolation of collagen from squid (*Illex argentinus*) skins

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Abstract

The solubility of collagen from squid (*Illex argentinus*) skin in salt solutions and the efficiency of removal of skin chromatophores were determined. Homogenization of minced squid skin in 5–15% NaCl solution at 0°C solubilized 35–24% of total amount of crude protein and caused 2–5% loss of collagen but was not effective in removing pigments from the skin. The treatment of whole squid skins in 5–15% NaCl solutions at room temperature led to separation of the chromatophores, but the loss of soluble collagen was 57–16%. Collagen, soluble in dilute acid solutions was isolated from squid skins by 24 h soaking in 10% NaCl solution at room temperature, washing with water and bleaching for 48 h in 1% H_2O_2 in 0.01 M NaOH. The yield of collagen was 53%. It could be increased to 90% by using NaOH solution at pH 11.5 instead of 10% NaCl but the isolate was less soluble in dilute acid and the viscosity of 0.5% dispersion of collagen was four times lower. The rancid off-odour could be prevented by adding 0.5% of a non-ionic detergent to all solution used in the procedure.

1. Introduction

The skins and collagenous membranes separated from the squid mantle during processing are not particularly suitable to be converted into fodder meal, as they contribute to a large increase in the viscosity of the mass of offals after heating and make the drying of the meal more difficult. On the other hand, they could serve as a valuable source of collagen.

Collagen preparations are usually produced from offals from slaughtered animals containing connective tissue. The preparations have very broad industrial applications, ranging from gelling materials, through edible and photographic gelatine, to sausage casings. Soluble collagen preparations are used in cosmetics. Special types of collagenous material serve in medicine in the form of surgical aids, in pharmacy and as supports of enzymes or biologically active compounds. Collagen hydrolysates have found various applications as functional food additives and can be regarded as a source of biologically active peptides.

Collagens from marine animals, mainly from fish skins, are soluble in salt solutions and in dilute acids and acid buffers (Yamaguchi, Lavety, & Love, 1976; Sikorski, Scott, & Buisson, 1984; Sadowska & Sikorski, 1987; Montero, Alvarez, Marti, & Borderias, 1995; Yoshinaka & Sato, 1990; Vis, Lammers, & de Wolf, 1996). The solubility is higher than that of collagen from the connective tissues of slaughtered animals. This property may be an asset, although it may also decrease the yield of the collagen preparation due to large losses at the stage of purification from noncollagenous material. Lipids contained in the tissues of marine animals are very prone to oxidation, thus leading to development of off-odours that are difficult to eliminate from the final product.

The objective of the investigations was to determine the solubility of collagen from squid skin in salt solutions of different acidities and the effect of these solvents on the colouring compounds present in the skin chromatophores, as well as to optimise the procedure leading to the preparation of odourless and colourless soluble collagen.

2. Materials and methods

The skins from mechanically separated mantle of squid, *Illex argentinus*, were obtained from the deep-sea fishing enterprise "Dalmor". They were kept frozen at

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−20°C before use in the experiments. Collagen was prepared from whole skins using the procedure presented in Fig. 1. In several other experiments disintegrated material was used.

The yield of collagen and loss at different stages of the process were measured by determining hydroxyproline in the materials and extracting solutions. The collagen isolates were characterized by the contents of nitrogen, hydroxyproline, dry mass, viscosity and electrophoretic pattern. The dry mass was determined by drying at 105°C, total nitrogen after Kjeldahl, and hydroxyproline, after hydrolysis of the material in 6 M HCl for 8 h at 110°C, using the colorimetric method recommended by ISO (1978). The viscosity of 0.5% of collagen in dilute HCl solution at pH 3.5 was measured in the Brookfield instrument DV-II, using a disc spindle LV and 60 rpm. The electrophoresis of collagen was performed, in principle, according to the methods of Weber and Osborn (1969) at 8 mA/gel on 5% SDS-polyacrylamide gel in 0.1 M phosphate buffer, pH 7.2, containing 0.1% SDS and 0.5 M urea. Samples of collagen for electrophoresis were dissolved in 0.01 M phosphate buffer containing 1% SDS and 0.5 M urea and denatured at 40°C for 10 min.

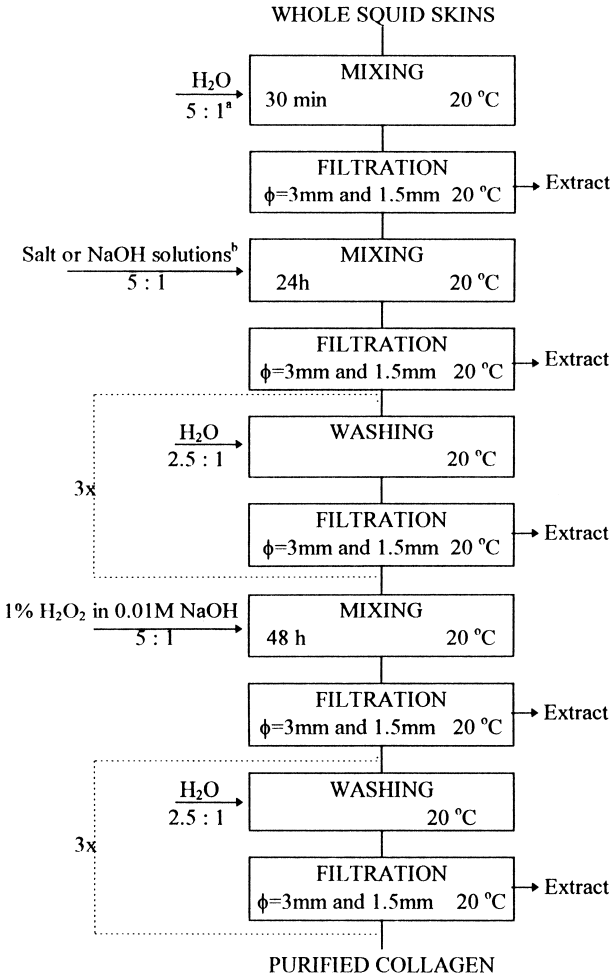

Fig. 1. Flow sheet of the procedure used for isolation of collagen from whole squid skins. (a) Solutions : skins; (b) NaCl in concentration 2.5–15%; $CaCl_2$, Na_2SO_4–ionic strength corresponding to 10% NaCl; 10% NaCl at pH 6.0, 6.5, 7.3; 0.016 M NaOH, pH 11.5.

3. Results and discussion

3.1. Isolation of collagen from squid skins

Homogenization of minced squid skin in 5–15% NaCl solution at 0°C resulted in solubilization of 35–24% of total amount of crude protein present in the raw material and only negligible loss of collagen (Table 1). This treatment was not effective in removing pigments from the skin. Neither could the pigments be removed by 24–48 h soaking of whole skins in NaCl solution at 4°C.

Increasing the time of soaking of whole skins in 10% NaCl solution at room temperature increased the intensity of the brown colour of the extracting solution and led to an accumulation in the soaking medium of dark-brown cells containing pigments. After 48 h, only few chromatophores were visible on the skin surface. However, the skin retained its light-beige colour with a slight violet hue and turned glassy and swollen. Further soaking reduced the volume and finally dissolved the skin completely. Such a result could not be obtained during soaking of cod skin under the same conditions.

It can be concluded that the physico-chemical changes in the connective tissue of squid skin due to soaking in NaCl solution at room temperature led to separation of the chromatophores, but the detailed conditions of treatment must prevent loss of collagen due to its solubility.

Soaking of whole squid skins in 10% NaCl solution during 24 h at room temperature resulted in solubilization of about 30% of the collagen contained in the skin (Table 2). Soaking in $CaCl_2$ solution at the same ionic strength led to 85% greater loss of collagen. Sodium sulphate treatment did not cause a significant loss of collagen, but was ineffective in removing the chromatophores. The effect of the salts was in agreement with their position in the Hofmeister lyotropic series.

Table 1
The contents of soluble proteins and hydroxyproline in extracts from homogenized squid skins[a]

Concentration of NaCl in extracting solution [%]	Soluble protein [% of the content in the raw material][b]	Hydroxyproline
5	35 ± 3.6	2.0 ± 0.4
10	27 ± 2.6	4.7 ± 0.9
15	24 ± 0.3	4.7 ± 0.2

[a] Extracting conditions: skins: solution = 1:10; homogenization: 2 min, 7000 rpm, 0°C; centrifuging: 30 min, 4500 × g, 0°C.

[b] Mean value ± standard deviation from 3 separate samples.

In order to find optimum conditions of bleaching of the skin with minimal loss of collagen, the material was soaked for 24 h in NaCl solutions ranging from 0 to 15%, washed 3 times with water (1:2.5) and bleached for 48 h in 1% H_2O_2 in 0.01 M NaOH. The largest amount of collagen, about 57%, was solubilized in 5% NaCl solution (Table 3a). This was accompanied by the most effective separation of the chromatophores. The sample that lost the minimum amount of collagen in 15% salt solutions released the largest quantities of collagen in the alkaline H_2O_2 solution. The optimum treatment with respect to minimal loss of collagen and optimal bleaching effect was with 10 or 15% NaCl solution. The skins soaked in a solution containing less than 2.5% NaCl were resistant to bleaching in the H_2O_2 solution. Prolonging the time of soaking in 10% NaCl solution to 48 h increased the loss of collagen by about 80%. Bleaching in alkaline H_2O_2 solution must be preceded by at least two washings of the skin with water (Table 3b). The loss of collagen could also be reduced by decreasing the pH of the soaking solution from 7.3 to 6 (Table 3c). However, this resulted in less effective removal of the chromatophores from the skin.

The yield of collagen could be increased by treating the skins first with NaOH solution at pH 11.5 for 24 h at room temperature and then, after washing with water, with 1% H_2O_2 in 0.01 M NaOH at pH 9.3. The total loss of collagen in this procedure did not exceed 10% and the effect of discoloration of the skins was similar to that obtained when a 10% NaCl soaking solution was used at the first stage.

Table 2
The contents of hydroxyproline in salt solution ($\mu = 1.7$) after 24 h soaking of whole squid skins at room temperature

Soaking solution	Hydroxyproline in the solution after soaking of skins and washing with water [% of the content in the raw material][a]
Na_2SO_4	6 ± 0.2
NaCl	32 ± 1.1
$CaCl_2$	59 ± 3.4

[a] Mean value ± standard deviation from 3 separate samples.

3.2. Characteristics of collagen isolate from squid skins

The conversion factors, f_N and f_{Hypro}, for calculating the content of squid skin collagen from nitrogen and hydroxyproline contents were 6–6.5 and 14.1–14.3, respectively (Table 4). The f_N is similar and f_{Hypro} is lower than the values found earlier for collagen from skin from squid of the same species (Sadowska & Sikorski, 1987). This indicates that the present procedure of isolation of collagen is effective in removing

Table 3
The contents of hydroxyproline in the solution after soaking of whole squid skins
(a) Effect of concentration of NaCl

Concentration of NaCl [%]	Hydroxyproline[a] in the solution after soaking and washing		
	10% NaCl + water[b]	1% H_2O_2 in 0.01 M NaOH[b] + water [% of the content in the raw material]	∑
0.0	2 ± 0.2	3 ± 0.1	5
2.5	39 ± 1.5	4 ± 0.2	43
5.0	57 ± 1.6	3 ± 0.3	60
10.0	31 ± 0.2	5 ± 0.2	36
15.0	16 ± 0.1	19 ± 0.9	35

(b) Effect of washing skins with water after soaking in 10% NaCl solution

Number of washings with water	Hydroxyproline[a] in the solution after soaking and washing		
	10% NaCl + water[b]	1% H_2O_2 in 0.01 M NaOH[b] + water [% of the content in the raw material]	∑
0	17 ± 0.6	42 ± 0.3	59
1	24 ± 1.1	31 ± 0.2	55
2	26 ± 0.8	13 ± 0.7	39
3	31 ± 0.2	5 ± 0.2	36

(c) Effect of pH of the NaCl solution

pH of NaCl solution	Hydroxyproline[a] in the solution after soaking and washing		
	NaCl + water[b]	1% H_2O_2 in 0.01 M NaOH[b] + water [% of the content in the raw material]	∑
6.0	16 ± 0.5	4 ± 0.2	20
6.5	24 ± 0.6	5 ± 0.5	29
7.3	32 ± 0.5	7 ± 0.2	39

[a] Mean value ± standard deviation from 3 separate samples.
[b] Soaking in NaCl solution for 24 h, followed by 3 washings with water (1:2.5), bleaching in H_2O_2 for 48 h at room temperature.
[c] Soaking in NaCl solution for 24 h, followed by 0–3 washings with water, bleaching in H_2O_2 for 48 h at room temperature.

noncollagenous components and that differences in f_{Hypro} rather result from intraspecies variation.

The collagen isolated by soaking in 10% NaCl solution, followed by washing with water and bleaching in 1% H_2O_2 in 0.01 M NaOH, was easily soluble in dilute HCl at pH 3.5. The thermally denaturated collagen was composed mainly of α-chains. The β component was present in significantly lower concentration (Fig. 2). A similar electrophoretic pattern was obtained by Kimura and Matsura (1974) from skin collagen from *Todarodes pacificus*. According to Mizuta, Yoshinaka, Sato, and Sakaguchi (1995, 1996) two distinct molecular species of collagen, type SQ-I and SQ-II are distributed, not only in the mantle and arm muscle, but also in the mantle skin.

Table 4
Characteristics of collagen isolate from squid skin[a]

Characteristics	Isolate I	Isolate II
Nitrogen [% of dry weight]	16.7	15.3
Hydroxyproline [% of dry weight]	7.1	7.0
Dry weight/nitrogen (f_N)	6.0	6.5
Dry weight/hydroxyproline (f_{Hypro})	14.1	14.3
Viscosity of 0.5% collagen in dilute HCl at pH 3.5 [cP]	1935	487

[a] The isolates were obtained according to the procedure presented in Fig. 1 using in the first stage as soaking solution 10% NaCl (Isolate I) and NaOH, pH 11.5 (Isolate II).

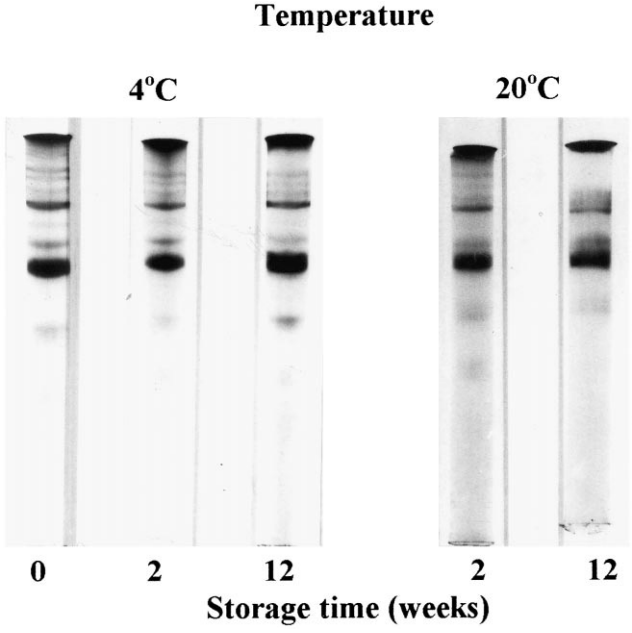

Fig. 2. Electrophoretic separation in 5% SDS-polyacrylamide gel of collagen from squid skin solubilized in dilute HCl, pH 3.5, stored at 4 and 20°C.

The isolates also contained low molecular weight products of hydrolysis that were visible on the chromatograms as bands of low intensity (Fig. 2). They also exist if much milder conditions are applied for collagen isolation (Mizuta et al., 1995) than in our experiments. In collagen solutions kept at pH 3.5 at 4°C no further hydrolysis was detected after 12 weeks (Fig. 2). At room temperature, the components of molecular weight higher than the β component depolymerized after prolonged storage.

The squid collagen, isolated according to the described procedure, had a slightly detectable, fish-oily and rancid off-odour. Its intensity increased after only 1 day at room temperature. Some antioxidants were not only ineffective in inhibiting the odour reversion, but also had an undesirable effect on the colour of the isolates. Cysteine, 0.05%, changed the colour of the isolate to yellow after 1 day at room temperature. The same effect was caused by BHA and a mixture BHA + phosphoric acid after a longer time of storage. Effective odour reversion suppressors were mixtures of citric acid with glucose, saccharose, glycine, glutamic acid and mannitol, although they could not totally arrest the development of off-odour. The most effective way of preventing the rancid off-odour was removal of lipids from the squid skins followed by adding the non-ionic detergent "Rokafenol N-6" to all solutions applied for the treatment of skins. The addition of "Rokafenol N-6" did not decrease the yield of collagen. The isolate obtained in the presence of this detergent was clear in an acid solution, while the products isolated in absence of the detergent were slightly opaque.

4. Concluding remarks

Collagen, soluble in dilute acid solutions can be isolated from squid skins by 24 h soaking in 10% NaCl solution at room temperature, washing with water and 24 h bleaching in 1% H_2O_2 in 0.01 M NaOH. The yield of the soluble collagen isolate is 53% of the collagen contained in the skins. The considerably high yield of the product is to a large extent the result of using whole skins. The yield of collagen isolate obtained by Vis et al. (1996) from minced plaice skins, in conditions limiting the solubility of collagen at 0–1°C and using water and dilute H_2O_2 for extraction, was 64%. The yield of collagen from squid skins can be increased to 90% by using NaOH solution at pH 11.5 instead of 10% NaCl. The isolate is, however, less soluble in dilute acid and the viscosity of the dispersion is four times lower (Table 4).

The rancid off-odour which tends to develop in the isolate can be effectively prevented by adding 0.5% of the non-ionic detergent "Rokafenol-6" to all solution used in the procedure. This detergent is more efficient than many commercial antioxidants.

The collagen isolate is white or slightly yellow. In respect to colour the isolate is equal to commercial products.

Acknowledgements

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