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Optimisation of conditions for precipitation of collagen from solution using κ-carrageenan. Studies on collagen from the skin of Baltic cod (*Gadus morhua*)

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Abstract

The effects of the concentration of collagen (0.025–3%), extracted from the skins of Baltic cod (*Gadus morhua*) and the concentration of κ-carrageenan (0.04–1%), in the presence of NaCl (0–5%), on the yield of precipitated collagen fibrils at 0 °C and at pH 2.2–8 were determined. The yield of precipitated collagen was directly proportional to the ratio, κ-carrageenan to collagen, within the range 0.015–0.4. Collagen dissolved in citric acid could be completely precipitated using κ-carrageenan if the weight ratio of dry reagents was 1:0.4 within a protein concentration of 1.5–0.08%. The maximal yield of precipitated collagen fibrils using κ-carrageenan could be achieved in the pH range 2.2–3. In this range of pH, the presence of NaCl in the system did not affect the efficiency of precipitation of collagen fibrils with κ-carrageenan. The yield of precipitated collagen was lower at 20 °C than at 0 °C.

Keywords: Baltic cod; Fish skins; Collagen; Collagen fibrils; κ-carrageenan

1. Introduction

Odourless and colourless collagen from the skins of fish can be isolated with a high yield by direct, exhaustive extraction with 0.5% M citric or acetic acid solutions (Ciarlo, Paredi, & Fraga, 1997; Montero, Alvarez, Martí, & Borderías, 1995; Montero, Borderıas, Turnay, & Leyzarbe, 1990; Montero, Gómez-Guillén, & Borderıas, 1999; Montero, Jiménez-Colmenero, & Borderías, 1991; Nagai & Suzuki, 2000; Sadowska, Kołodziejska, & Niecikowska, 2003). The collagen solutions obtained in this way are highly diluted, thus significantly limiting the practical application of this method. Precipitation of collagen fibrils from such solutions is necessary for preparing a viscous mass or highly concentrated collagen solution. Such material can be used successfully to manufacture different collagen products on a commercial scale.

Glycosoaminoglycans (GAG) are non-collagenous components of connective tissue which, in vivo, play a crucial role in the process of formation of the connective tissue matrix. Various types of GAG influence the generation of collagen fibrils, differing in length, diameter, and spatial orientation (Asghar & Henrickson, 1982; Bailey & Light, 1980; Einbinder & Schubert, 1951; Montes & Junqueira, 1988). Wood and Keech (1960) isolated collagen fibrils from solutions of calf tropocollagen using chondroitin 4-sulphate-A and chondroitin 6-sulphate-C. GAG prepared from bovine trachea cartilage (Łagocka, Sadowska, & Synowiecki, 1997) and commercial reagents, such as chondroitin sulphates A and C (Sigma), can be used to precipitate collagen extracted with HCl solution from bovine skins preceded by alkaline

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treatment (Sadowska, Gutowska, & Malesa, 2004). The interaction of GAG with collagen is due to ionic bonds between the carboxyl and/or sulphate groups of GAG and ϵ-amino groups of lysine and hydroxylysine residues, guanidyl groups of arginine residues, and N-terminal α-amino groups of collagen. However, this method of collagen precipitation is uneconomical on a commercial scale because of the high price of GAG.

Some polysaccharides, such as carboxymethylcellulose, sodium alginate, agar, pectin and carrageenans, are used to recover proteinaceous compounds from liquid industrial wastes. The yield of protein precipitation from its solution depends on the properties of the proteins and polysaccharide, pH, ionic strength of the medium, and the ratio of protein to polysaccharide (Ledward, 1994). Carrageenans are acid polysaccharides and their structure and properties are similar to those of chondroitin sulphate. κ-carrageenan is especially rich in sulphate groups.

The objective of the investigations was to check the usefulness of κ-carrageenan for the precipitation of fibrils from solutions of collagen isolated from the skins of Baltic cod.

2. Materials and methods

2.1. Raw material

The skins of fresh Baltic cod (*Gadus morhua*) were mechanically separated from the fish fillets. The residue of adhering tissues was 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 $\phi = 3$ mm. The dry weight, total nitrogen, and hydroxyproline contents in the raw material were determined and amounted to 30.3%, 4.34% and 1.39%, respectively.

2.2. Isolation of collagen from skins

Collagen was extracted from whole skins with 0.5 M citric acid solution or with 0.15 M HCl solution according to procedures described by Sadowska et al. (2003).

2.3. Precipitation of collagen fibrils

κ-carrageenan (Fluka) solutions, 0.04–1% in 0.5 M citric acid, were added to 0.025–3% collagen solutions in 0.5 M citric acid (1:1, v/v). The weight ratio of dry reagents, κ-carrageenan:collagen, ranged from 0.015 to 41.5. The carrageenan solution, cooled to 0 °C, was slowly added to vigorously stirred collagen solution kept in an ice bath. Precipitation of collagen from citric acid solutions was investigated within the pH range 2.2–8 and in the presence of NaCl (ranging from 0% to 5%). The pH was adjusted by means of 1 M NaOH solution. NaCl and carrageenan were introduced to the collagen solution simultaneously. After 30 min, the mixtures were centrifuged at 0 °C for 20 min at 2000*g*. In the control samples, citric acid solution was added instead of carrageenan solution. The hydroxyproline contents in all the supernatants were determined. The yield of precipitated collagen fibrils was calculated using the following formula:

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

where W is the yield of precipitated fibrils (%); A the concentration of hydroxyproline in the sample (%); B the concentration of hydroxyproline in the control (%).

2.4. Dry weight and total nitrogen content

The dry weight and total nitrogen were determined according to AOAC methods (1990).

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).

3. Results and discussion

3.1. Yield of precipitated collagen fibrils at different concentrations of collagen and κ-carrageenan

The introduction of κ-carrageenan dissolved in citric or hydrochloric acids into acidic solution of collagen (pH 2.2) resulted in an immediate precipitation of collagen fibrils. The amount of precipitated fibrils depended on the concentrations of κ-carrageenan and collagen. There was an inverse relationship between the concentration of collagen in solution and the amount of fibrils formed. The fraction of collagen precipitated from highly concentrated solutions was lower than that precipitated from low concentration solutions (Fig. 1). The yield of precipitated collagen increased with increase of κ-carrageenan concentration within the range studied. The efficiency of collagen fibril precipitation in the presence of κ-carrageenan depended on the ratio of the reagents and was directly proportional to that ratio within the range of κ-carrageenan to collagen ratio of 0.015 to 0.4 (Fig. 2). Collagen dissolved in citric acid could be completely precipitated using κ-carrageenan if the weight ratio of dry reagents was 1:0.4 within a protein concentration range of 1.5–0.08% (Fig. 1). Collagen fibrils could also be precipitated from solutions as low as 0.012%, but a weight ratio of collagen to κ-carrageenan

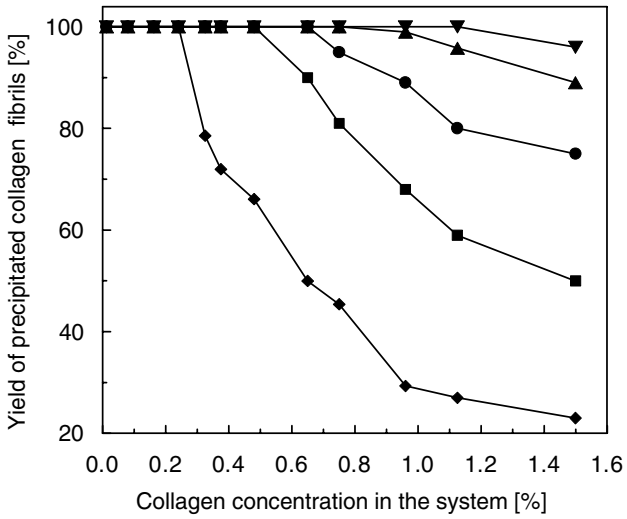

Fig. 1. Effect of collagen and κ-carrageenan concentrations on the yield of precipitated collagen fibrils. Concentrations of carrageenan: (♦) 0.1%, (■) 0.2%, (●) 0.3%, (▲) 0.4%, (▼) 0.5%.

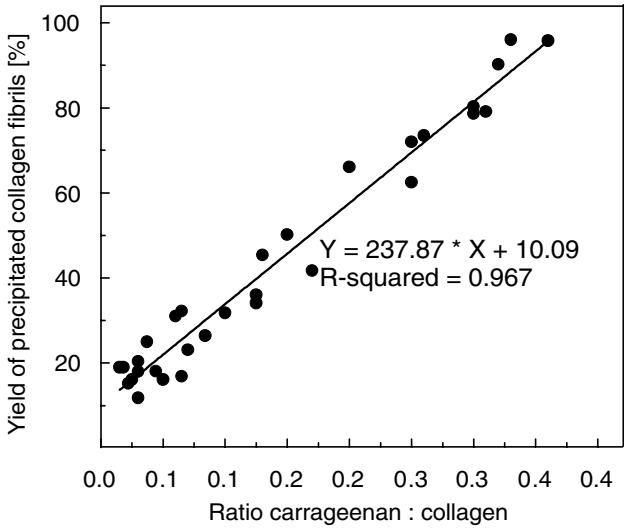

Fig. 2. Ratio of carrageenan to collagen and the yield of precipitated collagen fibrils.

of 1:8.3 had to be used (Table 1). The precipitation of bovine skin collagen (previously treated with 10% NaOH) dissolved in hydrochloric acid was more efficient at lower concentrations of κ-carrageenan than in the case of precipitation of collagen from Baltic cod skins dissolved in citric acid. Collagen from bovine skin could be completely precipitated using a collagen to κ-carrageenan ratio of 1:0.025 (Sadowska & Strojk, 1998). This discrepancy probably resulted from different concentrations of lysine, hydroxylysine, arginine and N-terminal α-amino groups in these two types of collagen, and different sizes of macromolecules in acid solution. Our previous experiments have demonstrated that fibril precipitation using GAG solution is a two-step process. First, GAG reacts directly with larger molecules, probably with tropocollagen aggregates, which is followed by fibril growth (Sadowska et al., 2004).

3.2. Effects of solvent, pH, and ionic strength on the precipitation of collagen fibrils with κ-carrageenan

Collagens isolated from Baltic cod skins using hydrochloric acid, showed different properties from those extracted with citric acid. Collagen dissolved in citric acid did not precipitate when the pH was changed using 1 M NaOH within the range 2.2–10. However, this solution formed a transparent gel at pH above 3 when samples were kept on ice. On the other hand, collagen dissolved in hydrochloric acid formed an opaque gel if the pH was increased above 4. The highest ability to form an opaque gel was observed with pHs ranging from 8.0 to 8.5, corresponding probably to the isoelectric point of collagen.

The maximal yield of collagen fibrils precipitated using κ-carrageenan could be achieved within the pH range 2.2–3 (Table 2). At pH values of 4, 5 and 6, collagen and κ-carrageenan made highly hydrated aggregates in the entire volume of the solution.

Sodium chloride, added to collagen dissolved in hydrochloric acid at concentrations corresponding to ionic strengths 0.72 and 0.9, precipitated collagen with yields of, respectively 22% and 84% (Table 3). A lower yield was obtained if sodium chloride was used to precipitate collagen extracted with citric acid. Only 11% of collagen could be precipitated at an ionic strength 0.9 at pH 2.2 (Table 3) and about 40%, 53% and 62%, respectively, at pHs 3, 4 and 5 (Table 4).

Table 1
Effect of κ-carrageenan, at low concentration, on the yield of collagen precipitated from 0.5 M citric acid solution

Concentration of collagen in the system (%)	Concentration of κ-carrageenan in the system (%)				
	0.02	0.04	0.06	0.08	0.1
	% Collagen precipitated[a]				
0.800	16.1 ± 3.6	16.1 ± 2.9	23 ± 5.8	31.8 ± 3.2	34.1 ± 5.1
0.080	62.5 ± 3.9	100 ± 0.0	100 ± 0.0	100 ± 0.0	100 ± 0.0
0.012	0.0	5.7 ± 2.4	17.5 ± 3.5	32.2 ± 4.5	100 ± 0.0

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

Table 2
Effect of pH, modified with NaOH, on the yield of precipitated collagen fibrils with κ-carrageenan[a]

pH	% Collagen precipitated[b]
2.2	60 ± 2.7
3.0	61 ± 3.8
4.1	25 ± 2.5
5.2	15 ± 1.9
6.0	7 ± 3.5
7.0	0.0
8.0	0.0

[a] Concentrations of collagen and κ-carrageenan in 0.5 M citric acid solution, respectively, 2.25% and 0.4%.
[b] Mean values ± SD from three separate samples.

Table 3
Effect of ionic strength on the yield of collagen precipitated from 0.7% solutions at pH 2.2

Concentration of NaCl (%)	Ionic strength	% Collagen precipitated from[a]	
		0.5 M citric acid	0.15 M hydrochloric acid
0	0	0.0	0.0
1	0.18	0.0	0.0
2	0.36	0.0	0.0
3	0.54	0.0	0.0
4	0.72	0.0	22.4 ± 3.4
5	0.9	11.2 ± 2.3	84.3 ± 4.5

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

Table 4
Effect of pH, modified with NaOH, at ionic strength 0.9, on the yield of collagen precipitated from 1% solution in 0.5 M citric acid

pH	% Collagen precipitated[a]
2.2	11.2 ± 2.3
3.0	40.8 ± 3.1
4.0	53.0 ± 1.9
5.0	62.5 ± 3.7

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

Sodium chloride, present in the system at a concentration corresponding to ionic strength about 0.6, had no impact on the efficiency of precipitated collagen fibrils with κ-carrageenan from citric acid solution at pH 2.2 (Table 5). At higher ionic strengths, κ-carrageenan precipitated out from the solution. Sodium chloride also did not stimulate precipitation from the solution of bovine collagen induced by GAG (Sadowska et al., 2004). The results of Wood (1960) suggested that an increase in the ionic strength in the system did not increase the yield of collagen precipitated from bovine skin dissolved in acetic acid with chondroitin sulphate but it delayed this process.

Table 5
Effect of ionic strength, modified with NaCl, on the yield of collagen fibrils precipitated with κ-carrageenan[a]

Ionic strength	% Collagen precipitated[b]
0	59.0 ± 2.6
0.18	66.1 ± 8.2
0.36	62.5 ± 3.4
0.54	63.4 ± 2.9

[a] Concentrations of collagen and κ-carrageenan in 0.5 M citric acid solution, respectively, 2.25% and 0.4%.
[b] Mean values ± SD from three separate samples.

Table 6
Effect of temperature on the on the yield of collagen fibrils precipitated with κ-carrageenan[a]

Temperature (°C)	% Collagen precipitated[b]
0	45.4 ± 6.7
20	16.3 ± 11.2

[a] Concentrations of collagen and κ-carrageenan in 0.5 M citric acid solution, respectively, 1.5% and 0.2%.
[b] Mean values ± SD from three separate samples.

3.3. Influence of temperature on yield of collagen fibrils precipitated with κ-carrageenan

The yield of collagen precipitated from the solution with κ-carrageenan was larger at 0 °C than that at 20 °C (Table 6). At 20 °C, in the presence κ-carrageenan, strongly hydrated aggregates, difficult to separate from the cloudy solution, were produced.

The temperature of denaturation of the collagen from cod skin is 15 °C. Disruption of hydrogen bonds and hydrophobic interactions results in destruction of the highly organized triple helix structure of collagen. Denatured collagen dissociates into single polypeptide chains (component α), dimers (component β), and trimers (component γ) with lower molecular mass than that of the native protein. This may explain the lower yield of fibril precipitated at 20 °C than at 0 °C. According to Ledward (1994), proteins of larger molecular mass are more effectively precipitated by anionic polysaccharides.

4. Conclusions

Collagen extracted from the skins of Baltic cod with 0.5 M citric acid can be precipitated from solutions at its initial concentrations, 0.08–3%, in the form of fibrils, with a yield of 100%, using κ-carrageenan at a weight ratio collagen: κ-carrageenan of 1:0.4. At higher concentrations of κ-carrageenan, the proportion of collagen in the precipitate decreases. This can limit the practical use of κ-carrageenan and increases the cost of separation of collagen.

Maximal yield of precipitated fibrils is between pH 2.2 and 3. In this range, the presence of NaCl at concentration, up to 5% does not increase the interactions of collagen with κ-carrageenan. The efficiency of precipitation of collagen with κ-carrageenan is higher at 0 °C than at 20 °C.

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