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EFFECT OF GLYCOSAMINOGLYCANS ON RECONSTITUTION OF COLLAGEN FIBRILS

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Changes in collagen structure resulting in etching connective tissue in concentrated NaOH solution and possible effects on reconstitution of fibrils and fibres from collagen solution under the influence of glycosaminoglycans (GAGs) were examined. Collagen was isolated from bovine hide scraps, etched for 24 h in a 10% NaOH solution saturated with anhydrous sodium sulphate. GAGs solutions were isolated from bovine tracheas. The effect of GAGs interactions with collagen in the systems at ionic strengths of 0–0.7 Debye, modified with NaCl at pH 3–8, was evaluated visually by comparing the quantity of fibres, measuring the absorbance of the system at 400 nm and determining the content of proteins in the supernatant after centrifugation. The addition of GAGs to the collagen solution resulted in immediate formation of fibres and determining the content of proteins in the supernatant after centrifugation. The quantity time of reconstitution of the fibres increased with the quantity of GAGs added and the concentration of collagen. The optimal medium for the reconstitution of fibrils or collagen fibres under the influence of GAGs, was pH 3 in the absence of NaCl. Collagen dissolved in chloride acid can be completely precipitated using crude GAGs isolate if the weight ratio of reagents, collagen:GAGs is 1:0.3. The increase in ionic strength of collagen solution did not stimulate the formation of fibres, but induced turbidity due to the presence of small flocks and an increase in viscosity of the system, regardless of the concentration of GAGs.

INTRODUCTION

Collagen is widely used in a variety of products in the food and leather industries as well as in cosmetic, biotechnological and pharmaceutical industries and in medicine.

The methods applied until now for obtaining products other than gelatine, glues or leather have been based on mechanical comminution of the weave of collagen fibres, swollen due to the action of acid, to the fibril bundles and fibrils. Because of the variability of connective tissue materials, however, the manufacture of products of standard quality is difficult. One of the methods used to avoid quality fluctuation of the intermediates is converting the fibrous collagen into tropocollagen and controlling the reconstitution of fibrils.

In the synthesis of collagen fibrils *in vivo*, a significant role is played by non-collagen components of the connective tissue matrix, mainly acid GAGs which are large and linear polymers. Their basic unit is a dimer, composed of an amino sugar and uronic acid. The amino groups are acetylated, and one of the hydroxyl groups of the amino sugar is most often estrified with sulphuric acid. The glycosaminoglycans, depending on their properties and quantity, stimulate the formation of fibrils and collagen fibres of various length, diameter and spatial orientation. Besides, they stabilise the collagen structure [Einbinder & Schubert, 1951; Wood, 1960a, b; Wood & Keech, 1960; Bailey & Light, 1989].

Until now investigations on the mechanism of *in vivo* synthesis of collagen fibrils have been applied to tropocollagen extracted from calf skins [Wood & Keech, 1960]. Young collagen is stabilised by hydrogen, hydrophobic and ionic bonds. This would allow extraction of collagen, without changes in its native helical structure from the connective tissue by neutral salt solutions of low ionic strength, phosphate buffer at pH 8, citrate buffer at pH 3, or 0.05 mol/L acetic acid. From such tropocollagen solutions fibres can be reconstitued *in vitro*. The quantity of collagen so extracted is small in comparison with its total amount present in the connective tissue, and it decreases with the age of the animal and also depends on the kind of tissue, species and breed of the animal [Sadowska, 1992].

Ripe, fibrous collagen cross-linked with intra- and intermolecular stable covalent bonds is insoluble in cold, dilute acids and neutral salt solutions. It can be dissolved only after destruction of crosslinks, stabilising its supermolecular structure, by applying proper mechanical, chemical or enzymatic treatment as well as their combinations. Drastic conditions of connective tissue pre-treatment can lead to the destruction of the native macromolecular structure and may significantly decrease the ability of self-reconstitution of collagen fibrils.

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This investigation was aimed at examining the feasibility of using GAGs for reconstitution of collagen fibres, previously chemically depolymerised. The effect of concentration of collagen, the ratio GAGs to collagen, the pH, ionic strength and temperature of the reaction mixture and of the time of reaction on the efficiency of the fibrils reconstitution was also evaluated. The products derived from collagen reconstituted with GAGs, being natural components of animal tissue, could be particularly useful for potential aplications in medicine and cosmetics.

MATERIALS AND METHODS

Material. Crude isolate of glycosaminoglycans was extracted from bovine tracheas, using the method of Łagocka *et al.* [1997]. The isolate contained approximately 27% of proteins and 54% of GAGs composed of chondroitin sulphate A and C, hyaluronic acid and keratin sulphate, on a dry weight basis [Łagocka *et al.,* 1997]. Chondroitin sulphates A and C purchases from Sigma Chemical Co. were also used. The source of collagen was non-hirsute bovine hide scraps, mainly from hypogastrium, stored for two months at -18°C. The material was etched for 48 h in a 10% NaOH solution saturated with Na_2SO_4. The collagen mass, free of non-collagen proteins and fat as well as other soluble compounds was solubilized in HCl at pH 3. After separation the solid collagen was precipitated with NaCl [Sadowska, 1992]. Isolated collagen was dehydrated with acetone and characterised, by determining dry weight, total nitrogen, hydroxyproline and sodium chloride.

Preparation of collagen solution. The collagen fibres, containing approximately 13% NaCl, 16% total nitrogen and 11% hydoxyproline, were allowed to swell for 24 h in HCl solution at pH 3 or in a Mc Ilvaine's buffer at 4°C. Then, the mixture was homogenised for 5 min in an ice bath using a homogenizer, and de-aerated by centrifugation at 350 g for 5 min.

Reconstitution of collagen fibrils. Reconstitution of collagen fibrils was carried out at room temperature and at 4°C at a weight ratio of dry GAGs to collagen within the range of 0.005 to 0.5, at pH 3, 5, 7, 8 set with Mc Ilvaine's buffer and at ionic strength from 0 to 0.7 modified with NaCl. The sample systems were prepared by mixing collagen solution of 0.2 to 1% concentration with the same volume of solutions containing or devoid of glycosaminoglycans and sodium chloride in a such quantity as was necessary for their required concentration in the mixture. After 45 min, the samples were centrifuged at 0°C for 20 min at 350 g. The efficiency of fibrils reconstitution was calculated using the following formula:

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

where Y is the efficiency of fibrils reconstitution (%); A the concentration of collagen in the system (%); B the concentration of collagen in the supernatant.

Physical and chemical assays. The reconstitution of collagen fibres was characterised on the basis of the content of proteins in the supernatants, collagen-glycosaminoglycans systems determined with the biuret method and on the basis of absorbance of the systems measured after 45 min reaction at the wavelength of 400 nm [Wood & Keech, 1960]. The method of preparing the material for the absorbance measurement, after Wood & Keech [1960], is based on preparing the examined systems in a volume of 1 mL directly inside the cuvette, was modified. The samples were prepared in large volume (50 mL) and only after thorough stirring samples of 1 mL were collected and placed in the cuvette. This enables decreasing the error which results from collecting a solution of large viscosity with simultaneous preservation of reproducibility of readings. The results presented in tables and figures represent mean values of 6 measurements of absorbance, each obtained for a separate sample. Total nitrogen was determined by the Kjeldahl's method; hydoxyproline was determined with the method recommended by ISO [International Standard ISO, 1978], after 6-h hydrolysis in 6 mol/L HCl, at 105°C [Sadowska, 1992]; sodium chloride content in the collagen material was determined after Mohr.

RESULTS AND DISCUSSION

Introduction of GAGs dissolved in 0.001 mol/L HCl into a collagen solution at pH 3 resulted in immediate separation of certain quantity of fibrils and fibres, depending either on the quantity of added or the concentration of collagen in the systems examined (Figure 1). Along with the increase in the quantity of reconstituted collagen there was a proportional increase in the absorbance of the system. A similar effect was observed while applying chondroitin sulphates A and C standards (Figure 2).

Total precipitation of collagen in the form of fibres was obtained in a mixture of crude GAGs isolate and collagen

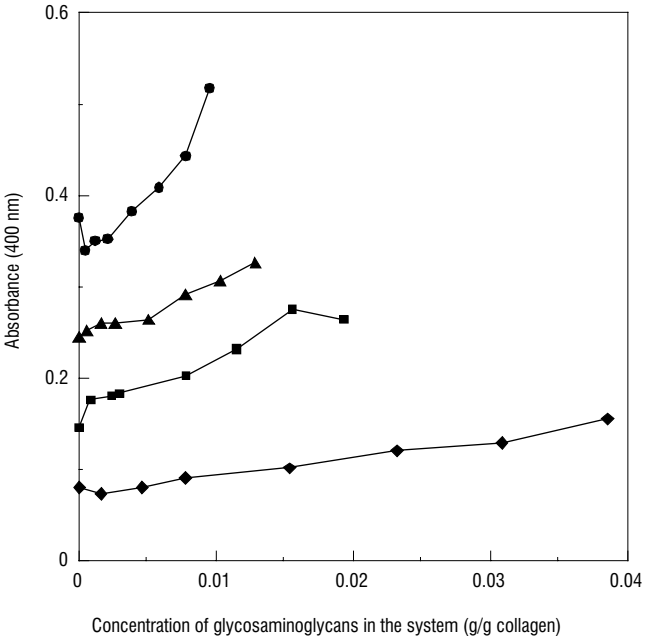

FIGURE 1. The effect of the concentration of glycosaminoglycans and collagen on the reconstitution of collagen fibrils expressed as absorbance of the samples at 400 nm. The concentration of collagen in the system: 0.5% (●–●); 0.375% (▲–▲); 0.25% (■–■); 0.125% (◆–◆).

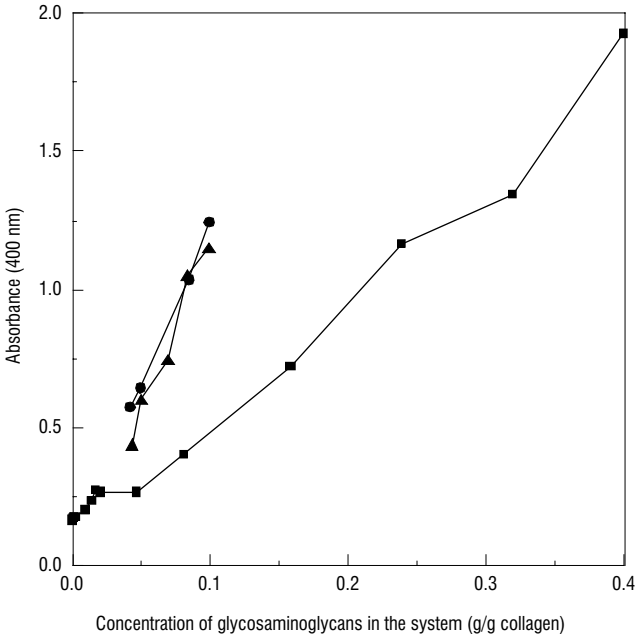

FIGURE 2. The effect of the concentration and quality of glycosaminoglycans on the reconstitution of collagen fibrils expressed as absorbance of the sample at 400 nm: (■–■)) crude isolate of glycosaminoglycans; (●–●); chondroitin sulphate A; (▲–▲) chondriotin sulphate C.

solution at the weight ratio of dry preparations at 0.3. In the event of using A and C chondroitin sulphates, 100% efficiency of reconstitution was obtained at a weight ratio of parent substances reaching 0.125 (Figure 3). This probably resulted from a better refinement of these GAGs and their affinity to collagen.

The best reproducibility of results was obtained with dilute collagen solutions, up to 0.5%. At higher concentrations, the de-aeration by rotation was of little efficiency due to high viscosity of the homogenate. The remaining minute air bubbles disfigure the result of the absorbance determination. Moreover, GAGs added to a concentrated collagen solution, in spite of intensive stirring, did not precipitate the collagen in the form of fibres, but formed with collagen membranes which immobilise in vacuoles the remaining collagen in the solution.

The higher the pH of collagen solution, the more difficult was to precipitate collagen fibres with GAGs (Table 1). Only at pH 3, was collagen separated in the form of fibres. The decrease in collagen solubility at pH 3 under the influence of GAGs arises from the decrease in pH at the isoelectric point of proteins as a consequence of pre-treatment with NaOH.

During visual assessment of the collagen-GAGs systems, the presence of small quantities of NaCl did not stimulate the formation of collagen fibrils (Figure 4). The results of absorbance measurements of these systems were higher than in the absence of NaCl, which resulted in stronger turbidity of the system with NaCl than in the increase in the quantity of fibres. The addition of larger amounts of salt caused precipitation of collagen bundles even before the GAGs solution was added. In the examined collagen systems, a rapid increase in turbidity appeared at the ionic strength of approximately 0.5 Debye, regardless of the presence of GAGs. It can also be presumed that a process similar to coagulation under the influence of salts appears, irrespective of the reconstitution under the influence of GAGs. Both these processes did not stimulate one another and can occur simultaneously. The content of proteins in supernatants also revealed that collagen was precipitated only at an ionic strength of above 0.5 Debye.

The addition of glycosaminoglycans solution to a stable collagen solution resulted in immediate separation of characteristic, distinct thin fibres. The length of fibres

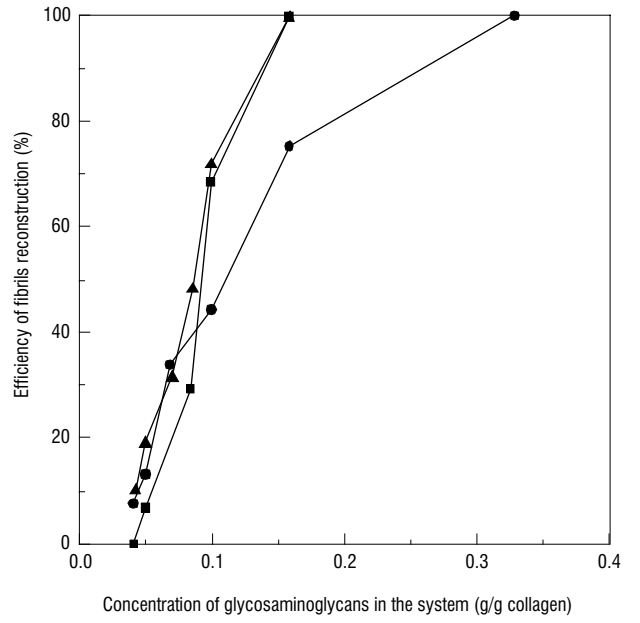

FIGURE 3. The effect of the concentration and kind of glycosaminoglycans on the efficiency of collagen fibrils reconstitution: (▲–▲) chondroitin sulphate A; (■–■) chondroitin sulphate C; (●–●) crude isolate of glycosaminoglycans.

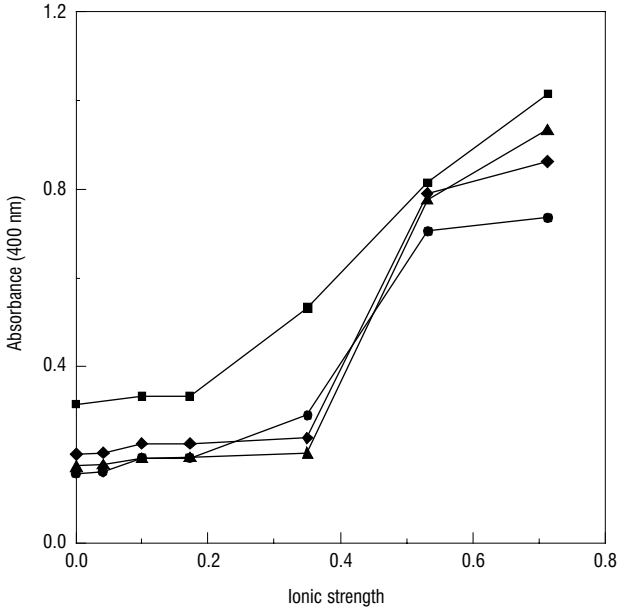

FIGURE 4. The effect of ionic strength and concentration of glycosaminoglycans on reconstitution of collagen fibrils expressed as absorbance of the samples at 400 nm. The concentration of glycosaminoglycans (g/g of collagen): (▲–▲) 0.00; (●–●) 0.004; (◆–◆) 0.01; (■–■) 0.02.

TABLE 1. The effect of medium acidity on reconstitution of collagen fibrils from 0.25 % solution by glycosaminoglycans expressed as absorbance of the system at 400 nm.

pH	Concentration of glycosaminoglycans (g/g of collagen)[a]			
	0.02	0.04	0.06	0.08
3	0.212±0.030	0.295±0.045	0.246±0.035	0.276±0.028
5	0.020±0.012	0.033±0.007	0.080±0.040	0.016±0.005
7	0.037±0.009	0.047±0.010	0.015±0.003	0.015±0.006
8	0.021±0.014	0.029±0.012	0.003±0.012	0.007±0.000

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

approached several centimetres. This made the absorbance measurement more difficult, resulting in deviations between following readings and prompting to conduct a greater number of determinations so as to achieve a representative average.

Registering the absorbance in the time of interaction of dissolved collagen with GAGs, conducted directly in the cuvette cell of the spectrophotometer, various reaction kinetics curves (Figure 5) were obtained. In some experiments, the absorbance of the system increased in a logarithmic manner, in others decreased, and in the remaining, after reaching its minimum or maximum, increased or decreased. The kinetics curve was never similar to that obtained by Wood [1960 a], who applied chemically unmodified tropocollagen, in spite of applying identical experimental conditions. The variability of the results is due to the aggregation of fibrils in time, which was stated visually, and the dislocation of aggregates in reference to the stream of incidencing light, depending on the viscosity of the dispersion medium.

Storage of the experimental system at 20°C and 4°C for 24 h did not significantly change the quantity and character

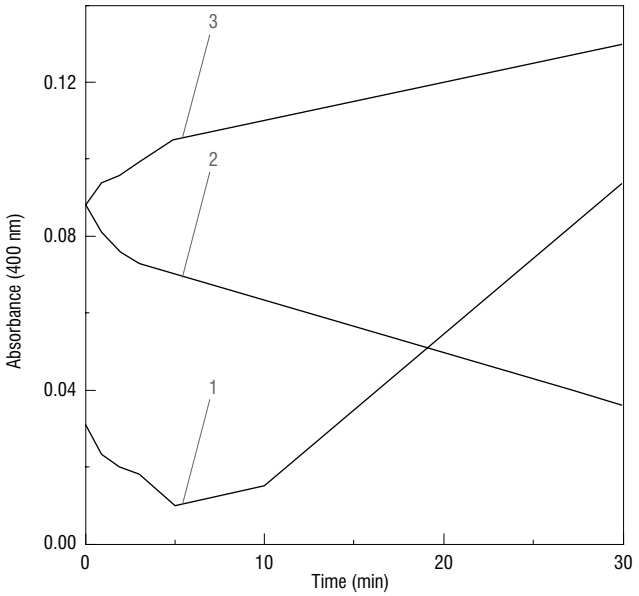

FIGURE 5. The influence of time on reconstitution of collagen fibrils expressed as absorbance of the collagen: glycosaminoglycans system at 400 nm. Curves: 1 – reconstitution conducted directly in the cuvette in samples stirred before the absorbance measurement, 2 – reconstitution conducted directly in the cuvette without stirring the samples before the absorbance measurement, 3 – samples were prepared in a large volume and the absorbance was measured after thorough stirring before the measurement.

TABLE 2. The effect of time and temperature of storage of collagen and glycosaminoglycans on fibrils reconstitution from 0.25% solution expressed as absorbance of the sample at 400 nm.

Concentration of glycosaminoglycans (g/g of collagen)	Absorbance at 400 nm[a] Time and temperature of storage		
	45 min, 20°C	24 h, 20°C	24 h, 4°C
0	0.128±0.055	0.131±0.006	0.136±0.011
0.006	0.138±0.021	0.137±0.017	0.136±0.004
0.018	0.184±0.022	0.189±0.025	0.145±0.020
0.030	0.179±0.025	0.226±0.032	0.177±0.042
0.060	0.350±0.025	0.343±0.010	0.326±0.026
0.090	0.411±0.008	0.394±0.015	0.410±0.018
0.120	0.546±0.093	0.913±0.328	0.651±0.037
0.150	0.604±0.068	0.772±0.086	0.669±0.075

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

of the remaining fibrils, conducted 45 min after from the moment of interaction (Table 2). The differences between the results were very small. Considering the deficiency of the spectrophotometric method for observing the reconstitution of fibrils, it cannot be unequivocally stated whether time and temperature had any effect on the efficiency of the process.

CONCLUSIONS

Collagen extracted under alkali conditions from bovine hide can be reconstituted from acid solutions in the form of fibrils formed with glycosaminoglycans. The maximum efficiency of reconstitution of fibrils from collagen solution under the influence of GAGs of crude isolate from bovine cartilages was observed at pH 3 after 45 min, both at 4°C and 20°C. The decrease in the acidity of the medium from pH 3 to 8 or the presence of NaCl inhibited the formation of fibres. Collagen dissolved in chloride acid can be completely precipitated using crude GAGs isolate if the weight ratio of reagents, collagen:GAGs, is 1:0.3. The interaction of GAGs with collagen is due to ionic bonds between the carboxyl, and/or sulphate groups of GAGs and ε-amine groups of lysine and hydroxylysine residues, guanidyl groups of arginine and N-termal α-groups of collagen.

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WPŁYW GLIKOZAMINOGLIKANÓW NA REKONSTYTUCJĘ FIBRYL KOLAGENOWYCH

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Celem pracy było sprawdzenie efektywności rekonstytucji fibryl i włókien z roztworu kolagenu pod wpływem glikozaminoglikanów (GAGs). Stosowano roztwory kolagenu, który wydzielono z nieodwłosionych ścinków skór bydlęcych, trawionych w 10% roztworze NaOH nasyconym Na_2SO_4 oraz roztwory GAGs, wyizolowane z bydlęcych tchawic. Efekt oddziaływań GAGs z kolagenem, w układach o sile jonowej modyfikowanej chlorkiem sodu w zakresie 0–0,7 oraz pH 3–8, oceniano wizualnie – na podstawie porównania ilości powstałych włókien, mierząc absorbancję układu przy długości fali 400 nm oraz oznaczając zawartość białka w supernatancie metodą biuretową.

Stwierdzono, że dodatek GAGs do roztworu kolagenu powoduje natychmiastowe powstawanie włókien, których ilość wzrasta wraz z ilością dodanych glikozaminoglikanów i stężeniem kolagenu (rys. 1, 2 i 3). Optymalne dla działania glikozaminoglikanów jest pH 3 i nieobecność chlorku sodu. Całkowite strącenie kolagenu z roztworu kwasu solnego w postaci fibryli i włókien uzyskuje się przy stosunku wagowym suchych reagentów (kolagen:GAGs) wynoszącym 1:0.3. Zwiększenie siły jonowej chlorkiem sodu nie stymuluje tworzenia włókien, lecz powoduje mętnienie układu, wynikające z pojawienia się drobnych kłaczków i zwiększenie lepkości układu bez względu na zawartość glikozaminoglikanów.