INFRARED SPECTRA OF CARTILAGE PROTEOGLYCANS

S. M. Bychkov, V. N. Bogatov, and S. A. Kuz'mina

UDC 612.751.2-088.1:547.963.1.06:543.422.4

KEY WORDS: infrared spectroscopy; cartilage; protein-chondroitin-keratan sulfate; aggregates of proteoglycans.

Characteristic groupings and bonds can be detected by infrared (IR) spectroscopy in glycosaminoglycan components of proteoglycans and the chemical individuality of these preparations can be identified, a matter of great importance when studying the structure of the glycosaminoglycans and their biological functions [1, 9, 11, 13-16]. The possibility of directly detecting proteoglycans present in the tissues by IR spectroscopy and of discovering changes in their macromolecules in the body in different states is of great interest in this connection.

The investigation described below was devoted to the study of cartilage in which proteoglycans are contained chiefly in the form of soluble (unaggregated) protein-chondroitin-keratan sulfate (PCKS) and as proteoglycan aggregates (PA), consisting of PCKS (slightly different from the unaggregated form), hyaluronate, binding protein, and sialic acid [2].

EXPERIMENTAL METHOD

Bovine tracheal cartilage, before spectroscopy and chemical investigation, was dehydrated by treatment twice with five times its weight of ethanol at 0°C, and then washed with ether. Before investigation, all preparations were kept *in vacuo* above $CaCl_2$ and paraffin.

The PCKS fraction [5] and PA were isolated from cartilage by the method of Hascall and Sajdera [12] with certain modifications (extraction with 3 M $MgCl_2$ solution instead of a 4 M solution of guanidine hydrochloride). The PCKS and PA were used as potassium salts for spectroscopy. Additional removal of proteoglycans left in the tissue after extraction of PCKS and PA was carried out by treatment with hyaluronidase (from Serva). The enzyme was added in a dose of 5 mg to 400 mg of cartilage remaining after extraction of PCKS and PA from it, and homogenized in 5 ml 0.05 M acetate buffer, pH 5.0. Incubation continued for 2.5 h at 37°C. The tissue residue obtained after treatment with hyaluronidase was washed with water and dehydrated as described above. To remove proteoglycans, in some experiments ascorbic acid, which breaks down glycosamine components of proteoglycans [17], also was used. To a homogenate of cartilage (400 mg tissue in 10 ml 0.05 M acetate buffer, pH 5.0) 400 mg ascorbic acid was added, the mixture was allowed to stand for 2.5 h at 20°C, after which the tissue residue was treated in the same way as after treatment with the enzyme. The proteoglycan preparations were subjected to quantitative analysis for nitrogen (by the micro-Kjeldahl method), amino sugars [3], and hexuronic [5] and sulfuric acids [4]. Only the content of amino sugars and sulfate was determined in cartilage.

IR spectra were obtained from dry preparations mixed with KBr in the ratio of about 1:300. Tablets 13 mm in diameter were pressed with a force of 10 t. The spectra were recorded at 20°C on a spectrophotometer (Perkin Elmer, model 577) in the frequency band 4000 200 cm^{-1}. The width of the slit was chosen so that the signal:noise ratio was not less than 100:1, and the scanning speed was 50 $cm^{-1} \cdot min^{-1}$.

Research Laboratory of Biological Structures, Ministry of Health of the USSR, Moscow.
(Presented by Academician of the Academy of Medical Sciences of the USSR S. S. Debov.) Translated from Byulleten' Eksperimental'noi Biologii i Meditsiny, Vol. 90, No. 11, pp. 561-563, November, 1980. Original article submitted May 15, 1980.

0007-4888/80/9011-1534$07.50

TABLE 1. Results of Analysis of Preparations (in %)

Preparation	Nitrogen	Amino sugars	Hexuronic acids	Sulfate groups	Protein
PA	3,80	29,40	26,20	11,22	16,50
PCKS	4,06	25,00	24,00	10,85	—
Cartilage:					
original	—	14,40	—	5,52	—
after extraction with 3 M $MgCl_2$	—	11,40	—	0,55	—
after action of hyaluronidase	—	11,00	—	0,34	—
after action of ascorbic acid	—	11,00	—	0,32	—

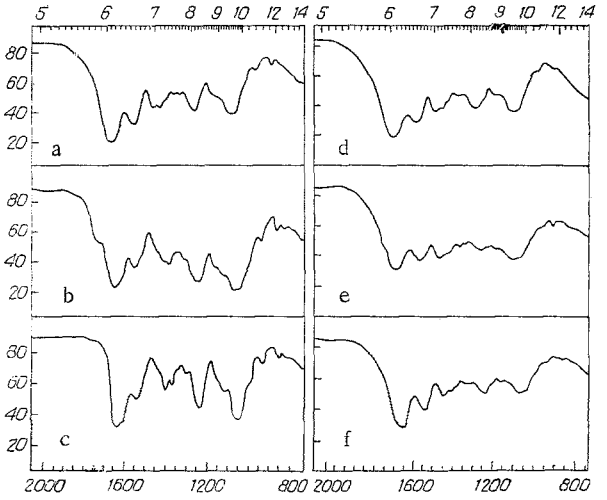

Fig. 1. IR-spectra of cartilage (a), PA (b), PCKS (c), and preparations obtained after removal of proteoglycans from cartilage by extraction with 3 M $MgCl_2$ (d) and by the action of hyaluronidase (e), and ascorbic acid (f). Abscissa: above — wavelength (in μ), below — wave numbers (in cm^{-1}); ordinate, transmission (in %).

EXPERIMENTAL RESULTS

The IR-spectra of whole cartilage (Fig. 1a) revealed a wide absorption band with a maximum of 1650 cm^{-1} ("amide I"), due to combined absorption of the carbonyl group of the acetamide residue, of amino sugars acetylated at the amino group, and the COOK group of hexuronic acids, and also C—N, C—C—O, and C—N—R bonds [7-9]. Alongside it was a band at 1550 cm^{-1} ("amide II"), due to deformation valence oscillations of the NH group [1, 8, 9]. Bands at 1245 and 850 cm^{-1}, connected with oscillations of S=O and C—O—S groups, respectively [9, 14], were clearly represented in the spectrum of this tissue. The first of these bands is also partly due to deformation oscillations of the NH group and valence oscillations of the C—N bond ("amide III"). The spectrum of whole cartilage also contains a distinct shoulder in the 1150-1100 cm^{-1} region, which has not yet been identified, despite the fact that clear bands at 1150 and 1125 cm^{-1} are present in this narrow frequency interval in the spectra of several proteoglycans [1].

Wide bands at 1650 cm^{-1} ("amide I"), 1550 cm^{-1} ("amide II"), 1245, and 850 cm^{-1}, due to the above-mentioned oscillations, were present in the IR spectrum of PA isolated from cartilage (Fig. 1b). This spectrum also contained a shoulder at 1740-1700 cm^{-1}, which could be attributed to oscillations of the carbonyl group of the sialic acid residues that are a component of PA. Finally, this spectrum had clearly distinguishable shoulders at 1150 and 1125 cm^{-1}. The spectrum of PCKS (Fig. 1c) is mainly characterized by the same

absorption bands as the spectrum of PA, except that two maxima (1650 and 1615 cm^{-1}) were clearly distinguishable in the complex, wide "amide I" band, and instead of the shoulders at 1150 and 1125 cm^{-1} mentioned above, the spectrum of PA contained only one small, but well defined, band at 1125 cm^{-1}. It can be concluded from the presence of two clearly defined shoulders at 1150 and 1124 cm^{-1} in the spectrum of PA, the first of which also is observed in the spectrum of hyaluronate [1] and the second in the spectrum of PCKS, and also from the discovery of sialic acid in the PA spectrum, that the sample of this macromolecular complex consisted of a proteoglycan of PCKS type, hyaluronate, and sialic acid, i.e., that it was isolated from cartilage in a chemically unchanged form. It also follows from the spectrum of PCKS that this sample of this proteoglycan was not contaminated by hyaluronate.

The spectra of samples of cartilage obtained after extraction of PA and PCKS from it (Fig. 1d) were characterized by a decrease in amplitude of all bands belonging to the spectrum of PA and PCKS. The decrease in amplitude of the 1245 cm^{-1} band was particularly considerable. Bands in the 850-800 cm^{-1} interval, however, were observed only in the form of shoulders. Treatment of the residual material of cartilage after extraction of proteoglycans from it by salt soltuions with hyaluronidase (Fig. 1e) or with ascorbic acid (Fig. 1f) led to disappearance of the 850-800 cm^{-1} band from the spectrum of the resulting preparation and to a marked decrease in amplitudes of the "amide I," "amide II," and 1245 cm^{-1} bands.

It follows from the results of chemical analysis (Table 1) that extraction of cartilage with 3 M $MgCl_2$ solution led to a decrease in the content of amino sugars in the residual tissue by 20%, whereas the content of sulfate was reduced by 90% as a result of the same treatment. The action of hyaluronidase and ascorbic acid on the cartilage preparation obtained after extraction of proteoglycans from it reduced the hexosamine content by 30% and the sulfate content by 94% or, in other words, led to the virtually complete removal of sulfated proteoglycans from the tissue.

Comparison of IR spectra of PA and PCKS isolated from cartilage revealed that all absorption bands characteristic of these biopolymers were well marked in the spectrum of whole cartilage also. The fact that total removal of proteoglycans from the cartilage, confirmed by chemical analysis of the treated tissue and disappearance of the bands at 850-800 cm^{-1} from the spectrum of this material, led only to a decrease in the intensity of the "amide I" and "amide II" bands, can be explained by the presence of the same groups and bonds in collagen, elastin, glycoproteins, and other protein substances as are present in proteoglycans. The weak band at 1250 cm^{-1} which was still present in the spectra of cartilage from which the proteoglycans had been removed was due to valence and deformation oscillations of the NH group and C–N bond, respectively in the 1305-1200 cm^{-1} range ("amide III"), which are present in proteins also.

The band at 1400 cm^{-1}, present in the spectra of untreated cartilage, PCKS, and PA, disappeared from the spectra of cartilage preparations obtained after complete removal of proteoglycans from it (Fig. 1e, f). The last two preparations gave a further band at 1380 cm^{-1}. The problem of identification of these bands in these cases requires additional investigation.

Hexosamine-containing substances remaining in cartilage after extraction of proteoglycans from it and additional treatment with hyaluronidase and ascorbic acid are biopolymers of the type of a structural glycoprotein present in considerable quantities in all forms of connective tissue [6], and which also possesses groups and bonds giving rise to absorption in the "amide I," "amide II," "amide III," and certain other regions of the spectrum.

It follows from these results that by means of IR spectroscopy it is possible to reveal the presence of proteoglycans in cartilage and, within certain limits, to determine their composition qualitatively. IR spectroscopy is thus fully applicable as a method of determining the completeness of extraction of proteoglycans from tissues and, possibly, as a method of studying the strength of the binding of these biopolymers with various tissue structures [10].

LITERATURE CITED

1. S. M. Bychkov, V. N. Kharlamova, Byull. Éksp. Biol. Med., No. 6, 680 (1980).
2. S. M. Bychkov and M. M. Zakharova, Vopr. Med. Khim., No. 3, 227 (1979).

3. S. M. Bychkov and M. F. Kolesnikova, Biokhimiya, 31, 533 (1966).
4. S. M. Bychkov and V. A. Fomina, Vopr. Med. Khim., No. 5, 528 (1960).
5. S. M. Bychkov and V. N. Kharlamova, Biokhimiya, 33, 840 (1968).
6. M. M. Zakharova and S. M. Bychkov, Byull. Éksp. Biol. Med., No. 12, 1430 (1976).
7. L. A. Kozitskaya and N. B. Kupletskaya, The Use of UV-, IR-, NMR-, and Mass-Spectroscopy in Organic Chemistry [in Russian], Moscow (1979).
8. A. Cross, Introduction to Practical Infrared Spectroscopy [in Russian], Moscow (1961).
9. A. M. Ovsepyan, V. V. Kobyakov, and V. P. Panov, Zh. Prikl. Spektrosk., 24, 302 (1977).
10. R. D. Campo, Calcif. Tissue Res., 22, 99 (1976).
11. K. S. Dodgson, A. G. Lloyd, and B. Spencer, Biochem. J., 65, 131 (1957).
12. V. C. Hascall and S. W. Sajdera, J. Biol. Chem., 241, 4261 (1969).
13. A. G. Lloyd, Biochem. J., 75, 478 (1960).
14. A. G. Lloyd, K. S. Dodgson, R. G. Price, et al., Biochim. Biophys. Acta, 46, 108 (1961).
15. S. F. Orr, Biochim. Biophys. Acta, 14, 173 (1954).
16. S. Roseman and J. Ludwieg, J. Am. Chem. Soc., 76, 301 (1954).
17. D. A. Swann, Biochem. J., 102, 896 (1967).