Characterization of Protein-Polysaccharides of Articular Cartilage from Mature and Immature Pigs

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Protein-polysaccharides of femoral articular cartilage from pigs of ages 9 months and 5 weeks were compared after extraction at pH 6.8 with iso-osmotic sodium acetate followed by 0.63 M-calcium acetate. The cartilage from the younger animals had a higher moisture content and contained considerably larger amounts of protein-polysaccharide, but less than half as much collagen/g. dry weight, than cartilage from the older pigs. There was notably less keratan sulphate in the fractions from the less mature animals. After gel filtration on 6% agarose, elution profiles of the calcium acetate extracts were similar to those of the sodium acetate extracts of the same tissue. Chemical analyses, however, showed that in both age-groups the extraction procedure had achieved a sequential solubilization of protein-polysaccharides in that the initial extracts contained a higher proportion of keratan sulphate than those that were extracted subsequently. Both extracts from the older animals contained up to 25% of a relatively small protein-polysaccharide that was retarded on 6% agarose and that had a lower protein content and less keratan sulphate than the larger protein-polysaccharides. In contrast, in extracts from the less mature cartilage only about 5% of the protein-polysaccharides were small enough to be retarded by 6% agarose, suggesting that the small components may not be precursors of the larger. The average length of chondroitin sulphate chains, as calculated from the analytical data, was the same in the smaller protein-polysaccharides as in the larger.

Protein-polysaccharides, as defined by Tsiganos & Muir (1969a), consist of a protein core to which chains of chondroitin sulphate and keratan sulphate are attached by covalent bonds. Pig laryngeal cartilage contains some relatively small, easily solubilized, protein-polysaccharides that may be separated from larger ones by electrophoresis (Muir & Jacobs, 1967) and gel chromatography (Tsiganos & Muir, 1969b). This smaller component differs from the larger ones in chemical composition (Muir & Jacobs, 1967; Tsiganos & Muir, 1969b) and antigenic determinants (Loewi & Muir, 1965; Tsiganos & Muir, 1969b), but its genesis and significance are not yet known. The present study was undertaken to determine whether a similar small constituent was present in pig cartilage from another site serving a different function and whether it was present in young immature cartilage of rapidly growing animals as well as of adults, as would be expected if it were a newly synthesized constituent analogous to neutral-

salt-soluble collagen rather than a later product of catabolism. A preliminary account of some of these results has been presented (Brandt & Muir, 1968).

MATERIALS AND METHODS

All reagents were of analytical grade, including acetone and ethanol (R.R. grade; James Burroughs Ltd., London S.E.11), with the exception of 9-aminoacridine hydrochloride, glucosamine hydrochloride, galactosamine hydrochloride, N-acetylneuraminic acid, authentic neutral sugars and acetylacetone, which was redistilled (b.p. 133–134°).

Hexuronic acid. Hexuronic acid was determined by a modification (Bitter & Muir, 1962) of the method of Dische (1947), with glucuronolactone as a standard.

Hexosamine. Samples were hydrolysed in 8M-HCl for 3 hr. at 95° (Swann & Balazs, 1966) and the total hexosamine content was determined as described by Tsiganos & Muir (1969a) by using a modification of the distillation procedure of Cessi & Piliego (1960). Glucosamine hydrochloride recrystallized to constant optical rotation was used as a standard.

Xylose. The method of Tsiganos & Muir (1966) was used with xylose as a standard and the interference by other sugars was accounted for as described by Tsiganos & Muir (1969b).

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Hexose. The method of Trevelyan & Harrison (1952) was used, with galactose as the standard. Interference by uronic acid was allowed for by addition of glucuronolactone to control tubes in amounts similar to those present in the sample.

Sialic acid. Sialic acid was determined after hydrolysis in 0.05M-H₂SO₄ at 80° for 1 hr. by the method of Warren (1959). *N*-Acetylneuraminic acid was used as a standard.

Hydroxyproline. Samples were hydrolysed in 8M-HCl at 105° for 24 hr. The acid was removed *in vacuo* over P₂O₅ and the residue made up to 1 ml. with water. Hydroxyproline was determined either by the method of Kivirikko, Laitinen & Prockop (1967) or by that of Stegemann (1958) with the use of an automated procedure.

Protein. Total protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

Molar ratios of glucosamine to galactosamine. A Locarte amino acid analyser was used to determine the molar ratios of the two hexosamines as described by Tsiganos & Muir (1969b). Samples were hydrolysed in 8M-HCl at 95° for 3 hr. and then concentrated to dryness by rotary evaporation at 40°. The residue was dissolved in water and samples containing about 1.5 μ moles of hexosamine were used for each determination.

Thin-layer chromatography of neutral sugars. Samples were hydrolysed in 1M-HCl at 100° for 3 hr. The solution was neutralized with Dowex 1 (acetate form) and the slurry poured on to a column (0.8 cm. $\times$ 20 cm.) of Dowex 50W (H⁺ form), and eluted with 3 column volumes of water. The eluent was evaporated to dryness, and the residue was taken up in water and applied to plates of silica gel (Chromalux; British Drug Houses Ltd., Poole, Dorset) impregnated with 0.3M-NaH₂PO₄ (Ovodov, Evtushenko, Vaskovsky, Ovodova & Soloveva, 1967). The plates were developed with propan-1-ol-pyridine-water (5:3:2, by vol.). Samples of authentic sugars were applied to the same plate alongside the hydrolysate. The dried plates were sprayed with an aniline reagent (Waldi, 1965).

Electrophoresis on cellulose acetate. The protein-polysaccharide fractions separated by gel filtration were digested with Pronase as described below. Samples (approx. 10 μ g.) were applied to cellulose acetate strips impregnated with 0.1M-phosphate buffer at pH 7.2. A potential difference of 8 v/cm. was applied for 1 hr., after which the strips were stained with aq. 0.1% Toluidine Blue. Samples of authentic chondroitin sulphate and hyaluronic acid were applied to the same strips.

Tissue. Seven normal knee joints from pigs aged 9 months and eight joints from pigs aged 5 weeks were obtained at slaughter and while still warm were frozen in solid CO₂. After being thawed to 4°, the joints were opened and the synovial fluid was wiped away. The cartilage was shaved from femoral condyles and femoral surfaces of the patellas and diced with a scalpel. Three portions (about 100 mg.) of diced cartilage from each age group were weighed immediately and then placed in acetone for 24 hr. during which time the acetone was changed once. The remainder of the cartilage was frozen in liquid N₂ and pulverized; 35 g. of granular powder was thus obtained from the older joints and 20 g. from the younger.

The weighed samples were removed from the acetone and dried to constant weight over P₂O₅ at 80°. The dry weight/100 mg. of original cartilage was thus determined in

triplicate, the values agreeing to within $\pm 4\%$. Samples (about 5 mg.) of dried cartilage were used to determine total hydroxyproline content after hydrolysis. The remainder of the dried cartilage was digested with Pronase as described below and the total glycosaminoglycans isolated in the same way as the protein-polysaccharides.

Extraction of protein-polysaccharides. Portions (15 g.) of pulverized cartilage were suspended in 100 ml. of 0.15M-sodium acetate, pH 6.8, and cooled to 2°. The suspension was homogenized at low speed for 5 min. with the temperature maintained below 5°. The combined homogenates were filtered through two layers of lint and washed three times with 50 ml. of 0.15M-sodium acetate. The clear filtrate and washings were combined and the protein-polysaccharides immediately isolated with 9-aminoacridine hydrochloride and purified as described by Tsiganos & Muir (1969a). The sodium salts thus obtained were dried to constant weight *in vacuo* over P₂O₅ at 80°.

The remaining cartilage residue was suspended in 200 ml. of 0.63M-calcium acetate, pH 6.8, to which a few drops of chloroform were added and stirred gently for 72 hr. at 4°. The suspension was filtered through two layers of lint, and the insoluble material was resuspended in 100 ml. of the calcium acetate solution and homogenized at low speed for 5 min. in the cold. The suspension was filtered and both filtrates were combined. The protein-polysaccharides were isolated and purified as described by Tsiganos & Muir (1969a). The glycosaminoglycans in the insoluble residues were obtained after digestion of the residue with Pronase from *Streptomyces griseus* (Nomoto & Narahashi, 1959) at 55° for 24 hr. in 0.1M-borate buffer, pH 8.2, containing 0.02M-CaCl₂. For each g. of tissue 10 mg. of enzyme in 50 ml. of buffer was used. The very small amount of undigested material remaining was removed by centrifugation. The glycosaminoglycans in the supernatant were isolated and purified in the same way as the protein-polysaccharides.

The amount of uronic acid remaining in the supernatants from the various precipitations with 9-aminoacridine was determined after removal of excess of acridine with Dowex 50 (8% cross-linking; Na⁺ form). The resin was filtered, and the filtrate was concentrated by rotary evaporation and made up to a known volume for analysis.

Gel chromatography of protein-polysaccharides. A column (45 cm. $\times$ 1.8 cm.) was packed with 75–125 μ m. beads of 6% agarose as described by Tsiganos & Muir (1969b) [6% agarose was a gift from Dr C. P. Tsiganos, and was prepared as described by Tsiganos & Muir (1969b)]. Samples (8 mg.) in 2.5 ml. of 0.5M-sodium acetate, pH 6.5, were dissolved with stirring overnight at 4° before application to the column, which was eluted with 0.5M-sodium acetate, pH 6.5; 5 ml. fractions were collected at 15 ml./hr. and the uronic acid content of each fraction was determined.

For preparative separation a larger column (100 cm. $\times$ 2.5 cm.) was used. Samples (30–35 mg.) in 4–5 ml. of sodium acetate solution were applied and 5–5 ml. fractions were collected. Tubes containing material that was retarded or excluded by the gel were pooled and concentrated by ultrafiltration (Tsiganos & Muir, 1969b). The concentrated fractions were filtered through grade 4 sintered-glass funnels, and the protein-polysaccharides were precipitated with ethanol, washed and dried (Tsiganos & Muir, 1969a). The elution profile of each fraction was examined on the small 6% agarose column, and the preparative chromatography was repeated once or twice more until each fraction

was wholly retarded or wholly excluded. The fractions were precipitated with 9-aminoacridine and finally obtained as dry sodium salt (Tsiganos & Muir, 1969a).

RESULTS

The whole cartilage from the two age-groups differed in several respects. The dry weight/g. of fresh cartilage was considerably higher in the older cartilage (Table 1), which contained twice as much hydroxyproline but 20% less uronic acid than the young cartilage. Collagen accounted for 59% of the dry weight of the older and 26% of the dry weight of the younger cartilage. The total glycosaminoglycans of the older tissue contained a much higher proportion of glucosamine relative to total hexosamine.

The cartilage from each age-group was, as far as possible, treated in the same way. The total uronic acid accounted for in the various fractions was approx. 75% in the older sample and approached 100% in the younger. All compounds containing uronic acid were precipitated by 9-aminoacridine,

since no significant amounts of uronic acid remained in the various supernatants. The protein-polysaccharides were not extracted quantitatively by the present procedures and those contained in adult cartilage were less readily extracted, by either sodium acetate or calcium acetate. Thus 32% of the total uronic acid of adult cartilage was extracted by sodium acetate, and a further 5.5% by calcium acetate, whereas from the young cartilage 43% of the total uronic acid was extracted by sodium acetate and a further 19% by calcium acetate (Table 2). The glycosaminoglycans that remained in the residue of adult cartilage had a higher molar proportion of glucosamine than that remaining in the young cartilage (Table 3).

In both age-groups the protein-polysaccharides in each extract differed in behaviour on gel chromatography and in chemical composition (Table 3) and hence were considerably heterogeneous. The sodium acetate extract of adult cartilage contained about 25% of material that was small enough to be retarded by 6% agarose (Fig. 1) and that was therefore comparable in size with a similar fraction from laryngeal cartilage of the same age (Tsiganos & Muir, 1969b). There was an appreciable amount of this material in the subsequent calcium acetate extract also (Fig. 1). On the other hand, sodium acetate extracts of young cartilage contained only about 5% of material retarded by 6% agarose, which was absent from the subsequent calcium acetate extract of this cartilage (Fig. 1). Analyses of the fractions of protein-polysaccharide separated by 6% agarose in the extracts from the older and the younger cartilage are given in Table 3. There was not enough of the retarded fractions from the young cartilage for full analysis. All the protein-polysaccharides contained similar amounts of uronic acid, hexosamine, hexose and xylose, but those retarded by the gel contained less protein and less glucosamine than those that were excluded.

There were also differences in the molar ratio of

Table 1. *Composition of articular cartilage of pigs aged 9 months and 5 weeks*

Acetone-treated cartilage was dried to constant weight to determine the dry weight. The total glycosaminoglycans were isolated after digestion of the cartilage with Pronase (see the text).

Age	Dry weight (mg./g. of wet tissue)	Hydroxyproline (g./100g. of dry cartilage)	Uronic acid (g./100g. of dry cartilage)	Glucosamine/galactosamine molar ratio of total glycosaminoglycans
9 months	320	8.4	2.65	1:18.5
5 weeks	200	3.7	3.20	1:24

Table 2. *Yields of protein-polysaccharides in sequential extracts of femoral articular cartilage*

The cartilage was extracted at pH 6.8 with 0.15M-sodium acetate followed by 0.63M-calcium acetate (see the text). The glycosaminoglycans remaining in the cartilage were isolated after digestion with Pronase (see the text).

Age	Protein-polysaccharides				Glycosaminoglycans in residue % of total uronic acid
	Sodium acetate extract		Calcium acetate extract		
	Yield (mg./g. of dry cartilage)	% of total uronic acid	Yield (mg./g. of dry cartilage)	% of total uronic acid	
9 months	25	32.2	5	5.5	37.8
5 weeks	46	43.1	20	18.8	36.8

Table 3. *Analyses of protein-polysaccharides extracted sequentially with 0.15 M-sodium acetate followed by 0.63 M-calcium acetate*

The remaining cartilage was digested with Pronase, the glycosaminoglycans were isolated and molar ratios of glucosamine to galactosamine were determined (see the text).

Age	Extract	6% Agarose fraction	Composition (% of dry wt.)					Glucosamine/galactosamine molar ratio
			Uronic acid	Hexose	Hexosamine	Xylose	Protein	
9 months	0.15 M-Sodium acetate, pH 6.8	Excluded	28.5	6.5	32.0	1.2	8.2	1:19
		Retarded	29.9	5.4	32.4	1.1	3.3	1:70
	0.63 M-Calcium acetate, pH 6.8	Excluded	29.1	6.3	32.7	1.1	6.1	1:29
		Retarded	28.2	5.2	31.3	—	3.9	1:47
	Cartilage residue	—	—	—	—	—	—	1:15
5 weeks	0.15 M-Sodium acetate, pH 6.8	Excluded	28.2	6.0	31.3	1.0	8.1	1:28
		Excluded	27.5	6.1	31.8	1.3	7.6	1:35
	0.63 M-Calcium acetate, pH 6.8	Excluded	—	—	—	—	—	1:21
	Cartilage residue	—	—	—	—	—	—	—

Fig. 1. Elution of protein-polysaccharides with 0.5 M-sodium acetate, pH 6.8, at 4° from a column (45 cm. × 1.8 cm.) of 6% agarose; 5 ml. fractions were collected and the extinction of the coloured product of the reaction of uronic acid with carbazole was determined in each fraction. IA, protein-polysaccharides from cartilage of pigs aged 9 months and IB, from cartilage of pigs aged 5 weeks, extracted with 0.15 M-sodium acetate, pH 6.8. IIA, protein-polysaccharides from the residue of IA, and IIB, those from the residue of IB, extracted with 0.63 M-calcium acetate, pH 6.8. V_0 is the void volume of the column.

glucosamine to galactosamine between the protein-polysaccharides in sodium acetate and calcium acetate from both age groups. In the calcium acetate extracts of adult cartilage those protein-

polysaccharides that were retarded by the gel contained more glucosamine than those extracted with sodium acetate (Table 3).

In contrast, the larger compounds of both age

groups that were extracted with sodium acetate contained more glucosamine than those in the corresponding calcium acetate extracts.

DISCUSSION

The protein-polysaccharides in articular cartilage of adult pigs were far less easily extracted than those in laryngeal cartilage of pigs of the same age. The articular cartilage in this study had to be pulverized in liquid nitrogen before appreciable amounts of protein-polysaccharides could be extracted, whereas laryngeal cartilage needed only brief low-speed homogenization before considerable amounts were brought into solution (Tsiganos & Muir, 1969a). This difference may be due to the greater amount of collagen in joint cartilage, since it has been found that the hydroxyproline content of pig femoral articular cartilage is about twice that of larynx at 9 months of age (K. D. Brandt, C. P. Tsiganos & H. Muir, unpublished work).

Comparisons of collagen content of articular cartilage of long bone of calves and fetuses (Campo & Tourtellotte, 1967), have indicated an increase in collagen content with maturation in the bovine species as well. The collagen content of 59% in the articular cartilage of mature pigs in our study is similar to that of human adult femoral articular cartilage found by Anderson, Ludowieg, Harper & Engelman (1964).

The decrease in water content of articular cartilage from 80% in 5-week-old pigs to 68% in 9-month-old animals differs somewhat from the results of Linn & Sokoloff (1965), who found a water content of approx. 80% in human patellas in early childhood, but a fall of only about 3% after the age of 30. Miles & Eichelberger (1964) likewise found no appreciable change in water content of human knee articular cartilage between the ages of 10 and 26 years. It therefore appears that the fall in moisture content of porcine femoral articular cartilage during maturation is not shared by human knee-joint cartilage.

Despite the greater difficulty of extracting the protein-polysaccharides from articular cartilage, the older tissue contained some of smaller size, comparable with those of laryngeal cartilage (Tsiganos & Muir, 1969b) in that both were retarded by 6% agarose. These were almost absent from immature cartilage, so that they may not be precursors of the larger compounds, particularly since they contained much less glucosamine and somewhat less protein than the larger compounds (Table 3). This was also true of protein-polysaccharides of laryngeal cartilage (Tsiganos & Muir, 1969b), and it therefore appears that, in general, the amount of glucosamine and protein increases with the size of the protein-polysaccharide.

As discussed by Tsiganos & Muir (1969b) there appear to be several different core proteins in protein-polysaccharides, as indicated by the presence of several *N*-terminal amino acids and several antigenic determinants, as well as by differences in the amino acid compositions of larger and smaller compounds. Differences in protein content shown in Table 3 may be explained if it is assumed that protein-polysaccharides of larger size possess longer protein cores. Hoffman, Mashburn & Meyer (1967) and Hoffman, Mashburn, Meyer & Bray (1967) have suggested that the keratan sulphate content is proportional to protein content. The increasing proportion of keratan sulphate in larger protein-polysaccharides would thus be explicable also if core proteins were of different lengths.

Keratan sulphate has been shown to be an integral part of protein-polysaccharides from laryngeal cartilage (Tsiganos & Muir, 1967) and nucleus pulposus (Heinegård & Gardell, 1967) and it would account for the glucosamine in all the fractions studied here. No material with the mobility of hyaluronic acid was detected when the glycosaminoglycans were subjected to electrophoresis on cellulose acetate, nor were serum proteins detected by immunological methods (Tsiganos & Muir, 1969b). Furthermore, since none of the fractions contained sialic acid, and fucose was not detected by t.l.c., no significant amounts of glycoprotein were present that would account for the glucosamine. The finding of xylose and galactose in all fractions may be explained by the neutral sugars of the linkage region (Rodén & Armand, 1966; Lindahl & Rodén, 1966) and by the galactose of keratan sulphate.

Although the basis for the variations in size is unknown, it is unlikely that the gel-filtration profiles reflect differences in the lengths of chondroitin sulphate chains of the chondroitin sulphate-protein. An estimate of the average length of chondroitin sulphate chains in the larger and the smaller compounds was obtained from the molar ratio of galactosamine to xylose, assuming that all xylose is engaged in linking chondroitin sulphate to protein (Lindahl & Rodén, 1966; Rodén & Armand, 1966) and that all galactosamine is accounted for by chondroitin sulphate, disregarding the possibility that a small proportion of galactosamine might be derived from the linkage region of keratan sulphate (Mathews & Cifonelli, 1965; Bray, Lieberman & Meyer, 1967). In the fractions analysed the chondroitin sulphate chains were thus calculated to average 20–26 repeating disaccharide units in length, with the small protein-polysaccharides in the sodium acetate extract of the older pigs having an average chain length of 26 repeating units. Tsiganos & Muir (1969b) likewise

found no difference in chain length of chondroitin sulphate in protein-polysaccharides of different size from laryngeal cartilage, and their calculations, which were based in addition on losses of hydroxy-amino acids after β -carbonyl elimination, gave chains of 28 disaccharide units.

It is improbable that the differences in size and composition of the protein-polysaccharides arose from the action of degradative enzymes, as discussed by Tsiganos & Muir (1969b), whose extraction procedure (Tsiganos & Muir, 1969a) was essentially employed here.

The larger, gel-excluded, protein-polysaccharides were not identical in the two age-groups. Those from mature cartilage had a higher proportion of glucosamine than those from immature cartilage and in this respect resembled those from mature laryngeal cartilage of the same age. Thus the larger compounds from cartilage of the same age serving different functions were more similar than those from cartilage at different stages of maturity serving the same function.

When compounds in corresponding extracts of young and mature cartilage were compared, those from immature cartilage invariably contained less glucosamine. This difference was extended to the glycosaminoglycans, still left in the cartilage residues, that were obtained after final proteolysis of the tissue. In this articular cartilage, therefore, the population of protein-polysaccharides changes with maturity, increasing in their keratan sulphate content with age. This is in accord with the results of Mathews & Glagov (1966), who found that the proportion of keratan sulphate of human costal cartilage increased markedly from birth to maturity.

The changing composition of protein-polysaccharides in each successive extract of both age-groups showed that there was a mixed population in the tissue and that some selection took place during each extraction. This is consistent with local topographical variations in the distribution of chondroitin sulphate and keratan sulphate in adult human articular cartilage (Stockwell & Scott, 1967; Maroudas, Muir & Wingham, 1969).

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