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PURIFICATION OF THE 'LINK PROTEINS' FROM BOVINE ARTICULAR CARTILAGE AND COMPARISON WITH 'LINK PROTEINS' FROM NASAL SEPTUM

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SUMMARY

Proteoglycan links are glycoproteins present in cartilage ground substance which are reportedly involved in stabilizing the interaction between two other components of cartilage, proteoglycan subunit and hyaluronic acid. These proteins have been isolated and purified in the past by equilibrium density centrifugation. Presented here, for the first time, is evidence for the purification of the links from bovine articular cartilage using a new method which does not involve equilibrium density centrifugation. The results indicate that the articular cartilage links have properties similar to those of the nasal septum links.

INTRODUCTION

Sajdera and Hascall (1) first demonstrated the ability of cartilage proteoglycan to aggregate in the presence of a cartilage fraction which they termed "glycoprotein link". This fraction was separated from the majority of the proteoglycans by equilibrium buoyant density centrifugation of a cartilage extract. The link proteins sedimented to the top, whereas the larger proteoglycans went to the denser regions of the gradient. Further work by Hardingham and Muir (2) showed that another cartilage fraction, which sedimented in the middle of the gradient, was also required for proteoglycan aggregation. The active component in this middle fraction was determined to be hyaluronic acid. They also demonstrated the specificity of this interaction (proteoglycan-hyaluronate) by using low levels of hyaluronic acid.

The abbreviations used are: SDS, sodium dodecyl sulfate. GdmCl, guanidine hydrochloride. Tris, tris(hydroxymethylaminomethane). The nomenclature "A1-D" is that of Hascall and Sajdera (1) and refers to the fractions of a cesium chloride dissociative buoyant density gradient of aggregate.

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Using the information from this and other work, Rosenberg et al. (3) reconstituted the aggregate by combining the three fractions (link protein, hyaluronic acid, and proteoglycan), then examined the product using electron microscopy. As a result of their investigations and more recent work by Heinegard et al. (4), it has been generally accepted that one proteoglycan molecule is bound to hyaluronate at approximately every 50 hyaluronate dissacharide units.

The role played by link proteins in aggregation is not well understood. It is generally agreed that they serve to stabilize the aggregate through some as yet undiscovered mechanism. Two glycoproteins are believed to be the active components present in the fraction stabilizing aggregation. These proteins have been purified from bovine nasal septum by several groups of investigators (5,6,7). The molecular weights of these proteins, as determined by sodium dodecyl sulfate-polyacrylamide gels, are reported to be 52,000 for one species, and 48-44,000 for the other.

There is some evidence to indicate that the two link proteins are structurally related and that one contains a glycoprotein extension. This has been extrapolated from data which show that after trypsin treatment of the link fraction only the lower molecular weight species is recovered. Since the amount of this link protein was increased after trypsin treatment, it was thought that the larger species had been converted into the smaller one (8). Baker and Caterson (6) support these results with further evidence that the two proteins are indeed structurally similar. Their data suggest that the major difference between the links lies in their carbohydrate contents rather than in their amino acid compositions. It is clear that additional investigation of both the structural relationship of these two proteins and their roles in the proteoglycan aggregate will be required before many intricacies of cartilage structure and metabolism will be elucidated.

Since a future goal of this laboratory is to study the cell-free synthesis and mode of action of these two proteins as well as their structural relationship, a method will be required to assay for their presence. Immunological indentification is one such method. This requires large quantities of highly purified antigen in large yield, which are difficult to obtain by the conventional method employing cesium chloride equilibrium density gradients. The purpose of this paper is two-fold: to describe a method for large-scale link purification which is less time consuming than the presently used method of cesium chloride equilibrium density gradients, and to compare the properties of links isolated from bovine articular cartilage to those of links from bovine nasal septum.

EXPERIMENTAL PROCEDURES

All procedures were carried out at 4° unless otherwise stated. Protein determinations were made using the method of Bradford et al. (9).

Isolation of Link. Calf articular cartilage resected in 1 cm slices from 6 ankle joints was placed in 500 ml of a solution containing 6 M GdmCl, 75 mM sodium acetate, pH 7.0, 10 mM EDTA, and 5 mM benzamidine and stirred for 48 hours. The non-solubilized cartilage was separated from the solution by passage thorugh two layers of cheesecloth. The filtrate was centrifuged at 200,000 x g for 6 hours at 4° to remove high molecular weight material. The upper two-thirds of each tube was removed and dialyzed for 12-18 hours against buffer (A) containing 1 M NaCl and 20 mM Tris, pH 7.5. The protein in the dialyzed extract was precipitated by adding ammonium sulfate to 80% saturation and collected after centrifugation at 10,000 x g for 20 minutes. The precipitate was dissolved in buffer (A) and dialyzed against this buffer containing 50% glycerol for 18 hours at 4°. To 2.5 ml of the dialyzed extract (28 mg of protein) was added 1 mg of hyaluronic acid (M.W. 1×10^6) and the solution was dialyzed for 6 hours against buffer (B) containing 4 M GdmCl and 20 mM Tris, pH 5.8. Finally, the extract was dialyzed for 4 hours against 0.5 M sodium acetate, pH 7.0.

The dialyzed material was applied to a CL Sepharose 6B column (2.6 x 35 cm, flow rate 15-20 ml/hour) which had been equilibrated with 0.5 M sodium acetate, pH 7.0 and eluted with the same buffer. One ml fractions were collected and those containing protein and eluting in the void volume were pooled (12 ml containing 4-5 mg of protein). The pooled fractions were dialyzed for 6 hours against buffer (B) containing 50% glycerol. The dialyzed solution (2-3 ml) was applied to a Sephacryl S-200 column (2.6 x 43 cm, flow rate 6 ml/hour) which had been equilibrated with buffer (B) and 2 ml fractions were collected. Protein-containing fractions eluting from the column just beyond the void volume (M.W. 100×10^3) were pooled and dialyzed against buffer (A) containing 50% glycerol. Samples were stored in this buffer at -20°.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of purified link. Gel electrophoresis in the presence of sodium dodecyl sulfate was performed according to the procedure of Weber and Osborn (10) with 10%

polyacrylamide gels. Samples (10 µg) were prepared by dissolving them in a solution of 1% 2-mercaptoethanol, 1% SDS and 10 mM $NaPO_4$, pH 7.0 in 25% glycerol and heated for 3 minutes at 90°. The samples were applied to the gel surface and electrophoresis was carried out at room temperature for 4 hours at a current of 6 mAmps/gel. The molecular weight standards were albumin (68,000), ovalbumin (45,000) and myoglobin (17,800).

Agarose immunodiffusion studies. These studies were performed on 0.8% agar according to standard procedures. Samples were applied to each well and diffusion allowed to procede at room temperature.

Amino acid analysis of link protein. Samples for amino acid analysis were hydrolyzed in 6 N HCl for 6 hours at 110° in vacuo before application to a Durham Amino Acid Analyzer.

Production of antisera to link protein. New Zealand white rabbits weighing from 2.0 to 2.4 kg were injected with homogenous link preparations over a 7 week period. The first injection into the foot pads contained 200 µg of link mixed with an equal volume of Freund's complete adjuvant (5 ml of protein solution added to 5 ml of Freund's). The second and third were subcutaneous and intramuscular injections of 100 µg of link mixed with an equal volume of Freund's incomplete adjuvant. All injections were at 2 week intervals. One week after the final injection the rabbit's serum was tested for reactivity with homogenous link on an agar immunodiffusion plate (see above). If the reaction appeared positive, the rabbit was bled and the gamma globulin fraction partially purified with 40% ammonium sulfate precipitation of the serum. The precipitated protein was dialyzed against phosphate buffered saline containing 0.2% sodium azide and stored in this buffer at -40°. Final protein concentration was 40 mg/ml.

Treatment of proteoglycan subunit with chondroitinase. Proteoglycan subunit (5 mg/ml) in 0.1 M sodium acetate, pH 7.0 was incubated with chondroitinase ABC (Sigma, 0.02 U per mg dry weight proteoglycan) at 37° for 20 minutes. The digested sample was then tested for reaction with link antibody in an agar immunodiffusion plate.

RESULTS AND DISCUSSION

The procedure described here for the purification of link protein from articular cartilage yields a highly purified preparation of these factors (see figure 1). The most important advantage this method offers over the standard procedure employing equilibrium buoyant density gradients is its relative simplicity. This method, unlike any other purification method, does not call for the isolation of aggregate (proteoglycan subunit, hyaluronate, and other factors). Instead, the majority of the aggregate components and other cartilage proteins are separated from the lower molecular weight link fractions by brief centrifugation. The lower molecular weight components present in the upper portion of the tubes are further fractionated by sieve chromatography, using link's capacity for binding to hyaluronic acid. This step separates most of the low molecular weight cartilage proteins, which do not bind to hyaluronic acid, from the link proteins.

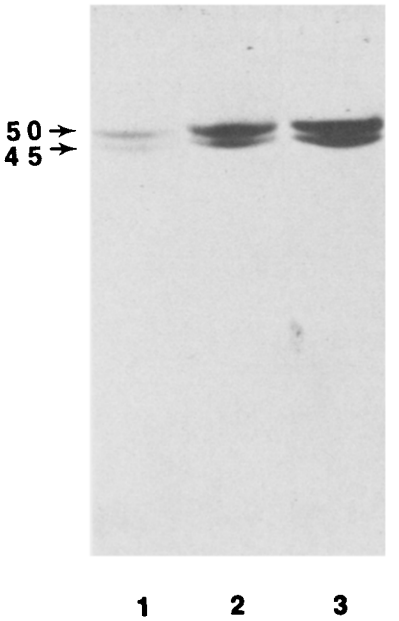

Figure 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of link proteins. Link proteins (5-10 μg) were subjected to electrophoresis in 10% polyacrylamide gels in sodium dodecyl sulfate and stained with Coomassie blue. Link fractions run on individual gels were as follows:
1. 5 μg of bovine nasal link prepared from fraction A1-D5 (7).
2. 10 μg of calf articular cartilage link.
3. 5 μg of bovine nasal link plus 5 μg of calf articular cartilage link.
The numbers indicated by the arrows represent molecular weight x 10^{-3}.

As is shown in table 1, the time required to purify link with conventional methods is considerably longer than with this procedure. The yield of pure link is also lower with the conventional methods.

That these proteins are link can be demonstrated from several lines of evidence. First, the amino acid composition of the link purified by this method is similar to that reported by Bonnet et al. (5) and Baker and Caterson (6) for link obtained from bovine nasal septum by conventional methods. Second, rabbit antisera prepared against homogenous link obtained from articular cartilage in the manner described in this paper formed a line of identity between artiuclar cartilage link and bovine nasal septum link. These results are shown in figure 2. Third, bovine nasal link prepared by conventional methods and articular cartilage link co-migrate on sodium dodecyl sulfate-polyacrylamide gels, as shown in figure 1.

Table 1. Comparison of the amount of time required to purify link using the procedure described in this paper and the equilibrium density gradient method (1).

Cartilage Extract (100 mg protein)	
Method described in text	Equilibrium density method
Centrifuge 6 hours 100,000 x g.	Dialyze 18 hours against associating buffer.
Dialyze upper two-thirds of each tube against 0,5 M NaCl for 18 hours.	Add cesium chloride and centrifuge for 60 hours.
Add ammonium sulfate to 80% saturation. Collect precipitate by centrifugation.	Remove lower one-sixth of each tube. Adjust to 4 M GdmCl.
Add high molecular weight hyaluronic acid (4 mg) to precipitate dissolved in a minimum volume of 4 M GdmCl. Dialyze for 3 hours against associating buffer.	Centrifuge for 60 hours.
Place sample on Sepharose 6B column. Collect excluded fractions, dialyze against 4 M GdmCl for 4 hours.	Remove upper one-sixth of each tube. Dialyze against 4 M GdmCl for 4 hours.
Place dialyzed fraction on Sephacryl S-200 column. Collect 90,000 mol. wt. protein.	Place dialyzed fraction on Sephacryl S-200 column. Collect 90,000 mol. wt. protein.
yield: 0.8 mg	yield: 0.4 mg
Time required to purify link: 2 days.	Time required to purify link: 7 days.

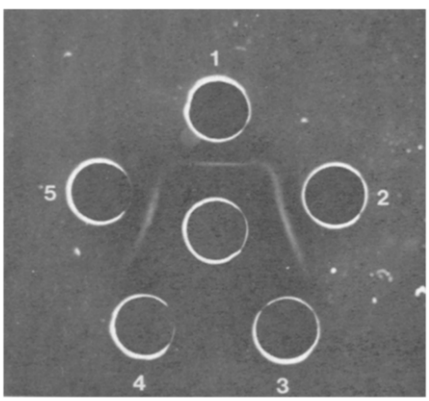

Figure 2. Agarose immunodiffusion studies of link proteins and proteoglycan fractions. The center well contained 8 ul of rabbit antisera prepared against homogenous articular cartilage link. The other wells contained the following:
1. 2.5 ug articular cartilage link
2. 2.5 ug bovine nasal link
3. 10 ug chondroitinase treated proteoglycan subunit (fraction A1-D1)
4. 50 ug chondroitinase treated proteoglycan subunit (fraction A1-D1)
5. 7 ug fraction A1-D5

Table 2. Amino acid analysis of link from bovine nasal septum and link from bovine articular cartilage.

Amino Acid	Bovine Nasal Septum Link	Articular Cartilage Link
	residues/1,000 residues	
Aspartic Acid	119	127
Threonine	47	45
Serine	61	52
Glutamic Acid	92	82
Proline	50	48
Glycine	105	99
Alanine	80	77
Valine	69	73
Methionine	3	2
Isoleucine	36	33
Leucine	85	86
Tyrosine	53	62
Phenylalanine	52	58
Histidine	27	24
Lysine	56	58
Arginine	67	72

As a check on the procedure reported here, link was purified from articular cartilage using the method of Sajdera and Hascall (1). Two major bands present in the top fifth (fraction A1-D5 (1)) of a dissociative buoyant density gradient of the aggregate could be seen after electrophoresis on SDS gels. The molecular weights of these two proteins are identical to those of both the proteins purified by the procedure described here and to those of the proteins purified from bovine nasal septum by the buoyant density gradient (7) In addition, rabbit antisera prepared against the proteins purified by the new procedure reacts with the A1-D5 proteins, as shown in figure 2.

In light of a report indicating a common antigenic site present in link and the hyaluronate binding site region of the proteoglycan subunit (12), it was of some interest to determine whether antisera to link would form a precipitant line with proteoglycans. As demonstrated in figure 2, no precipitant line formed between proteoglycan subunit which had been chondroitinase treated and link antisera, which argues against a common antigenic site. These results indicate that link proteins isolated from articular cartilage are structurally different from proteoglycans isolated from this tissue.

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