CHARACTERISATION OF THE MICROFIBRILLAR COMPONENT OF BOVINE LIGAMENTUM NUCHAE

A. Serafini-Fracassini, J. M. Field and C. Armitt

Department of Biochemistry, St. Andrews University, St. Andrews, Scotland

Received June 16, 1975

SUMMARY: The microfibrillar component of bovine ligamentum nuchae has been solubilised with dithiothreitol in guanidine and purified using collagenase. Equilibrium sedimentation analyses carried out on the carboxymethylated material indicated a molecular weight of about 14,000 daltons. Glycine was the unique N-terminal at a concentration of 65.9 moles/ 10^6g. Amino acid analyses revealed the presence of three thiol groups per molecule. Mannose and glucose were found at a concentration of 3.0 and 3.5% by weight, respectively.

INTRODUCTION

Elastic fibres examined by electron microscopy in tissue sections have been shown to consist of two morphologically distinct components, namely an elastin core and a surrounding layer of microfibrils which are approximately 11 nm in diameter (1). The latter structures are solubilised by reagents which cleave disulphide bonds yielding a cysteine-containing protein which in the present communication will be referred to as microfibrillin. Two conflicting reports have been published on the composition of this protein (1,2) but in neither case was any assessment made of the degree of purity of the preparation. In this article we describe the isolation of microfibrillin from adult bovine ligamentum nuchae by a procedure similar to that originally proposed by Ross and Bornstein (1) but utilising collagenase purified by affinity chromatography. The protein was characterised, as the S-carboxymethyl derivative, with respect to its amino acid and carbohydrate composition, amino end-group content and molecular weight distribution profile.

MATERIALS AND METHODS

Purification of collagenase. A commercially available preparation of Clostridium histolyticum collagenase (EC 3.4.4.19) (Sigma Chemical Co., Type 1) was purified by affinity chromatography under experimental conditions identical to those previously detailed (3) utilising thermally-treated elastin (4) as the affinity support.

Isolation of microfibrillin. The ligamentum nuchae of three-year old cattle was defatted and dehydrated in chloroform-methanol (3:1, by vol.) for 24 hr at 4°. The material was powdered and the 100-200 mesh fraction extracted in 1% NaCl for 24 hr at 4°, in the presence of toluene. The residue was collected by centrifugation and washed with water prior to lyophilisation. An aliquot (100 g) of this material was suspended in 1,000 ml of 5M guanidine-0.1M Tris (pH 7.4) and extracted with continuous stirring for 24 hr at 4°. Guanidine was purified according to Nozaki (5). The extraction was repeated for a total of six 24-hr periods, after which the residue was suspended in 500 ml of 5M guanidine-0.1M Tris (pH 7.4) containing 0.4% EDTA and placed under N_2 barrier. Dithiothreitol was added (final concentration 0.05M) and the suspension stirred at 37° for 48 hr after which time it was centrifuged at 23,000 g for 30 min under N_2 barrier. Iodoacetic acid was added in a four-fold molar excess over dithiothreitol to the supernatant, the pH being adjusted and maintained at 8.6, and the reaction mixture was stirred for 45 min. After addition of a five-fold molar excess of mercaptoethanol over iodoacetate, the solution was dialysed against water for 48 hr when a white precipitate formed within the dialysis sac. Three volumes of ethanol were added to the retentate and the precipitate collected. Contaminating collagen was removed by treatment with collagenase. Aliquots of S-carboxymethylated material were suspended in 0.01M $CaCl_2$, the pH adjusted to 7.5 and collagenase added to give a substrate to enzyme ratio of 500:1. The digestion was carried out at 37° and pH 7.5 in a Radiometer pH stat. When no indication of proteolytic activity was detectable upon further addition of small aliquots of enzyme, the suspension was centrifuged at 38,000 g for 90 min, the residue resuspended in 0.01M $CaCl_2$ and the treatment with collagenase repeated. S-carboxymethylated microfibrillin was collected by centrifugation and washed with water. Contaminating elastin fibrils, revealed by electron microscopic examination, were removed from the preparation, dissolved in 5M guanidine-0.1M Tris (pH 7.4), by centrifugation at 240,000 g for 2 hr. The S-alkylated protein was chromatographed on Sephadex G-75, equilibrated with 5M guanidine. The major fraction which was eluted in a position slightly preceding that of lysozyme, as determined in a trial run, and which accounted for over 90% of the loaded material was recovered after dialysis and lyophilised.

Chemical analyses. Samples for amino acid analysis were hydrolysed in constant-boiling HCl at 110° in sealed tubes under N_2 for 24, 36 and 72 hr. Amino acids were quantitated on a Locarte single-column analyser. Both hydroxyproline (6) and tryptophan (7) were assayed independently. Hexosamines were determined (8) after hydrolysis in 4M HCl at 110° for 8 hr under N_2. Sialic acid was quantitated according to Warren (9). Neutral sugars were estimated by gas chromatography (8) and by reaction with anthrone (10) after resin hydrolysis (11). Amino end-group analysis was performed by the 2-chloro-3,5-dinitropyridine method (12), as previously detailed (3).

Equilibrium sedimentation. Aliquots of the S-carboxymethylated protein were dissolved in 5M guanidine-0.1M Tris (pH 7.4) at concentrations ranging from 0.3 to 1 mg ml^{-1} and dialysed exhaustively against several changes of the same buffer. Sedimentation analyses were carried out on a Spinco model E ultracentrifuge using interference optics. Molecular weight determinations were performed at 20° by the meniscus depletion technique (13), as described by Chervenka (14). Fringe displacements were analysed according to the procedure of Roark and Yphantis (15) with a computer program kindly supplied by Dr. Roark. The partial specific volume was calculated from compositional data (16).

RESULTS AND DISCUSSION

End-group analyses revealed, after correction for regeneration losses (17), 65.9 moles of glycine per 10^6g of microfibrillin, a value which corresponds to a Mn of about 15,000 daltons. Aspartic acid, threonine, serine and alanine were also detected, but at concentrations not exceeding 1 mole per 10^6g.

The apparent molecular weights (18) determined by equilibrium ultracentrifugation at an initial sample concentration of 0.3 mg ml^{-1} and calculated using a partial specific volume of 0.706 ml g^{-1} are reported in Table 1. The narrow spread of the moments Mn, Mw and Mz at mid-point suggests a low degree of polydispersity in the preparation. At vanishing concentration, the three moments converge to a value of about 14,000 daltons. The accuracy of these extrapolations is supported by the coincidence of their intercepts with those of the ideal moments My4, My5, My6 and My7.

The results of amino acid analyses are reported in Table 2. Neutral sugar determinations revealed only glucose and mannose which accounted for 3.5 and

TABLE 1

APPARENT MOLECULAR WEIGHTS

Moments at mid-point:	
Mn	13,657 ± 279
Mw	14,493 ± 156
Mz	16,235 ± 136
Moments at vanishing concentration:	
Mn	13,866 ± 836
Mw	13,797 ± 557
Mz	13,518 ± 557
My4	14,075 ± 1,881
My5	14,981 ± 1,533
My6	14,075 ± 2,090
My7	15,539 ± 2,021

3.0%, respectively, of the preparation on a moisture- and ash-free basis. Glucosamine was found at a concentration lower that 0.3% and sialic acid was not detected in samples sufficiently large to allow the quantitation of one unit per 1,000 amino acid residues.

The amino acid composition of microfibrillin, reported in this paper, differs substantially from published analyses (1,2). Preliminary investigations conducted in this laboratory suggest that other thiol-containing proteins present in bovine ligamentum nuchae may contaminate preparations of microfibrillin if the tissue is not exhaustively extracted with guanidine. In this respect, it is unfortunate that the extreme insolubility of S-carboxymethyl microfibrillin in solvents of low ionic strength, even after succinylation or introduction of

TABLE 2

AMINO ACID COMPOSITION OF MICROFIBRILLIN FROM BOVINE LIGAMENTUM NUCHAE

Values are expressed as residues/1,000 total amino acid residues and have been corrected for hydrolytic losses.

Hydroxyproline	0.0
Aspartic acid	105.8
Threonine	59.1
Serine	77.6
Glutamic acid	101.8
Proline	43.9
Glycine	82.4
Alanine	66.8
Valine	59.1
Half-cystine	24.9
Methionine	8.6
Isoleucine	49.2
Leucine	100.5
Tyrosine	32.8
Phenylalanine	51.1
Hydroxylysine	0.0
Lysine	67.0
Histidine	17.2
Arginine	42.5
Tryptophan	9.7

aminomethyl functions, has prevented us carrying out further checks on the homogeneity of the preparation. However, the presence of only one amino acid in N-terminal position and the results of molecular weight analyses seem to

exclude the possibility of any gross contamination.

It is worth noting that the protein moiety of microfibrillin might be considered to be genetically related to those of other connective tissue structural glycoproteins as it exhibits a similar total content of amino acids coded by the triplet X-cytosine-X (review, ref. 19). However, the presence of only five units of hexose per molecule is not in keeping with the general structure of glycoproteins which usually possess much larger hetero-polysaccharide chains.

ACKNOWLEDGMENTS

The authors are grateful to Mr. J. C. Hunter for amino acid analyses. This work was supported by a grant from the Scottish Home and Health Department.

REFERENCES

1. Ross, R., and Bornstein, P. (1969) J. Cell Biol. 40, 366-381.
2. Ross, R., and Bornstein, P. (1970) Chemistry and Molecular Biology of the Intercellular Matrix, Vol. 1, pp. 641-655, Balazs, E. A., ed., Academic Press, New York.
3. Serafini-Fracassini, A., Field, J. M., Rodger, G. W., and Spina, M. (1975) Biochim. Biophys. Acta 386, 80-86.
4. Partridge, S. M., Davis, H. F., and Adair, G. S. (1955) Biochem. J. 61, 11-21.
5. Nozaki, Y. (1972) Meth. Enzymol. 26, 43-50.
6. Blumenkrantz, N., and Asboe-Hansen, G. (1970) Anal. Biochem. 55, 288-291.
7. Opienska-Blauth, J., Charezinski, M., and Berbec, H. (1963) Anal. Biochem. 6, 69-76.
8. Bhatti, T., Chambers, R. E., and Clamp, J. R. (1970) Biochim. Biophys. Acta 222, 339-347.
9. Warren, L. (1959) J. Biol. Chem. 234, 1971-1975.
10. Scott, T. A., and Melvin, E. H. (1953) Anal. Chem. 25, 1656-1661.
11. Anastassiadis, P. A., and Common, R. H. (1958) Can. J. Biochem. 36, 413-424.
12. Signor, A., Previero, A., and Terbojevich, M. (1965) Nature 205, 596-597.
13. Yphantis, D. A. (1964) Biochemistry 3, 297-317.
14. Chervenka, C. H. (1970) Anal. Biochem. 34, 24-29.

15. Roark, D. E., and Yphantis, D. A. (1969) Ann. N. Y. Acad. Sci. 164, 245-278.
16. Zamyatnin, A. A. (1972) Prog. Biophys. Mol. Biol. 24, 109-123.
17. Signor, A., and Biondi, L. (1964) Ric. Sci. 4, 165-168.
18. Yphantis, D. A., and Roark, D. E. (1972) Biochemistry 11, 2925-2934.
19. Mathews, M. B. (1975) Connective Tissue - Macromolecular Structure and Evolution, pp. 86-92, Springer-Verlag, Berlin.