AN APPROACH TO THE STUDY OF THE STRUCTURE OF DESMOSINE AND ISODESMOSINE CONTAINING PEPTIDES ISOLATED FROM THE ELASTASE DIGEST OF ELASTIN*

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Each desmosine or isodesmosine can cross-link four peptides. To study the structure of desmosine and isodesmosine containing peptides isolated from elastase digest of elastin, it is, therefore, necessary to cleave the cross-linkages in a manner that the peptides can be isolated in a relatively unaltered form and that the points of cleavage can be identified. Such a cleavage scheme has been developed and applied to a desmosine and isodesmosine containing peptide. This scheme involves the reduction of the pyridinium rings with $NaBH_4$ followed by cleavage with permanganate-periodate reagent.

Introduction

Elastin is a highly insoluble connective tissue protein. Its methods of purification involve extraction, by various means, of other compounds present in the tissue. The residue remaining is taken as elastin (1). Therefore, it is difficult to establish the purity of elastin preparation. However, elastin contains two unique amino acids, desmosine and isodesmosine, not known to be present in other proteins (2,3). In order to study the structure of elastin, we therefore set out to isolate the desmosine and isodesmosine containing peptides from the elastase digest of elastin.

Since each desmosine or isodesmosine residue can cross-link four peptide chains, the study of the structure of desmosine and isodesmosine containing peptides presents a new problem; namely, cleavage of the pyridinium ring to separate the cross-linked peptides in relatively unaltered

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form. A technique for cleaving the pyridinium ring of desmosine and isodesmosine has been developed and applied to an isolated desmosine and isodesmosine containing peptide. These results are reported in this communication.

Experimental

Elastin was prepared from bovine ligamentum nuchae according to the method of Partridge and Davis (4). Twice crystalline elastase was purchased from Worthington Biochemical Corporation. Dowex 50W X 2 and cellulose phosphate (cellex-P) were obtained from Cal. Biochem. Research. Sephadex G-25 was the product of Pharmacia. Reagents for acrylamide gel electrophoresis were purchased from Eastman Organic Chemicals. All other reagents used were analytical grade. Amino acid analyses were carried out with a Beckman-spinco amino acid analyser. Desmosine and isodesmosine were determined as described by Anwar (3). Polyacrylamide gel electrophoresis was performed at pH 9.0 as described by Davis (5). N-terminal residues were identified by the cyanate method of Stark and Smyth (6).

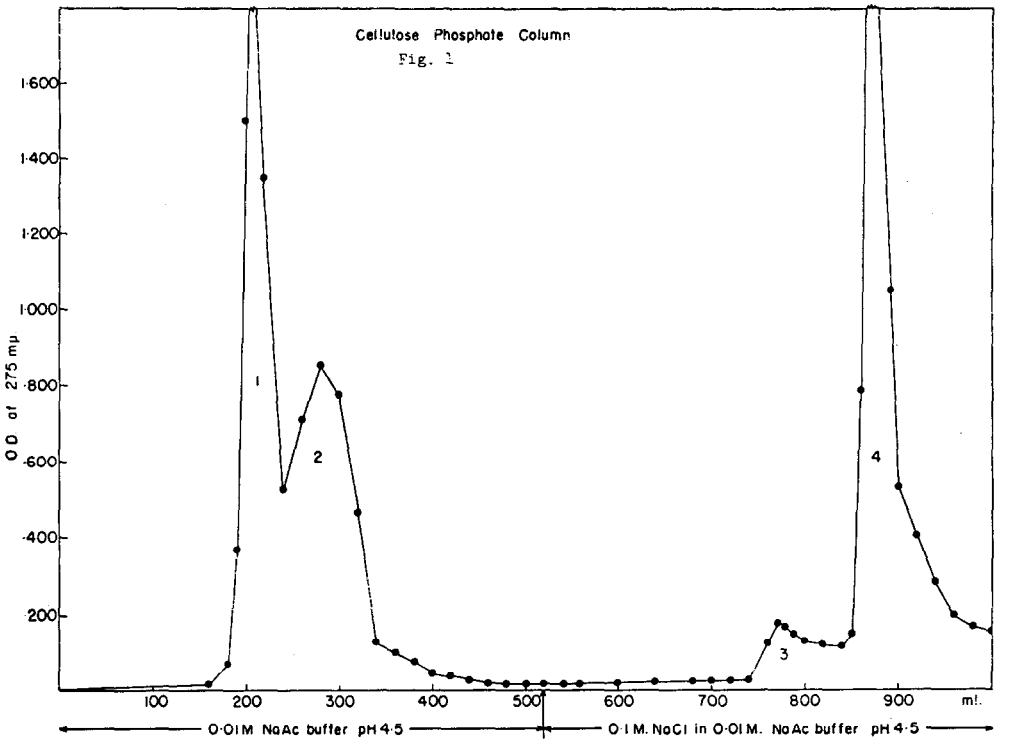
Results and Discussion

Digestion of elastin with elastase: Elastin (5 gm) was suspended in 500 ml of 0.05 M $(NH_4)_2CO_3$ buffer pH 8.8 and stirred for 2 hours prior to the addition of 50 mg of crystalline elastase. The digestion was allowed to proceed for 6 hours at which time 25 mg of fresh elastase were added to the reaction mixture and the reaction mixture allowed to dialyse against 0.05 M $(NH_4)_2CO_3$ buffer, pH 8.8 for 12 hours. During this period, buffer was changed four times. The digestion was stopped by bringing the reaction mixture to boil.

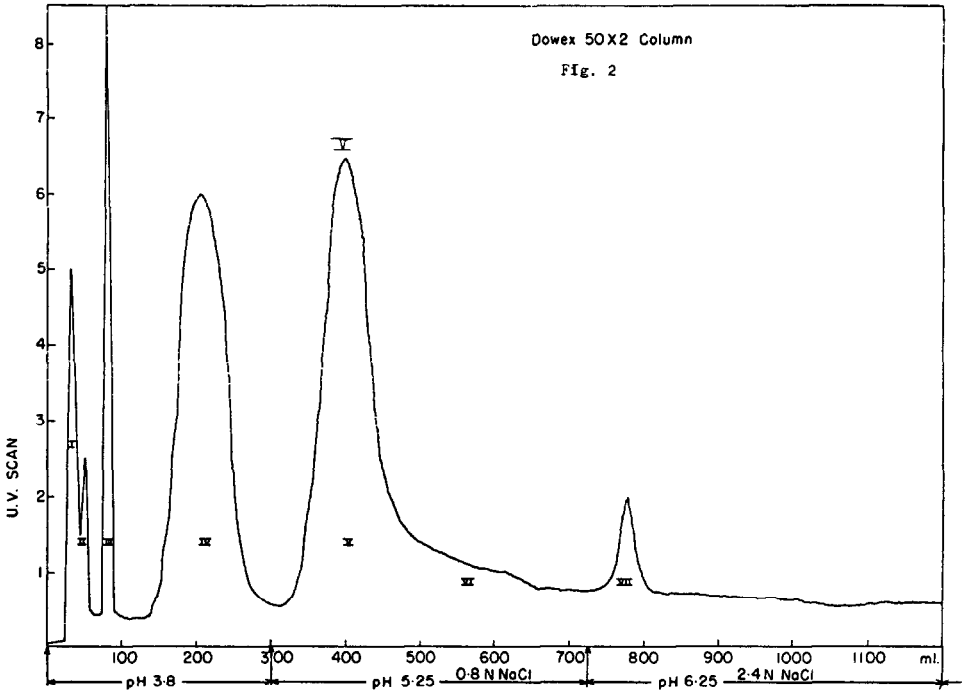
Separation of the peptides: The digest (50 ml) was placed on a sephadex G-25 column (4 x 90 cm) and the column was eluted with 0.05 M $(NH_4)_2CO_3$ buffer, pH 8.8. The fraction which was excluded contained over 90% of the desmosine and isodesmosine present in the digestion mixture applied to the column. This fraction was further separated on cellulose phosphate and

Dowex 50W x 2 column as described below.

Cellulose phosphate chromatography: The fraction excluded from sephadex G-25 column (combined fractions from two runs) were dried under reduced pressure, taken up in about 6 ml of 0.01 M sodium acetate buffer, pH 4.5 and applied to a cellulose phosphate column (2 x 70 cm). The column was then eluted with 500 ml of 0.01 M sodium acetate buffer, pH 4.5 followed by 500 ml of 0.1 M NaCl in 0.01 M sodium acetate buffer, pH 4.5. The elution pattern is shown in fig. 1. The fraction '4' was found to be the richest in desmosine and isodesmosine content and was further fractionated on Dowex 50W x 2 column.



Dowex 50W x 2 column chromatography: The fraction '4' from cellulose phosphate column (combined from two cellulose phosphate runs) was desalted on sephadex G-25 column, dried and applied to a Dowex 50W x 2 column (1 x 70 cm) in about 5 ml of 0.2 M sodium citrate buffer pH 2.2. The column was then eluted sucessively with: 300 ml of 0.2 N sodium citrate buffer, pH 3.8; 425 ml of 0.8 M NaCl in 0.2 N sodium citrate buffer, pH 5.0; 475 ml



of 2.4 M NaCl in 0.2 N sodium citrate buffer, pH 6.1, as shown in fig. 2.

The fraction V (fig. 2) was found to be homogeneous on high voltage paper electrophoresis at pH 3.5 and polyacrylamide gel electrophoresis at pH 9.0. The amino acid compositions at different stages of purification are shown in Table I (i.e. Fraction excluded from sephadex G-25; Fraction 4 from cellulose phosphate column and fraction V from Dowex 50W x 2 column).

The N-terminal analysis of peptide V (fraction V from Dowex) revealed the presence of one glycine (1.1) residue and five alanine (5.2) residues as the N-terminal residues per residue of desmosine present in peptide V, suggesting the presence of 6 crosslinked peptides. This peptide was subjected to the cleavage reactions.

Cleavage of the pyridinium ring of desmosine and isodesmosine residues: To separate the crosslinked peptides the pyridinium rings were cleaved according to the reactions shown in fig. 3.

Reduction of pyridinium rings of desmosine and isodesmosine by

Table 1

Amino acid compositions of desmosine and isodesmosine containing peptides at different stages of purification

Residues per 1000 residues

Amino Acid Residue	Seph. G-25	CP 4	Dowex V
Aspartic acid	12	4	4
Threonine	10	2	5
Serine	15	3	5
Glutamic acid	14	11	9
Proline	136	125	140
Glycine	279	273	251
Alanine	316	396	408
Valine	74	58	61
Isoleucine	14	12	10
Leucine	44	50	34
Tyrosine	10	12	11
Phenylalanine	24	28	19
Lysine	8	11	13
Arginine	7	6	2
Isodesmosine	4 (3.9)	11	11
Desmosine	6 (5.9)	15	16
Lysinonorleucine	2 (1.7)	7	3

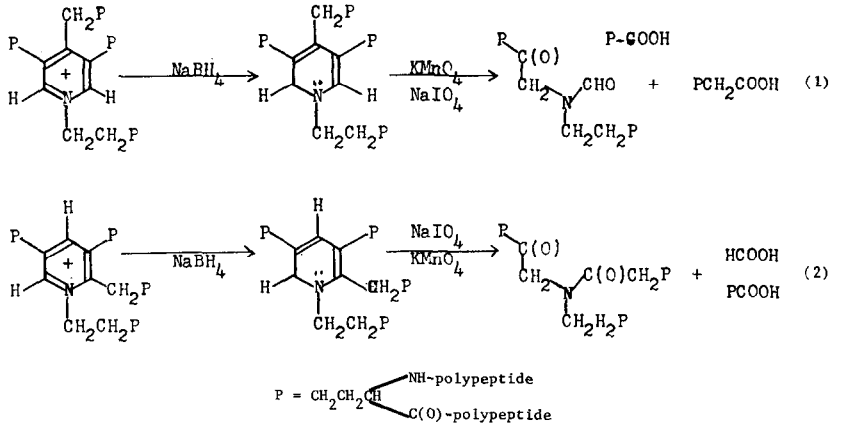


Figure 3: Crosslink Cleavage Reactions; (1) Desmosine Derived Peptides; (2) Isodesmosine Derived Peptides.

$NaBH_4$ treatment of elastin was reported by Lent and Franzblau (7). Earlier, Lyle et al had shown that the 3,5 - substituted pyridinium ions on reduction with $NaBH_4$ form 1,6 dihydropyridine. The $NaBH_4$ reduction of the pyridinium

rings of desmosine and isodesmosine residues was carried out to destroy the aromaticity of the rings and thereby to render them susceptible to the action of permanganate-periodate reagent for the specific cleavage of the rings.

The permanganate-periodate reagent was first described by Lemieux (8). With this reagent the sequence is hydroxylation of the double bonds with permanganate in low concentrations and cleavage of the resulting cis glycols with periodate. Thus, in the case of desmosine and isodesmosine, the bonds between C_2 and C_3, between C_3 and C_4 and between C_4 and C_5 of pyridinium rings will be cleaved, thereby liberating the cross-linked peptides. The peptide involving the side chain at C_4 of the pyridinium ring of desmosine will be liberated and will contain a residue of α-amino adipic acid. The peptides involving the side chains at C_3 of the pyridinium rings of desmosine and isodesmosine will be liberated and will contain glutamic acid residues. These glutamic acid residues thus formed can be easily distinguished from other glutamic acid residues present in the peptides by modification of these residues prior to the cleavage of the rings. Therefore, it is possible to locate the positions of different peptides.

To carry out these reactions, peptide V was treated with $NaBH_4$ according to the method of Spiro (9). The reaction was allowed to proceed for 24 hours at room temperature. The pH of the reaction mixture was then adjusted to 7.5 with 1 N HCl and the reaction mixture evaporated to dryness under reduced pressure. The dried material was subjected to periodate-permanganate oxidation as outlined by von Rudloff (10). The oxidation was allowed to proceed at 30° C for about 48 hours. At the end of this period, the reaction mixture was acidified with 10% H_2SO_4 and treated with sodium bisulfite to reduce all the periodate, iodate and iodine to iodide. The colorless solution was evaporated to dryness under reduced pressure. The residue was dissolved in 2-3 ml of distilled water and applied to a Dowex 50W x 2 column (1 x 10 cm in H^+ form) to remove the salts present as described previously (3). The peptides eluted from the column were tested for the

cleavage of pyridinium rings as follows:

1. A portion was hydrolysed in 6N HCl and analysed for amino acid composition. No desmosine or isodesmosine could be detected but the formation of α-amino adipic acid and glutamic acid was observed (new peak corresponding to standard α-amino adipic acid).
2. A second portion was subjected to high voltage paper electrophoresis at pH 3.5. Three ninhydrin positive bands were observed.
3. A third portion was analysed for N-terminal residues. The results showed the presence of glycine and alanine in the ratio of 1:5 as the only N-terminal residues, indicating that the peptide bonds were not hydrolysed during the reaction.

β elastin has also been subjected to the above treatment (cleavage reaction) with similar results.

These results show that pyridinium rings of desmosine and isodesmosine were cleaved without effecting the peptide bonds. This makes it possible to study the structure of cross-linked peptides. The work is now in progress to isolate the products of cleavage in a preparative scale and thus further study the structure of these peptides.

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