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ISOLATION AND CHARACTERIZATION OF CALCIUM OXALATE CRYSTAL GROWTH INHIBITORS FROM HUMAN URINE*

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Summary: Four acidic polypeptides which inhibit the growth of calcium oxalate crystals have been isolated from normal human urine, and two of these have been characterized with respect to their amino acid and carbohydrate compositions. SDS-Polyacrylamide gel electrophoresis of either of the two latter inhibitors revealed one prominent band that migrated with an apparent molecular weight of 17,500 daltons. γ-Carboxyglutamate is present in these inhibitors, and they contain a total of more than 25% glutamic and aspartic acids and less than 10% of basic amino acids.

INTRODUCTION

Evidence supporting the presence of calcium oxalate crystal growth inhibitors in human urine appearing to be acidic polypeptides has been reported recently, and poly-L-aspartic acid and poly-L-glutamic acid have been found to be effective model inhibitors (1). We now wish to report that we have succeeded in isolating four inhibitory polypeptides from normal human urine, purifying two of these to homogeneity and characterizing them with respect to their amino acid and carbohydrate compositions

MATERIALS AND METHODS

One normal donor's 24 urine (about 1.5 ℓ) was collected in a plastic bottle containing sodium azide. During the collection of urine, the container was stored in a refrigerator.

DEAE-Cellulose (Whatman DE-52) was recycled with acid and base, and Biogel P-100 (Bio-Rad) was equilibrated before use as recommended in the accompanying instructions. Preparative polyacrylamide gel electrophoresis was carried out by the use of a Canalco "Prep-Disc" apparatus with an LKB 2103 power supply. The electrophoresis equipment was maintained at 10° during each run by the use of a Lauda low temperature bath. Protein was monitored during column chromatography at 280 or 230 nm employing either LKB recorders (Uvicord II or Uvicord III) or a Beckman DU-2 spectrophotometer.

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Calcium oxalate crystal growth inhibition was followed using the assay method reported by Ito and Coe (1). The molecular weights of the inhibitors isolated were determined by SDS polyacrylamide gel electrophoresis, as described by Osborn and Weber (2). Dialysis tubing with a molecular weight cut off of 8,000 was obtained from Arthur Thomas Co. and prepared by boiling in 2% $NaHCO_3$, 10 mM EDTA and water for 30 min each, then stored in 50% ethanol at 4°.

Amino acid analyses were performed by the use of Beckman 121 or Durrum D-500 analyzers after 24 hr acid hydrolysis of samples at 110° in evacuated tubes containing 6 N HCl. γ-Carboxyglutamic acid was determined on the amino acid analyzer after 22 hr hydrolysis by 2.0 M KOH, followed by neutralization with $HClO_4$ as described by Hauschka (3). Amino-terminal determinations were carried out using dansyl chloride, following the method of Gross and Labouesse (4). The solvent systems used were water:formic acid (100:1.5, v/v) for the first dimension and, subsequently, benzene:acetic acid (9:1, v/v). Carbohydrate analyses were performed by the use of a Perkin-Elmer Model 910 gas chromatograph, and the method described by Clamp et al. (5) was followed.

RESULTS

The urine collected was dialyzed against 12 ℓ of deionized water for 24 hr at 4° with three changes. The dialyzed urine was adjusted to pH 7.3 by the addition of 1 N NaOH, and NaCl was added to a concentration of 0.05 M. To reduce the volume of the sample, 1/10 volume of DEAE-cellulose equilibrated with 0.01 M Tris-HCl, pH 7.3, containing 0.05 M NaCl, was added to the dialyzed urine and stirred for 30 min. The resultant mixture was filtered through a glass filter with gentle suction and washed with the same buffer (about 4 ℓ) until the filtrate became colorless. The washed DEAE-cellulose cake was suspended in 300 ml of 0.01 M Tris-HCl, pH 7.3, containing 0.6 M NaCl, and stirred magnetically for 30 min. The slurry obtained was filtered through a glass filter by suction, and the cake produced was washed with 200 ml of 0.6 M NaCl buffer. The 0.6 M NaCl filtrates and wash were combined, then dialyzed thoroughly against water. After dialysis, the solution was adjusted to a concentration of 0.05 M NaCl by the addition of solid NaCl, and then subjected to DEAE-cellulose column chromatography. The proteins were eluted by a linear NaCl gradient from 0.05 M to 0.3 M in 0.01 M Tris-HCl, pH 7.3. Two inhibitory fractions, A and B, were collected, eluting from the column at conductivities of approximately 12.2 mmho, and 14.6 mmho, respectively. These fractions were purified separately by passing them through a Biogel P-100 column (2 x 110 cm) using 0.01 M Tris-HCl, pH 7.3, containing 1 M NaCl and 0.02% NaN_3. Two inhibitory fractions, I and II, were isolated from each of the DEAE-cellulose fractions by

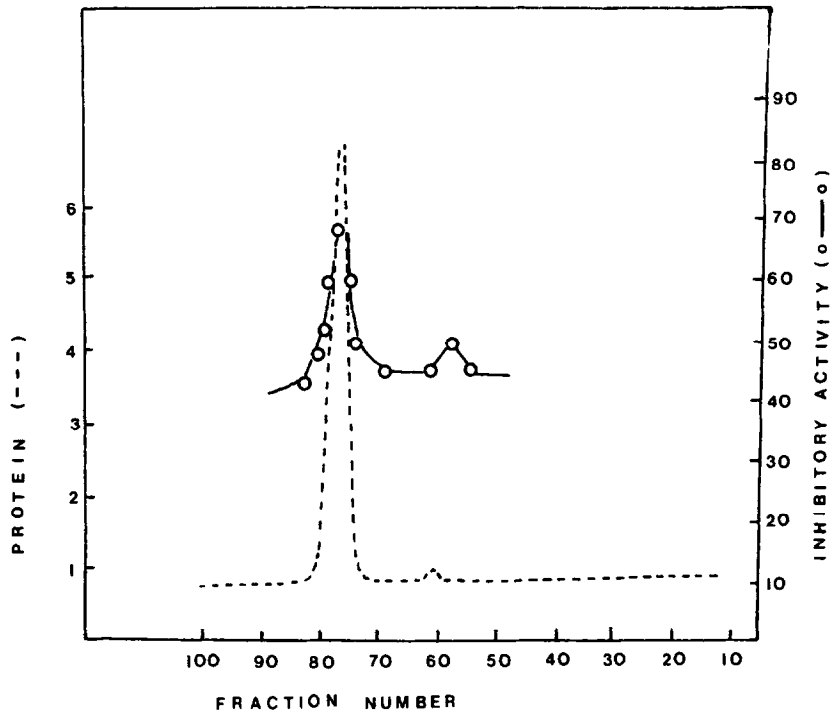

Figure 1.--Elution profile of A-I inhibitor on preparative polyacrylamide gel electrophoresis. The column height was 6 cm and the gel was 10% cross-linked. Electrophoresis was performed at 10°C and 1490 V. Twenty drops/tube were collected. The buffer was 0.04 M Tris-HCl at pH 9.0.

this procedure. The fractions eluting first, A-I and B-I, were colorless; however, the fractions eluting later, A-II and B-II, were contaminated with a brown pigment. The purification and characterization of the latter fractions are in progress. Both fractions A-I and B-I were purified further by preparative gel electrophoresis, and a typical elution pattern for A-I is shown in Figure 1. The inhibitory activity and the protein peak match, providing good evidence for purity. Subsequent SDS-polyacrylamide gel electrophoresis was performed for both A-I and B-I and revealed in each case one prominent band corresponding to a molecular weight of 17,500 daltons. The N-terminal amino acids were identified as their dansyl derivatives (4); phenylalanine was found for A-I and serine for B-I.

The results of amino acid and carbohydrate analyses performed on the A-I and B-I fractions are summarized in Tables 1 and 2. Five residues of γ-carboxyglutamic

TABLE 1

AMINO ACID COMPOSITIONS OF THE INHIBITORS (% OF TOTAL AMINO ACIDS PRESENT)*

	25-3-A-I**	25-4-B-I
Lys	3.1%	2.9%
His	1.7	2.7
Arg	2.8	0
Asp	12.9	15.0
Thr	8.2	9.5
Ser	7.7	13.5
Glu	16.9	17.0
Pro	2.1	
Gly	13.6	13.1
Ala	6.3	7.5
Val	5.9	6.6
Met		
Ile	1.7	2.2
Leu	6.1	5.5
Tyr	2.8	
Phe	8.2	4.4

*Because of the limited amounts of the inhibitors which have been isolated, Cys and Trp have not been determined.

**Employing alkaline hydrolysis following the method of Hauschka, 5.2 residues of γ-carboxyglutamic acid/1,000 residues of glutamic acid were detected for the A-I sample. γ-Carboxyglutamate acid was detected for the B-I fraction but was not determined quantitatively.

acid per 1,000 residues of glutamic acid were found in the A-I fraction. γ-Carboxyglutamic acid was detected also in the B-I fraction; however, the sample used was not sufficient for quantitative analysis. The γ-carboxyglutamate content found for the A-I fraction corresponds to considerably less than one residue per inhibitor molecule. Whether this means that not all of the A-I inhibitor molecules contain γ-carboxyglutamate or whether the low γ-carboxyglutamate content observed is due to decarboxylation of this residue during isolation of the inhibitor remains to be de-

TABLE 2

CARBOHYDRATE COMPOSITIONS OF THE INHIBITORS (WEIGHT %)

	25-3-A-I	25-4-B-I
Man	2.0	1.2
Gal	16.8	3.1
Gal NAc	1.2	1.7
Glu NAc	1.0	1.0
Neu Ac	2.2	3.2

termined. The total amount of glutamic and aspartic acids measured was more than 25% with less than 10% of basic amino acids present in these crystal growth inhibitors. Mannose, galactose, N-acetyl galactosamine, N-acetyl glucosamine and N-acetyl neuraminic acid were detected in both inhibitors. The A-I fraction contained a substantially greater amount of galactose than did the B-I fraction; also, in contrast to A-I, arginine was not found in B-I.

DISCUSSION

That calcium oxalate crystal growth inhibitors are present in human urine has been known for a long time (6). However, the nature of the inhibitory materials has been the subject of controversy, and many proposals have been made concerning their composition (7). In our work, four inhibitory proteins have been detected in the urine of a non-stone former. Two of the proteins have been purified by us, and their chemical compositions determined. Although we are now attempting to remove the brown pigment which is present in the remaining two protein fractions, in some cases it has not been possible to remove such contaminants from urinary proteins (8). A particularly intriguin aspect of the composition of the purified proteins A-I and B-I we have isolated is that they contain γ-carboxyglutamic acid. The presence of free γ-carboxyglutamic acid in human urine has been reported (9), and the acid has also been detected in kidney

stones (10). The amino acid compositions of the urinary proteins A-I and B-I are different from those of a number of calcium binding proteins including bovine dentin phosphoprotein (11), statherin isolated from saliva (12), and γ-carboxyglutamate-containing proteins isolated from various types of bone (13-15). Furthermore, none of the latter proteins has been reported to contain carbohydrate, a feature appearing to be unique to the γ-carboxyglutamic acid-containing glycoproteins A-I and B-I.

The dependence of γ-carboxyglutamate formation in plasma and bone proteins on vitamin K has been shown in several instances (13-16). We are currently investigating not only the mechanism by which the urinary proteins we have isolated inhibit calcium oxalate crystal growth but also the relationship of our inhibitors to proteins which involve vitamin K in their biosynthesis.

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