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Purification and Characterization of a Calcium Oxalate Monohydrate Crystal Growth Inhibitor from Human Kidney Tissue Culture Medium*

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Yasushi Nakagawa‡**, Henry C. Margolis‡†, Shinji Yokoyama‡, Ferenc J. Kezdy§, Emil Thomas Kaiser‖ and Fredric L. Coe‡**

From the ‡Renal Division, Michael Reese Hospital and Medical Center, Chicago, Illinois 60616 and the †Department of Chemistry and the ‖Department of Biochemistry, University of Chicago, Chicago, Illinois 60637

A calcium oxalate monohydrate crystal growth inhibitor has been isolated from human kidney tissue culture medium by using DEAE-cellulose batch chromatography followed by DEAE-cellulose and Sephacryl S-200 chromatography. The isolated inhibitor was found to be a glycoprotein with a \( M_r = 1.33 \times 10^5 \), as determined by analytical ultracentrifugation, and a carbohydrate content of 38.4 wt %. Amino acid analysis showed that the protein portion consists of 73 amino acid residues; it is rich in acidic amino acids and contains few aromatic acid residues, resulting in an atypical ultraviolet spectrum with an absorption maximum at 272.5 instead of 280 nm.

Kinetic studies, using a seeded crystal growth system, suggest a surface inhibition mechanism where the inhibitor binds to specific growth sites on the calcium oxalate monohydrate surface. The binding obeys a Langmuir adsorption isotherm and shows a dissociation constant for the inhibitor-surface complex of 1.9 \( \times 10^{-8} \) M. Supporting evidence for the adsorption type mechanism of inhibition is provided by the effect of the inhibitor on the electrophoretic mobility of calcium oxalate crystals. With respect to carbohydrate and amino acid composition, as well as with regard to inhibitory activity, the kidney tissue culture inhibitor is very similar to the inhibitor isolated from human urine (Nakagawa, Y., Kaiser, E. T., and Coe, F. L. (1978) Biochim. Biophys. Res. Commun. 84, 1038-1044). The concentration of inhibitor in urine is sufficient to prevent calcium oxalate monohydrate crystal growth.

The inhibitor forms an insoluble monolayer at the air-water interface and has an unusually high surface activity with a collapse pressure of 45 dynes/cm. From analysis of the force-area curve of the monolayer, \( M_r = 1.39 \times 10^5 \) is determined, in good agreement with that obtained by analytical ultracentrifugation.

During the course of our studies of macromolecular inhibitors of kidney stone formation, we have reported on the isolation and purification of glycoprotein inhibitors of calcium oxalate monohydrate crystal growth from human urine (7). The difficulty in obtaining large enough quantities of inhibitor from human urine, needed for detailed mechanistic studies, has made it necessary to investigate new sources of similar inhibitory glycoproteins.

We now wish to report on the isolation, purification, and molecular characterization of a glycoprotein inhibitor of calcium oxalate monohydrate crystal growth from human kidney tissue culture medium, and to present evidence for the mechanism of the inhibition process. Unlike the urinary inhibitor, sufficient quantities of glycoprotein inhibitor are readily obtained from kidney tissue culture medium. The similarity of composition and inhibitory characteristics ensures that the kidney tissue culture inhibitor is an appropriate one for the investigation of the mechanism of calcium oxalate monohydrate crystal growth inhibition in the urinary tract.

EXPERIMENTAL PROCEDURES

Materials

Frozen human kidney tissue culture medium was obtained from Collaborative Research, Waltham, Mass. We are indebted to Dr. P. R. Kelly for supplying us with this material.

DEAE-Cellulose was obtained from Whatman and recycled according to the manufacturer's procedure using 0.5 N HCl and 0.5 N NaOH prior to equilibration with the appropriate buffer. Sephacryl S-200 was suspended in buffer solution and desalted before use. Tris-base (Trizma base) was purchased from Sigma and \( \mathrm{U}^{[4]} \) oxalic acid, 75 mCi/mmole, was obtained from Amersham. All other reagents were the highest grade available and water was deionized and distilled in an all-glass apparatus. Dialysis tubing was obtained from Arthur Thomas (molecular weight cut off, 8000) and boiled in solutions of 2% NaHCO3 and 10 mM EDTA (disodium salt) for 20 min each, then finally boiled in deionized distilled water and stored in 50% ethanol at 4 °C. Reagents for SDS-polyacrylamide electrophoresis were electrophoresis purity reagents purchased from Bio-Rad. Drimarene brilliant blue K-Bi was obtained from Pierce.

Methods

Calcium Oxalate Monohydrate Crystal Growth Inhibition

Kinetic Studies—Using the purified inhibitor, the kinetics of calcium oxalate monohydrate inhibition were investigated using a seeded crystal growth system. Seed crystals of calcium oxalate monohydrate were prepared by the slow addition of oxalic acid to a suspension of calcium carbonate at 85 °C, according to the procedure of Nancollas and Gardner (8). The structure of the crystals obtained was confirmed as calcium oxalate monohydrate both by elemental and x-ray diffraction.

The abbreviation used is SDS, sodium dodecyl sulfate.
tion analyses. Alternatively, crystals were prepared by the method of Pak et al. (9). These crystals were used routinely for the assay of the inhibitors. The crystals prepared by the two methods were crystallographically identical, and they were indistinguishable as far as their kinetic behaviors are concerned. The same batch of seed crystals was used throughout the kinetic studies. Seed crystal slurries were prepared by the suspension of 1.5 mg/ml of the crystals in a 50 mM sodium acetate buffer, pH 5.7, containing 96 mM NaCl; this was used instead of the 5 mM sodium acetate buffer (as used in the routine assay) due to its better buffering capacity over longer periods of time needed for the kinetic studies. Essentially no effect of the increase in sodium acetate concentration was observed on the rate of seeded crystal growth. Slurries were allowed to equilibrate in buffer, with stirring, for 2 h prior to use. The crystal growth reaction was initiated by the addition of 3 ml of slurry to 33 ml of a metastable calcium oxalate solution containing 0.83 mM CaCl₂ and 0.167 mM sodium oxalate along with a tracer of [14C]oxalate in the same buffer. The final seed crystal concentration was 0.125 mg/ml. The solutions were magnetically stirred in a constant temperature bath at 37 °C. At appropriate time intervals, 1-ml aliquots were removed and filtered through Millipore filters (0.2 μm) and the level of [14C]oxalate radioactivity was determined by means of a Beckman liquid scintillation counter, LS-230. The instrument was standardized using a 0.01% C14 Beckman standard. Calcium oxalate monohydrate seed crystals were prepared by mixing equal volumes of 0.01 M calcium chloride and sodium oxalate aqueous solutions dropwise while stirring magnetically, followed by continuous stirring for 1 week at 4 °C (9). The crystals were collected by centrifugation and dried after washing with water and methanol. Seed crystals were suspended in 0.05 M barbiturate-acetate buffer, pH 5.7, to give a concentration of 1.5 mg/ml. We have shown (5, 10), as others have (11), that calcium oxalate-seeded crystal growth follows a second-order rate law according to the following equation, where [C] and [Cₐ] represent the concentration of oxalate at any given time and at equilibrium, respectively.

\[
\frac{d[C]}{dt} = k_{\text{exp}} ([C] - [Cₐ])²
\]  

(1)

Upon integration and rearrangement, Equation 2 is obtained.

\[
Cₐ = C + aCₐ t + \frac{a}{1 + a t}
\]

(2)

where a, kₑ₀, (Cₐ - C); Cₐ, initial concentration of [14C]oxalate; kₑ₀, experimental second-order rate constant. Using a nonlinear regression analysis computer program (12), the time course data were fit to Equation 2 and the rate constant, kₑ₀, was determined along with the calculated values of C and Cₐ. The calculated value of Cₐ was found constantly to be 4-5% lower than the "theoretical" C value measured prior to the addition of seed crystals. This small difference may represent a rapid pre-steady state precipitation of [14C]oxalate on the crystal surface. Such a pre-steady state must represent the deposition of the first molecular layer of radioactive oxalate on the seed crystal, since during that process the reverse reaction is not in a steady state. Nevertheless, except for the first few seconds on the crystal growth reaction, the entire time course is successfully fit to Equation 1. This initial burst is of interest but has essentially no effect on the results as discussed in this report.

Rate constants were then determined in the manner described above in the absence (k₀) and presence (kₑ₀) of various amounts of inhibitor added to the metastable crystal growth solution prior to the addition of seed crystals.

If the binding of the inhibitor obeys a simple Langmuir adsorption isotherm (10), then the relationship between the concentration of unoccupied binding sites (s), surface complex (c), and inhibitor concentration (I) is determined by the dissociation constant, K:

\[
K = \frac{I(a)}{(c)}
\]

(3)

since (s) + (c) = (s₀), i.e., the analytical concentration of binding sites, we obtained

\[
s = s₀\frac{1 + (I/K)}{K}
\]

(4)

If binding of the inhibitor does not alter appreciably the concentration of the inhibitor in solution, and if the rate of crystal growth is first order with respect to the surface unoccupied by the inhibitor, then

\[
kₑ₀ = \frac{k₀}{1 + (I/K)}
\]

Transformation of this expression yields Equation 3, from which K can be determined as the slope of a plot of kₑ₀/(k₀ - kₑ₀) versus 1/[I].

Determination of Crystal Growth Inhibitory Activity—As shown in the previous study (10), we have derived a practical expression (Equation 4) from the Langmuir adsorption isotherm that varies linearly with inhibitor concentration. The expression I/K can therefore be determined using a simple experimental protocol. It is a useful measure of inhibitory activity.

\[
I₀ = \frac{(Cₐ - C)}{(Cₐ - Cₐ)} \times \frac{(Cₐ - C)}{(Cₐ - Cₐ)}
\]

(4)

where I₀, inhibitor concentration; Cₐ, ¹⁴C cpm in a solution at zero time; Cₐ, ¹⁴C cpm in a solution at time, t; Cₐ, ¹⁴C cpm at 24 h; K, dissociation constant. In Eq. (4), the second term, (Cₐ - Cₐ)/constant, is a constant, and Equation 4 is simplified as follows:

\[
I₀ = \frac{(Cₐ - C)}{(Cₐ - Cₐ)} \times \text{constant}
\]

(5)

Since the value of the binding constant K is constant for a given inhibitor, one can assay for the specific inhibitory activities of the isolated fractions during the course of purification. Radioactivities determined after a 40-min incubation were converted to an I₀/K value using Equation 5 and expressed as specific activity units/mg of protein.

In the chromatographic steps used for purification of inhibitor, the position of the inhibitor was detected both by UV absorption and by assaying fractions for inhibitory activity in a calcium oxalate monohydrate crystal growth system as described above. The calcium oxalate crystal growth assay was carried out according to the method of Ito and Coe (5) using a Beckman liquid scintillation counter. The instrument was standardized using a 0.01% C14 Beckman standard. Calcium oxalate monohydrate seed crystals were prepared by mixing equal volumes of 0.01 M calcium chloride and sodium oxalate aqueous solutions dropwise while stirring magnetically, followed by continuous stirring for 1 week at 4 °C (9). The crystals were collected by centrifugation and dried after washing with water and methanol. Seed crystals were suspended in 0.05 M barbiturate-acetate buffer, pH 5.7, to give a concentration of 1.3 mg/ml. The assay solution contained 5 ml of 2 × 10⁻³ M CaCl₂ in 0.05 M barbiturate-acetate buffer, pH 5.7, 1 ml of calcium oxalate monohydrate seed crystal slurry (1.5 mg of the crystal/ml), and appropriate amounts of test solution (usually 200 μl). The assay was initiated by adding 5 ml of 0.4 × 10⁻³ M sodium oxalate with [¹⁴C]oxalate as a tracer in a 0.05 M barbiturate-acetate buffer, pH 5.7, after the temperature was equilibrated at 37 °C. After 40 min of incubation, a 1-ml aliquot was removed from the reaction mixture, filtered through a Millipore filter (Metricel GA-8; pore size, 0.2 μm), and 100 μl of the filtrate were added to 5 ml of Beckman liquid scintillation mixture (Ready-Solv GP) and counted.

Isolation of the Inhibitor—The isolation and purification of calcium oxalate monohydrate crystal growth inhibitor from kidney tissue culture medium followed the method for isolation of the inhibitors from human urine reported previously from this laboratory (7). The elution of the protein during column chromatography was monitored by measuring adsorption at 280 or 230 nm with either a Beckman DU-2 spectrophotometer or a Zeiss M4 QII spectrophotometer. The protein concentrations of combined inhibitory fractions after each purification step were determined by alkaline hydrolysis (14) using bovine serum albumin as a calibration standard.

Salt gradients were monitored by conductivity measurements using a Radiometer conductivity meter, type CDM 2e. Buffer solutions were prepared by dissolving all required reagents in deionized-distilled water, followed by adjustment of pH with acid or base using a Corning Research pH meter with a combination glass electrode and temperature compensator.

SDS-Polyacrylamide Gel Electrophoresis—SDS-polyacrylamide gels (0.6 x 10 cm), 10% cross-linked, were prepared following the method of Weber et al. (15). Staining was done with 0.25% Coomassie brilliant blue R 250, but since staining of inhibitor proteins with Coomassie blue was not stable, Drimarene brilliant blue K- BL was substituted as described by Bosshard and Datyner (16). Gel electrophoresis was carried out using Bio-Rad gel electrophoresis equipment with 10 mA/gel, and destaining was performed with a Bio-Rad diffusion destainer, model 172A. A molecular weight calibration was
Calcium Oxalate Crystal Growth Inhibitor

Amino Acid Analyses—Amino acid analysis was carried out using a Beckman 118 Cl analyzer with a single-column system (W-3H for 24 h, neutralization using Bio-Rad AG 3-X4A (100-200 mesh OH made by using bovine serum albumin, ovalbumin, chymotrypsinogen, and lysozyme. To avoid this interference of the hexosamines, the following method was developed. The column was extended to 35 cm (original length glass tubing) with nitrogen as the carrier gas and the temperature program was set from 170 to 250 °C at a rate of 2 °C/min. The resulting chromatograms were analyzed using a Sigma 10 analyzer, with mannitol as the internal standard.

Amino Acid Analyses—Amino acid analysis was carried out using a Beckman 118 Cl analyzer with a single-column system (W-3H resin). The inhibitor was hydrolyzed in 6 N HCl in evacuated tubes at 110 °C for 24, 48, and 72 h. Cysteine was quantitated after carboxymethylation by iodooacetic acid followed by 6 N HCl hydrolysis. Carboxyglutamic acid was determined after alkaline hydrolysis as described by Haaschka (18). Tryptophan and tyrosine residues were quantitated spectrophotometrically in 0.1 N NaOH, following the method of Beaven and Holiday (19) using a Cary 15 spectrophotometer.

It should be noted that the original method for the Beckman 118 Cl analyzer was designed to give a 1-h analysis time using an acid detector. However, it was found that hexosamines were eluted between the alanine and methionine region in broad peaks, thus making the quantitative analysis of the protein hydrolysate impossible. To avoid this interference of the hexosamines, the following method was developed. The column was extended to 35 cm (original specification was 22 cm) with Beckman W-3H resin and the pH values of the sodium citrate buffer solutions were adjusted to 3.33 (0.20 N Na%), 4.25 (0.40 N Na%), and 6.05 (1.00 N Na%). The flow rate was adjusted to 60 ml/h for buffer only and 89.9 ml/h for buffer and nitrogen solutions. The initial temperature was 40 °C, and it was programmed to rise to 65 °C 10 min after sample injection. The total run time was 135 min. Hexosamines were eluted after phenylalanine and lysine. It should be noted that the original method for the Beckman 118 Cl analyzer was designed to give a 1-h analysis time using an acid detector. However, it was found that hexosamines were eluted between the alanine and methionine region in broad peaks, thus making the quantitative analysis of the protein hydrolysate impossible. To avoid this interference of the hexosamines, the following method was developed. The column was extended to 35 cm (original specification was 22 cm) with Beckman W-3H resin and the pH values of the sodium citrate buffer solutions were adjusted to 3.33 (0.20 N Na%), 4.25 (0.40 N Na%), and 6.05 (1.00 N Na%). The flow rate was adjusted to 60 ml/h for buffer only and 89.9 ml/h for buffer and nitrogen solutions. The initial temperature was 40 °C, and it was programmed to rise to 65 °C 10 min after sample injection. The total run time was 135 min. Hexosamines were eluted after phenylalanine and lysine.

Molecular Weight Determination—The molecular weight of inhibitor was determined by a meniscus-depletion equilibrium method using a Beckman model E analytical ultracentrifuge (21). An Yphantis multiple-channel centerpiece with sapphire windows was used. The inhibitors were dissolved in 0.05 M Tris-HCl, pH 7.3, containing 0.1 M NaCl, and dialyzed at 4 °C overnight against the same buffer. Running conditions were 44,000 rpm with An-D rotor at 20 °C for 25 h. Protein concentrations were 0.3, 0.2, and 0.1 mg/ml, and were determined by alkaline hydrolysis as described in the previous section. Photographs were taken on Kodak metaloplate type I1 and the fringes were measured on a Nikon comparator with digital readout, Ektromike (Ehrenreich Photo-Optical Industries). After the centrifugation, the baseline was determined using the same samples at 5000 rpm.

Surface Activity Measurement—Surface properties of the isolated inhibitor at the air-water interface were investigated by using a Lauda film balance. An aliquot of inhibitor solution (30-100 µg of protein), dissolved in 0.01 M Tris-HCl, pH 7.2, containing 0.1 M NaCl to make 0.11 mg/ml, was spread on the surface of the buffer solution using a Hamilton syringe with an air slide of the surface. The surface pressure, \( \pi \) (dynes/cm), was measured as a function of the area of the monolayer (A) by the compression and expansion of the monolayer between 600 and 200 cm\(^2\) at a rate of 2.2 cm\(^2\)/s, at 22 °C.

Effect of the Isolated Inhibitor on Calcium Oxalate Monohydrate Mobility—Mobility (µ/s/V/cm) was measured directly using a Zeta meter (Zeta Meter Inc., New York), housed in a constant temperature chamber. Calcium oxalate monohydrate crystal slurries were prepared (0.315 g/l) and adjusted to the desired pH with diluted HCl or NaOH. The diluted slurries were allowed to equilibrate at 37 °C with shaking in a constant temperature shaker bath (100 stroke/min) for 1 or 2 h. The inhibitor solution was then added and allowed to equilibrate for 2 more h before making mobility measurements.

RESULTS

Isolation of Calcium Oxalate Crystal Growth Inhibitors—Calcium oxalate crystal growth inhibitors were isolated from human kidney tissue culture medium by a method similar to that previously reported for the isolation of inhibitors from human urine (7).

Kidney tissue culture medium (750 ml) was dialyzed against 12 liters of deionized water for 24 h at 4 °C with two changes. During the dialysis, -4% of protein was lost with a 7% decrease in inhibitory activity. The loss of inhibitory activity is most likely due to the removal of known small molecular weight inhibitors, such as citrate, phosphate, and pyrophosphate. The dialysate was adjusted to 0.05 M NaCl and pH 7.3, with the addition of NaCl and diluted base, respectively. The dialysate was then mixed with DEAE-cellulose that had been previously equilibrated with 0.05 M Tris-HCl-0.05 M NaCl, pH 7.3. The suspension was magnetically stirred at room temperature for 30 min, and filtered through a sintered glass filter. The inhibitor bound to DEAE-cellulose tightly, other proteins were eluted, and an 8-fold purification was achieved (Table I). The filter cake was washed with 4 liters of the same buffer. The inhibitors were finally eluted from the DEAE-cellulose cake with 0.05 M Tris-HCl (pH 7.3) containing 0.6 M NaCl, successively washing the cellulose cake with 300 and 200 ml of this buffer. The eluates were combined and dialyzed against 12 liters of deionized water for 24 h at 4 °C with two changes.

After dialysis, the inhibitor solution was readjusted to pH 7.3 and 0.05 M NaCl and subjected to DEAE-cellulose chromatography (2 × 15 cm). The column was developed using a linear sodium chloride gradient from 0.05 to 0.5 M in 0.05 M Tris-HCl, pH 7.3 (600 ml of each solution). The fractions were collected in test tubes containing 1 drop of 1% NaN\(_3\) aqueous solution. The conductivity and inhibitory activity of every fifth fraction were determined. A typical chromatographic pattern is shown in Fig. 1. Inhibitory activities could be separated into four fractions by conductivities: A, 11.0-12.0 mmho (0.144-0.164 M NaC\(_1\)); B, 12.2-15.0 mmho (0.167-0.218 M NaCl); C, 15.1-18.0 mmho (0.220-0.273 M NaCl); D, 18.1-20.0 mmho (0.275-0.309 M NaCl). The most active inhibitor fraction, B, was dialyzed and lyophilized to obtain a protein concentrate.

<table>
<thead>
<tr>
<th>Step</th>
<th>Total volume</th>
<th>Protein(^{m})</th>
<th>Protein recovery</th>
<th>Inhibitory activity</th>
<th>Specific activity</th>
<th>Purification</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Concentrated medium</td>
<td>750</td>
<td>6.9</td>
<td>5178</td>
<td>100.0</td>
<td>7.4</td>
<td>1.07</td>
</tr>
<tr>
<td>2. Dialysis</td>
<td>800</td>
<td>6.2</td>
<td>4960</td>
<td>95.8</td>
<td>6.2</td>
<td>1.00</td>
</tr>
<tr>
<td>3. Batch DEAE-cellulose 0.6 M NaCl eluate</td>
<td>400</td>
<td>0.28</td>
<td>112</td>
<td>2.2</td>
<td>2.4</td>
<td>8.57</td>
</tr>
<tr>
<td>4. DEAE-cellulose column chromatography, NaCl gradient, Fraction B</td>
<td>70</td>
<td>0.90</td>
<td>63</td>
<td>1.2</td>
<td>10.4</td>
<td>11.5</td>
</tr>
<tr>
<td>5. Sephacryl S-200 column chromatography, Fraction B</td>
<td>125</td>
<td>0.26</td>
<td>32.5</td>
<td>0.6</td>
<td>7.85</td>
<td>30.2</td>
</tr>
<tr>
<td>6. Sephacryl S-200 column chromatography after EDTA treatment</td>
<td>130</td>
<td>0.17</td>
<td>22.5</td>
<td>0.4</td>
<td>15.0</td>
<td>88.2</td>
</tr>
</tbody>
</table>

* Protein concentration determined by alkali hydrolysis.
Calcium Oxalate Crystal Growth Inhibitor

A serine protease inhibitor, found in human kidney tissue culture medium using DEAE-cellulose column chromatography (2 x 15 cm). Elution was carried out using a linear NaCl gradient from 0.05 to 0.5 M in 0.05 M Tris-HCl, pH 7.3 (600 ml of each solution). The fractions (3 ml/tube) were collected in test tubes containing 1 drop of 1% NaN₃ aqueous solution. Protein was monitored by absorption at 280 nm, and salt concentration was determined using a Radiometer conductivity meter.

give a volume of ~3 ml and then thawed. It was chromatographed on a Sephacryl S-200 column (4 x 113 cm) using 0.05 M Tris-HCl (pH 7.3), containing 0.2 M NaCl and 0.02% NaN₃. The elution pattern is shown in Fig. 2. The first inhibitory peak (estimated \( M_r = 1.0 \times 10^6 \)) was rechromatographed on the same column. The chromatogram is shown in Fig. 3. In comparing Figs. 2 and 3, it can be seen that two inhibitory peaks (elution volumes of 245 and 472.5 ml) were separated with low absorbance at 280 nm. The molecular weight of the main peak was estimated by SDS gel electrophoresis. In preliminary experiments, protein bands were stained with Coomassie blue and several diffuse bands were always observed on the SDS gels. These bands correspond to \( M_r = 1.5 \times 10^3, 3.0 \times 10^4, 4.5 \times 10^4, \) and \( 6.0 \times 10^4 \). However, it was difficult to determine the exact molecular weight. During gel column chromatography, the apparent largest molecular weight fraction always separated into two protein peaks that were inhibitory. This elution pattern is quite reproducible, and suggests a possible dissociation of a larger molecular weight species into smaller molecules. Since this inhibitor protein binds to calcium oxalate crystals as described later, aggregation could be caused by Ca²⁺ or other divalent metal ions. The major fraction was thus reduced in volume to 20 ml by lyophilization after dialysis, and adjusted to 0.1 M disodium EDTA. After stirring for 12 h at 4 °C the protein solution was gel-filtered on Sephacryl S-200 (4 x 113 cm). The elution pattern is shown in Fig. 4. The large molecular weight inhibitor (elution volume, 245 ml) was converted to the smaller molecular weight inhibitor (elution volume, 472.5 ml) as judged by elution volume. The optical density at 280 nm was also greatly decreased, so protein elution had to be followed by the absorbance at 230 nm. The fractions under the major inhibitory peak were combined and thoroughly dialyzed against deionized water, and then lyophilized. The protein recovery and purification of the inhibitor at each step have been summarized in Table I. The inhibitory activity after each step was determined by calculating the value of \( I_0/K \) (see "Methods"). The inhibitor, the characterization of which is described below, was purified 82-fold after the last Sephacryl S-200 column chromatography. This inhibitor was difficult to stain with Coomassie blue after SDS-polyacrylamide gel electrophoresis, although it could be modified with Drimarene brilliant blue K-B1 prior to SDS gel electrophoresis. The results showed a single band; however, estimation of a molecular weight on SDS was impossible since modified standard proteins do not migrate according to their molecular weights. The effect of Ca²⁺ on this inhibitor's aggregation and dissociation is under investigation.

Molecular Weight Determination

A meniscus-depletion equilibrium ultracentrifugation of the inhibitor was carried out with three different protein concentrations. The inhibitor treated with EDTA showed straight log \( y \) versus \( r^2 \) (cm²) plots with all concentrations, indicating a homogeneous preparation. The molecular weight was calculated to be \( 1.33 \times 10^4 \) based on a specific volume of 0.68, which was calculated from the amino acid (22) and carbohydrate compositions (23).

Amino Acid and Carbohydrate Compositions

Gas chromatographic analysis of carbohydrate after methanolic HCl hydrolysis followed by trimethylsilylation showed
that the carbohydrate content in the inhibitor was 33.4 weight % (This value does not include hexosamines but only neutral sugars). The carbohydrate composition of the inhibitor obtained by gas chromatography (neutral sugars) and amino acid analysis (hexosamines) is shown in Table 3. When pure hexosamines are hydrolyzed under the same conditions as for amino acid analysis (i.e., in 6 N HCl at 24, 48, and 72 h), decomposition takes place within the hydrolysis time. Thus, the amounts of glucosamine and galactosamine measured from amino acid analyses at different times were plotted on semilogarithmic graph paper for estimation of initial quantities (zero time of hydrolysis).

Amino acid composition was determined using an amino acid analyzer under modified conditions after 24, 48, and 72 h of hydrolysis. The number of amino acid residues was calculated for \( M_r = 1.33 \times 10^6 \) that was obtained by analytical ultracentrifugation and a carbohydrate content of 38.4 weight %. The results are summarized in Table III. Analysis of tryptophan was performed spectrophotometrically and one residue/molecule was found. \( \gamma \)-Carboxyglutamic acid was analyzed for after alkaline hydrolysis and was not detected. Two residues of cysteine, determined as carboxymethyl cysteine, were found. Threonine and serine were graphically extrapolated to zero time, and the 72-h hydrolysis value was used for valine. From the analysis, this inhibitor is composed of 73 amino acid residues with a protein \( M_r = 8194.5 \).

This inhibitor contains 26 residues of neutral and amino sugars: 2 fucose, 9 galactose, 3 glucose, 8 N-acetylgalactosaminic acid, 3 glucosamine, and 1 galactosamine.

**UV Absorption Spectrum**

The isolated inhibitor (EDTA treated), 0.19 mg/ml, was dissolved in 0.01 M Tris-HCl buffer, pH 7.2. The UV absorption spectrum of the inhibitor was measured using a Cary 15 spectrophotometer and the results are shown in Fig. 5. The absorption maximum was observed at 272.5 nm and the calculated \( E_{272.5}^1 \) was 0.568 (\( \epsilon_m = 736 \) absorbance units/mol/cm). As seen in this figure, the inhibitor shows weak absorption at 280 nm where typical proteins show strong absorption.

**The Effect of Inhibitor on the Rate of Calcium Oxalate Monohydrate Crystal Growth**

The effect of increasing concentrations on inhibitor on the time course of calcium oxalate monohydrate crystal growth is shown in Fig. 6. Using Equation 2 and the iterative computer procedure described previously, \( (k_{\text{exp}}) \) and \( (k_0) \) were determined. Increasing inhibitor concentration causes a dramatic reduction in the rate constant, \( k_{\text{exp}} \). The rate data have been successfully fit to a form of the Langmuir adsorption isotherm (Equation 3) by plotting \( k_0/(k_0 - k_{\text{exp}}) \) versus \( 1/I \), as seen in Fig. 7. The slope of this line, the dissociation constant for the crystal-inhibitor complex, is equal to \( 1.9 \times 10^{-6} \) M (r = 0.996). This dissociation constant represents the concentration of inhibitor needed to produce a 50% reduction in \( k_0 \) for calcium oxalate crystal growth in the absence of inhibitor.

**Surface Properties of Inhibitor at the Air-Water Interface**

Since the inhibition mechanism of calcium oxalate monohydrate crystal growth by this inhibitor is suggestive of an absorption type, surface properties of the inhibitor were investigated. The inhibitor protein formed an insoluble monolayer at the air-water interface. The monolayer was stable for at least several hours, over a range of surface pressure of 0-30 dynes/cm. The force \( (\pi) \) - area \( (A) \) curve showed a discontinuity at a pressure of 45 dynes/cm, indicating the collapse of
Calcium Oxalate Crystal Growth Inhibitor

Fig. 5 (left). UV absorption spectrum of the isolated inhibitor. The inhibitor was dissolved in 0.01 M Tris-HCl, pH 7.2, to give 0.19 mg/ml, and the spectrum of the resultant solution was scanned between 220 and 300 nm using a Cary 15 spectrophotometer with 0.1 scale expansion (solid line). Background absorption of the buffer solution was also recorded (dotted line).

Fig. 6 (right). Time course of calcium oxalate monohydrate crystal growth with increasing inhibitor concentration. Reactions were performed at 37 °C in a 50 mM acetate buffer, pH 5.7, containing 96 mM NaCl. Initial concentration of calcium and oxalate were 0.833 and 0.167 mM, respectively, with a trace amount of [14C]oxalate. Seed crystal concentration was equal to 0.125 mg/ml. Details of crystal growth experiments are described under “Methods.”

Points are experimentally obtained, and the theoretical curves represent the fit of the data to Equation 4 using a nonlinear regression analysis computer program. Curve 1, 1 × 10⁻¹ M inhibitor; Curve 2, 5 × 10⁻¹ M inhibitor; Curve 3, 2 × 10⁻⁹ M inhibitor; Curve 4, 1 × 10⁻⁹ M inhibitor; Curve 5, no inhibitor added.

![Graph](image-url)

The Effect of Inhibitor on the Electrophoretic Mobility of Calcium Oxalate Monohydrate

Fig. 8 shows the effect of varying inhibitor concentration on the electrophoretic mobility of calcium oxalate monohydrate crystals at pH 5.7 and 37 °C, with an analytical calcium oxalate concentration of 0.315 g/liter. In the absence of the
inhibitor, the mobility of calcium oxalate monohydrate crystals was +1.3 μs/V/cm. As the inhibitor concentration is increased, the mobility is reduced, and then reverses sign at tals was +1.3 p/s/V/cm. As the inhibitor concentration is increased, the mobility is reduced, and then reverses sign at +1.3 μs/V/cm. As the inhibitor concentration is increased, the mobility is reduced, and then reverses sign at

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\frac{k_{in} - k_{ex}}{k_{ex}} \times 100
\]

obtained, as obtained from the seed crystal growth experiments, is also shown in Fig. 8. To emphasize the relationship between inhibition and the effect of inhibitor on electrophoretic mobility.

**DISCUSSION**

We have described the isolation and characterization of a new glycoprotein purified from human kidney tissue culture medium. The purification was performed by DEAE-cellulose batch chromatography followed by DEAE-cellulose column chromatography with a linear NaCl gradient and Sephacryl S-200 chromatography. Under "Results," we have described the 82-fold purification of the major inhibitor fraction.

This glycoprotein, a potent inhibitor of calcium oxalate monohydrate crystal growth, contains 38.4% carbohydrate, consisting of fucose, glucose, galactose, N-acetylgalactosaminic acid, glucosamine, and galactosamine. Such a composition is commonly found in glycoproteins of animal origin. The amino acid composition of this glycoprotein inhibitor is, however, less common. Acidic amino acids make up about one third (54.2%) of the total amino acid composition, with lesser amounts of nonpolar (26.0%) and hydroxyamino acid residues (16.4%). Basic amino acid residues comprise only 10.1%. The aromatic amino acid residues (5.5%) and proline (5.5%) were also found in relatively small amounts.

The urinary glycoproteins have been reported to contain little or no aromatic amino acid residues (29). This is consistent with our amino acid analysis and with the observation that the purified kidney tissue culture inhibitor absorbs weakly at 280 nm, where typical proteins exhibit an adsorption maximum (30). As stated earlier, the kidney tissue culture inhibitor has an absorption maximum at 272.5 nm with

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E_{272.5nm} = 0.568
\]

in complete agreement with our other observations, this suggests that this inhibitor contains fewer aromatic amino acid residues than typical proteins. The fact that the purified inhibitor contains few aromatic amino acid residues explains the decrease in absorption at 280 nm as purification progressed, the difficulty in performing protein determination by the Lowry method, and the weak staining of SDS-polyacrylamide gel with Coomassie blue.

Some compositional differences between the kidney tissue culture inhibitor and that isolated from human urine (7) were observed. With respect to carbohydrate composition, the major difference is that the inhibitor isolated from human urine contains 1–2 weight % of mannose, but this carbohydrate is not detected in the inhibitor isolated from human kidney tissue culture medium. Many known glycoproteins such as immunoglobulins, ovomucoid, and fetuin have branched structures at mannose residues through a (1 → 3) and/or a (1 → 6) linkage to the next mannose residues (31). Thus, the presence of mannose residues in human urinary inhibitor may indicate the presence of such a branched carbohydrate structure. However, the carbohydrate chains of kidney tissue culture inhibitor may not have such a branched structure as seen in the immunoglobulins, or other glycoproteins of animal origin.

No major differences in amino acid composition of the urinary and kidney tissue culture inhibitors were observed. However, γ-carboxylglutamic acid is present in the urinary inhibitor, whereas we have been unable to detect its presence in the kidney tissue culture inhibitor. Recently, the biological formation of this amino acid has been studied by Stenflo and his co-workers (32, 33), and Suttie et al. (34), and they have shown that Vitamin K is an essential factor for the posttranscriptional carboxylation of glutamic acid to form γ-carboxylglutamic acid. Since no Vitamin K is present in the growth medium used for kidney tissue culture we obtained (information provided by Dr. P. Kelly, Collaborative Research, Waltham, MA.), posttranscriptional carboxylation of glutamic acid could not have occurred during incubation.

The molecular weight of this protein after treatment with EDTA was found to be 1.33 × 10^4 by analytical ultracentrifugation and 1.39 × 10^4 from the monolayer experiment. During the course of purification, we noticed that the inhibitor eluted from a column of Sephacryl S-200 had an apparent Mr = 1.00 × 10^4 (Fig. 3) (elution volume of 245 ml). However, when this inhibitor was incubated with EDTA and rechromatographed, it was eluted at an elution volume of 472.5 ml (corresponding to Mr = 1.5 × 10^4) (Fig. 4). In our attempts to examine the purity of the isolated proteins (before EDTA treatment) by SDS-polyacrylamide gel electrophoresis, we found multiple bands with Mr = 1.5, 3.0, 4.5, and 6.0 × 10^4, suggesting protein association to form dimers, trimers, and tetramers. Upon treatment of the inhibitor with EDTA, a single band was observed after modification of the inhibitor with Drimarene brilliant blue followed by SDS-polyacrylamide gel electrophoresis. Thus, we believe that Ca^{2+} (or another divalent metal ion) is involved in the aggregation process to yield oligomers. It is also interesting to note that Lawrie and Kemp (35) have observed reversible conformational changes at the COOH terminus of the γ-chain of human fibrinogen with Ca^{2+}, resulting in multiple bands on SDS-polyacrylamide gel electrophoresis. We are now focusing our attention on this point.

The successful fit of the obtained rate data to the Langmuir adsorption isotherm (Fig. 7) strongly suggests an adsorption type mechanism of inhibition; that is, the inhibitor serves to
block specific growth sites on the seed crystal surface, thus preventing crystal growth at these sites. This observation, in fact, supports the idea that crystal growth takes place at a limited number of growth sites on the crystal surface. (Other evidence for an adsorption type mechanism of inhibition is presented later in this section.)

As described earlier, the dissociation constant for the kidney tissue culture inhibitor-surface complex, \( K \), has a value of \( 1.9 \times 10^{-8} \) M. This parameter, \( K \), is quite useful for comparing the potency of various inhibitors. Although large quantities of urinary inhibitors are difficult to obtain, we have isolated and purified small amounts from normal subjects. Using a purified inhibitor (No. 34-B) isolated from an individual’s three 24-h urine collections,\(^2\) the inhibitory activity was assessed in precisely the same manner as for the kidney tissue culture inhibitor. The fit of these rate data to a Langmuir adsorption isotherm is illustrated in Fig. 9. From Fig. 9, a dissociation constant of \( 1.9 \times 10^{-8} \) M is obtained. Due to the limited data for the urinary inhibitor, the value of \( K \) should be considered approximate, but it is indeed remarkably similar to the value obtained with the kidney tissue culture inhibitor. We have therefore demonstrated that the kidney tissue culture inhibitor is similar to the urinary inhibitor with respect to both composition and inhibitory activity. Although our work on urinary inhibitors is continuing, based on the results presented here, we feel that the kidney tissue culture inhibitor is an excellent model for the urinary inhibitor.

We have previously shown the use of Equation 4 in assessing the inhibitory activity of urine, using varying concentrations of dialyzed urine in our assay system. Rate constants can be obtained in the same fashion as for the isolated inhibitors, and fit to the same Langmuir equation. A dissociation constant is then obtained in terms of a urine volume/liter of assay solution, enabling comparison of urine inhibitory activities without knowing the molar concentration of inhibitor present. Using the same urine, No. 34-B, prior to isolation of the inhibitor, we determined a dissociation constant \( K \) equal to 20 ml of urine/liter of assay solution, the concentration of urine needed to obtain 50% inhibition in our crystal growth assay. By equating this value to the value obtained using purified inhibitor \( (1.9 \times 10^{-8} \) M), we can estimate that the concentration of inhibitor in urine is equal to \( 9.7 \times 10^{-5} \) M, or 14.3 mg of inhibitor/liter of urine. (This urinary inhibitor was isolated from a 6-year-old boy. As children have higher levels of calcium oxalate monohydrate crystal growth inhibitor activity than adults, we predict the inhibitor concentration in both normal adults and adult patients would be lower than this.) By examining Fig. 9, it can be seen that a urinary concentration of inhibitor of \( 9.7 \times 10^{-5} \) M would be more than sufficient to protect the urine from any precipitation. This observation indicates that the urine is protected against calcium oxalate precipitation.

In addition to its ability to inhibit calcium oxalate monohydrate crystal growth, this glycoprotein isolated from kidney tissue culture medium shows strong surface activity represented by an abnormally high collapse pressure (~45 dynes/cm) and a relatively small limiting area/weight of protein \( (3.5 \text{ cm}^2 / \text{mg}) \). These properties suggest that the inhibitor molecule is strongly amphiphilic in nature and that it has a highly organized structure at the air-water interface. The relationship between surface activity and inhibition of crystal growth is intriguing, and we are investigating this point further. It should be recalled, however, that when 1 mg of the inhibitor in 1 ml of Tris-HCl buffer (pH 7.2) was incubated with 2 and 27.6 mg of calcium oxalate monohydrate crystals, respectively.

\(^2\) Y. Nakagawa, unpublished results.

**Fig. 9.** Langmuir adsorption isotherm type plot showing the effect of human urinary inhibitor on the rate of calcium oxalate monohydrate crystal growth. Values of \( k_in \) and \( k_{ex} \) represent the rate constants in the absence and in the presence of inhibitor, respectively. The line drawn represents a linear regression fit to the data \( (r = 0.998) \).

**REFERENCES**

Calcium Oxalate Crystal Growth Inhibitor

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