Elafin: An Elastase-specific Inhibitor of Human Skin

PURIFICATION, CHARACTERIZATION, AND COMPLETE AMINO ACID SEQUENCE*

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A potent inhibitor of human leukocyte elastase (EC 3.4.21.37) and porcine pancreatic elastase (EC 3.4.21.36) was purified to homogeneity from human horny layers. It inhibits human leukocyte elastase and porcine pancreatic elastase in a 1:1 molar ratio and shows equilibrium dissociation constants of $6 \times 10^{-10}$ M and $1 \times 10^{-9}$ M, respectively. Inhibition of plasmin, trypsin, α-chymotrypsin, and cathepsin G was not observed. This inhibitor proved to be an acid stable basic peptide with an isoelectric point of 9.7. The complete amino acid sequence appears to be unique with 38% homology to the C-terminal half of antileukoprotease.

The sequence shows that the inhibitor is composed of 57 amino acids and predicts a $M_r$ of 7017. The high affinity as well as the apparent specificity for elastases suggests a functional role in preventing elastase-mediated tissue proteolysis. It is suggested that the term "elafin" be used to designate this inhibitor.

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‡The abbreviations used are: HLE, human leukocyte elastase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
Elastase Inhibitor from Human Skin

TABLE I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_i$</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human leukocyte elastase</td>
<td>$6 \times 10^{-10}$ M</td>
<td>2.0 mM AAPVpNA</td>
</tr>
<tr>
<td>Porcine pancreatic elastase</td>
<td>$1 \times 10^{-9}$ M</td>
<td>0.1 mM AAPV-AFC</td>
</tr>
<tr>
<td>Human cathepsin G</td>
<td>NI</td>
<td>0.5 mM AAPFpNA</td>
</tr>
<tr>
<td>Rovine α-chymotrypsin</td>
<td>NI</td>
<td>0.5 mM AAPFpNA</td>
</tr>
<tr>
<td>Porcine trypsin</td>
<td>NI</td>
<td>0.1 mM TGFLpNA</td>
</tr>
<tr>
<td>Human plasmin</td>
<td>NI</td>
<td>0.1 mM TGFLpNA</td>
</tr>
</tbody>
</table>

$^a$ $K_i$, dissociation constant according to the method of Green and Work (20).


$^c$ NI, no inhibition.

TABLE II

Amino acid analysis of elafin

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Elafin</th>
<th>Antileukoprotease*</th>
</tr>
</thead>
<tbody>
<tr>
<td>D/N/C$^a$</td>
<td>15.4</td>
<td>23.6</td>
</tr>
<tr>
<td>T</td>
<td>3.7</td>
<td>3.8</td>
</tr>
<tr>
<td>S</td>
<td>7.7</td>
<td>5.7</td>
</tr>
<tr>
<td>E/Q$^b$</td>
<td>7.6</td>
<td>6.6</td>
</tr>
<tr>
<td>P</td>
<td>12.3</td>
<td>12.3</td>
</tr>
<tr>
<td>G</td>
<td>13.0</td>
<td>7.5</td>
</tr>
<tr>
<td>A</td>
<td>5.3</td>
<td>2.6</td>
</tr>
<tr>
<td>V</td>
<td>5.3</td>
<td>4.7</td>
</tr>
<tr>
<td>M</td>
<td>2.8</td>
<td>3.8</td>
</tr>
<tr>
<td>I</td>
<td>4.9</td>
<td>0.9</td>
</tr>
<tr>
<td>L</td>
<td>6.0</td>
<td>4.7</td>
</tr>
<tr>
<td>F</td>
<td>1.9</td>
<td>1.9</td>
</tr>
<tr>
<td>K</td>
<td>1.9</td>
<td>14.2</td>
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<tr>
<td>R</td>
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<td>4.7</td>
</tr>
<tr>
<td>Y</td>
<td>0.0</td>
<td>1.9</td>
</tr>
<tr>
<td>W</td>
<td>0.0</td>
<td>0.9</td>
</tr>
</tbody>
</table>

$^a$ Calculated from sequence (11).

$^b$ The reason for grouping D/N/C and E/Q is given in the "Mini-print Section."

FIG. 1. Polysulfoethyl aspartamide chromatography (Poly LC-HPLC) of elastase inhibitor containing fractions purified by cation exchange and reversed-phase C$_18$ chromatography. Protein content was recorded at 215 nm. Peak V (hatched area) showing approximately 20% of total inhibitory capacity was collected and further purified by reversed-phase C$_18$ chromatography. $y$ axis: % inhibition of HLE activity (% HLE-I) using 100 ng of HLE and 5 µl fraction/ml assay.

FIG. 2. Reversed-phase C$_18$ chromatography (Nucleosil 5 C$_18$-HPLC) of the major elastase inhibitory component derived from Poly LC-HPLC, subjected to a second rechromatography. The hatched area represents the purified elastase inhibitor, called elafin. This fraction was used for further characterization. $y$ axis: % inhibition of HLE activity (% HLE-I) using 100 ng of HLE and 5 µl fraction/ml assay.

psoriasis. This skin disease is characterized by hyperproliferating keratinocytes, as well as an inflammatory infiltrate consisting partly of neutrophils migrating into the affected epidermis. Previous studies have revealed considerable amounts of enzymatically active HLE present on the surface of psoriatic lesions (8). However, at present, no definitive conclusion can be drawn concerning the role of HLE in this skin condition.
The potent proteolytic activity of HLE is known to be balanced to a certain extent by the antiproteolytic activity of α1-antitrypsin and α2-macroglobulin. In addition to this, an acid stable low molecular weight inhibitor of human leukocyte proteases (antileukoprotease, HUSI-I, SLPI) could be detected in seminal fluid (13), cervical mucus (14), bronchial secretion (15), parotid secretion (16), and in human serum (17).

The elastase inhibitor "elafin" described here differs from these well-characterized inhibitors in several aspects. As a leading feature elafin is apparently specific for serine elastases (Table I) in contrast to antileukoprotease and α1-antitrypsin, which are very potent inhibitors of trypsin.

Two human elastase inhibitory activities apparently specific for HLE have been reported. The 9-kDa inhibitory activity from psoriatic skin with specificity for HLE (18) appears to be different from elafin, since this is not able to inhibit porcine pancreatic elastase. Furthermore, this inhibitory activity differs from elafin by its pI of 7.4. A low molecular weight inhibitory activity specific for HLE and porcine pancreatic elastase has been detected in bronchial mucus (19). This inhibitor is reported to have a molecular mass of 11 kDa, a K, of $5 \times 10^{-10}$ M with HLE and $9 \times 10^{-9}$ M with porcine pancreatic elastase. The relation of this inhibitor to elafin remains to be clarified.

The structure established (Fig. 3) is that of a small predominantly basic polypeptide. It has an unusually high content of proline and the glycine and cysteine contents also indicate a tightly charged molecule. The directions of the four disulfide bonds have not yet been established. A thorough search of protein data bases revealed the structure to be unique with no close homologues. The only structure found in the literature to have any significant homology was antileukoprotease. Presumably, those elements of the structure giving the high specificity for serine elastases are to be found in the regions in which the two molecules differ and remain to be established.

The HLE inhibitor described here shows a dissociation constant of $6 \times 10^{-10}$ M and could therefore be an effective regulatory element in inflammatory tissue damage and probably a compound of therapeutical value in elastase-mediated disorders.

Acknowledgments—We wish to thank A. Rakers for collecting scales, B. Bargmann for excellent technical assistance, and I. Brandt for her help in preparing the manuscript.

REFERENCES


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Supplemental Material to ‘Elastin. An Elastase Specific Inhibitor of Human Skin’ by Oliver Wiedow, Jens-Michael Schöder, Harry Gregory, Janice A. Young and Klaus Christopoulos.

Extraction of Human Epidermis

Samples of patients with psoriasis (1 - 100 g) were suspended in 30 - 200 ml epa (m. 120°C) (100°C, DSM, Germany) using a gradient of 0.01 M ammoniumacetate pH 4.0. Buffer A: 0.01 M ammoniumacetate pH 4.0, buffer B: 0.01 M ammoniumacetate pH 4.0, buffer C: 0.01 M ammoniumacetate pH 2.0. The chromatographic run was performed using a gradient of 0.01 M ammoniumacetate pH 2.5. The run was performed using a gradient of 0.01 M ammoniumacetate pH 4.0. Buffer A: 0.01 M ammoniumacetate pH 4.0, buffer B: 0.01 M ammoniumacetate pH 4.0, buffer C: 0.01 M ammoniumacetate pH 2.5. The chromatographic run was performed using a gradient of 0.01 M ammoniumacetate pH 4.0. Buffer A: 0.01 M ammoniumacetate pH 4.0, buffer B: 0.01 M ammoniumacetate pH 4.0, buffer C: 0.01 M ammoniumacetate pH 2.5.

Column Exchange-HPLC

The samples were chromatographed on a TSK HW 250 column (7.5 x 150 mm, 100 Å, Bio-Rad, Sweden) using a gradient of 0.01 M ammoniumacetate pH 2.5. The run was performed using a gradient of 0.01 M ammoniumacetate pH 4.0. Buffer A: 0.01 M ammoniumacetate pH 4.0, buffer B: 0.01 M ammoniumacetate pH 4.0, buffer C: 0.01 M ammoniumacetate pH 2.5. The chromatographic run was performed using a gradient of 0.01 M ammoniumacetate pH 4.0. Buffer A: 0.01 M ammoniumacetate pH 4.0, buffer B: 0.01 M ammoniumacetate pH 4.0, buffer C: 0.01 M ammoniumacetate pH 2.5.

Isoelectric Focusing

Inhibition of human leukocyte elastase (HLE)

The inhibition of HLE was determined by the use of the synthetic peptide Ac-SDKP, a known inhibitor of human neutrophil elastase. Activity was determined in a 96-well plate assay. The inhibitor concentration was determined by absorbance at 405 nm.

Aprotinin

Aprotinin was used as a control. Aprotinin concentration was determined by absorbance at 405 nm.
Inhibition of Trypsin and Plasmin

Phenylalanyl-arginine-4-nitroanilide (PRAA) and 0.1 M porcine trypsin (1 mg/ml) or human plasmin (1 mg/ml) were incubated for 10 minutes before adding the substrate. 0.1 M Tris–HCl–glycine–pyrrolidine–1-thione–EDTA–malachite green buffer, pH 7.5 was used. 0.1 M HCl, pH 2.5.

Direct Sequencing

Approximately 5 μg of the purified material was subjected to sequence analysis in the Applied Biosystems Model 473A automated Edman sequencer, followed by the Edman sequencer with modified cycle. The automated Edman sequencer was equipped with a 550-nm light analysis of the phenylthiohydantoin. This gave a sequence of thirty residues but with cysteine inferred by the absence of any identified residue. The initial yield of amino acid residues was compatible with a single chain polypeptide of molecular size 6 × 8 k Daltons.

Carnoy's methylation

Purified elastin (500 μg) was dissolved in water (40 ml) and mixed with 6 M guanidine hydrochloride, 0.1 M Tris hydrochloride pH 8.5 buffer (100 ml). The tube was flushed with nitrogen and a 10% solution of formaldehyde added (1 ml of 25 M formaldehyde buffer). The tube was again flushed with nitrogen, closed and kept at 80°C for 1 hr. After cooling to room temperature, formaldehyde was added to 1 M with 0.18 aqueous trifluoroacetic acid and applied directly to a Vydac C4 column (46 × 150 mm). The column was developed at a rate of 1 ml/min. The eluate was methanolized with acetic acid in a microwave oven and then analyzed in the same conditions. However, the affinity for the adsorbent was not clear. Thus, this was not used.

Amino Acid Analysis

Half of the minor component I (approx. 5 μg) was hydrolyzed with 6N hydrochloric acid containing 1% phenol in vacuo at 110°C for 16 hrs. The nitrosochloride was hydrolyzed using 6N HCl and a plus amino acid analyzer. Each fraction of the eluate was collected and evaporated to dryness. The residue was redissolved in 50 μg of CD. The residue was redissolved in 50 μg of 0.3 M HCl and hydrolyzed to Arg and Glu is hydrolyzed to Glu. Since carboxymidomethylated Cys coelutes in this analysis with Arg, it was only possible to obtain a single combined figure for Arg/Asp/Cys as well as Cys/Glu.

Digestion with chymotrypsin

The remaining half of component I was taken up in 0.1 M ammonium bicarbonate (0.1 ml) and 0.2 M trichloroacetic acid (1 ml) and treated with chymotrypsin (wt/wt, 1:4) at 37°C for 4 hr and then divided with 0.01 trifluoroacetic acid in water (200 μl) for direct loading on to a Vydac C8 column. A linear gradient of 0.1% trifluoroacetic acid in water for 30 min at a rate of 1 ml/min. The elution peaks were collected and assayed for amino acid content and were found to be present as the same extent with peaks 1 and 2 of the second digests. The additional peaks from C2, i.e., 3.5, 5.6, 7.9 were sequenced. Peak 7 proved to have the same sequence as 1 so far as it was taken. The sequence of C2 and C2+ and C2+ were shown in Fig. 3.

Digestion with Trypsin

The remaining portion of component II was taken up in 0.1 M ammonium bicarbonate (0.1 ml) and 0.2 M trichloroacetic acid (1 ml) and treated with trypsin (100 mg in 10 μl 0.1 M ammonium bicarbonate). The solution was maintained at 37°C for 30 min, diluted with 0.1% aqueous trifluoroacetic acid and loaded immediately on to a Vydac C8 column which was developed for 90 min with the same gradient as before. The relevant sequence data is shown in Fig. 3.

Sequencer Determination

Direct sequencing gave the first 30 residues and this was confirmed by the Edman sequencer. The sequence determined. Digestion with trypsin fragments on the major products, i.e., with all lysine bonds were broken and these fragments encompassing the full structure are given in Fig. 3. These points were limited to basic residues. By contrast chymotrypsin did not cleave after one arginine residue but did after two arginines, and also after leucine +1. In addition to Phe, phenylalanine was observed after leucine +2. The C-terminal glutamate was assumed on the basis of a very slow but detectable residue found in both the T3 and C2+3 peptides following strong flow peak. Most of the major peaks, however, were anticipated from such a C-terminal glutamate sequence. The C-terminal glutamic acid was not included in Fig. 3, and provided further confirmation of the structure.