

Evidence for the presence of different alpha-1-proteinase inhibitor genes products in mouse plasma*

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The plasma alpha-1-proteinase inhibitor (API) of three mouse species *Mus domesticus*, *M. caroli* and *M. pahori* was isolated. Each of the species isoforms were then separated by chromatofocusing; however, no significant differences in association rate constants toward human neutrophil elastase and bovine chymotrypsin were observed. The amino-acid sequence of the P₁-P₁₅ C-terminal fragments of the API variants indicate that mouse plasma contains at least two different active API isoforms in the case of *M. domesticus* (five API genes) but only one active API isoform in *M. pahori* and *M. caroli* (one API gene).

Alpha-1-proteinase inhibitor (API, also known as alpha-1-antitrypsin) and contrapsin are the two major plasma serine proteinase inhibitors (serpins) in mice [1]. Contrapsin is the murine equivalent to human alpha-1-antichymotrypsin but has been shown to inhibit trypsin-like enzymes in mouse. Murine alpha-1-proteinase inhibitor inhibits elastase, chymotrypsin, thrombin and bovine but not mouse trypsin [1-5]. The most interesting, recently studied feature of API is the presence of multigene families of this inhibitor [5-7]. This phenomenon may originate through single or multiple duplications of an ancestral gene with products of the resulting genes possibly acquiring new or related functions [8]. Analysis of genes in mice indicates that multiplication occurred at some time during the evolution of the *Mus* genus, leading to differentiation into a family

of three to five genes in *M. domesticus* and *M. saxicola* but only a single gene in *M. caroli* and *M. pahori* [4, 7]. A comparative computer analysis of mouse and other animals API genes suggests that the amplification of genes occurred prior to genus *Mus* speciation. Studies have also shown that the sequence of the reactive center of API, which determines target proteinase specificity, diverged in mice more rapidly than in other mammalian species [5, 9]. One can thus speculate that this reactive center divergence is a genus *Mus* response to some sort of positive Darwinian selection. In addition in the mouse one can also observe the multiplication of gene copy number as well as rapid reactive center divergence.

Apart from these genetic and evolutionary considerations all studies must be supported by confirmatory data obtained through protein

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Abbreviations: API, alpha-1-proteinase inhibitor; DFP, diisopropyl fluorophosphate; PVDF, polyvinylidene difluoride membrane; SDS/PAGE, sodium dodecyl sulphate polyacrylamide gel electrophoresis; TPCK, tosyl-L-phenylalanine chloromethyl ketone.

chemistry analysis. In this case, the first step in characterization of different mouse API genes products was performed by Paterson & Moore [10] who expressed all five genes of *M. musculus* API in mammalian cells and characterized the resulting inhibitors. Nevertheless, there are still no data indicating their actual presence in the plasma of different mouse species. As described here, we have isolated such proteins from *M. domesticus*, *M. caroli* and *M. pahori* and, in addition, characterized their structure within the reactive site of each isolated isoform.

MATERIALS AND METHODS

Materials. Bovine TPCK-treated trypsin, bovine alpha-chymotrypsin, Suc-Ala-Ala-Pro-Phe-pNA and MeOSuc-Ala-Ala-Pro-Val-pNA were from Sigma (St. Louis, MO, U.S.A.). Human neutrophil elastase and human API were purified by previously published procedures [11, 12], respectively. Blue Sepharose CL-6B, Polybuffer 74, Mono Q HR 5/5 and Mono P HR 5/20 columns were from Pharmacia (Uppsala, Sweden). S-2238 was from Kabi (Franklin, Ohio, U.S.A.). The polyvinylidene difluoride (PVDF) membrane was from Millipore (Bedford, CA, U.S.A.). All others reagents used were at least of analytical grade.

Purification of API from mouse plasma. The citrate-treated plasma from inbred strains of *M. domesticus*, *M. caroli* and *M. pahori* were a kind gift of Dr F.G. Berger, University of South Carolina, Columbia, SC, U.S.A. Two milliliters of plasma was diluted 5 times with 0.033 M phosphate buffer, pH 7.0, at 4°C, and passed through a Blue Sepharose CL-6B column (5 ml), equilibrated with the same buffer. The void volume was collected, concentrated, loaded to a Mono Q column equilibrated with 20 mM Tris/HCl, pH 8.0, and salt gradient applied. The fractions eluting at 0.15–0.18 M NaCl and showing inhibitory activity simultaneously toward elastase, chymotrypsin and trypsin were pooled, subjected to buffer exchange into 25 mM Bis/Tris, pH 6.4, concentrated, and applied to a Mono P column equilibrated with the above buffer. A pH gradient elution was performed using Polybuffer 74 adjusted with HCl to pH 4.0.

Active site titration. Specific activities were determined by titration with *p*-nitrophenyl-

guanidinobenzoate (trypsin) [13], *p*-nitrophenylacetate (chymotrypsin) [14] and human API (elastase).

Assays of enzymatic activity. The activity of the various proteinases was determined in 0.2 M Tris/HCl, pH 8.0, using a 4.8 nM final enzyme concentration and a 0.2 mM final substrate concentration. The substrates used were as follows: S-2238 for trypsin, Suc-Ala-Ala-Pro-Phe-pNA for chymotrypsin and MeOSuc-Ala-Ala-Pro-Val-pNA for elastase. The reaction was followed by spectrophotometric measurement of released *p*-nitroaniline at 405 nm.

Measurement of association rate constant (k_{on}). The determination of k_{on} was performed according to Bieth [15]. In brief, equimolar mixtures of enzyme and inhibitor (based on the active site titration of each protein) were incubated for increasing time periods at room temperature in a total volume of 0.75 ml of 0.1 M Tris/HCl, pH 8.0. Residual enzyme activity was then determined by addition of saturated amounts of a suitable chromogenic substrate and measurement of the amount of released *p*-nitroaniline at 405 nm. The data obtained were subjected to computer calculations to determine k_{on} .

Amino-acid sequence analysis. Amino-acid sequence analysis was performed on an Applied Biosystems 470A automatic gas-phase sequencer (Applied Biosystems, Foster City, CA, U.S.A.), using the program designated by the manufacturer.

Miscellaneous techniques. Electrophoresis of native proteins (8–12% gradient gels without SDS) and SDS/PAGE (12% gels, reducing conditions) was performed in Mini Protean II cell (Bio Rad, Hercules, CA, U.S.A.) according to the protocols in [16]. The sequences of peptides released from the reactive center of inhibitors were obtained after incubation at room temperature of equimolar amounts of proteinase and inhibitor in 0.2 M Tris/HCl, pH 8.0, for 5 min. The reaction was then stopped by adding diisopropyl fluorophosphate (DFP) to a final concentration of 2.5 mM and protein complexes and fragments were separated on 16.5% Tris/Tricine gels [17]. Polypeptides were transferred electrophoretically to a polyvinylidene difluoride (PVDF) membrane in 10 mM 3-cyclohexylamino-1-propanesulphonic acid (CAPS), pH 11, containing 10% methanol. The membranes were stained in Coomassie Brilliant Blue

G-250, and the stained peptide cut out and subjected to sequencing. Protein content was determined using the bicinchoninic acid assay (BCA) method.

RESULTS AND DISCUSSION

The alpha-1-proteinase inhibitor was purified from various mouse plasmas on the basis of protocols described previously [12]. Briefly, the plasma was depleted of albumin on a Blue Sepharose column and then subjected to ion-exchange chromatography on a Mono Q column. The column was washed with the equilibrating buffer and a NaCl gradient in the buffer was applied. API was eluted as one broad peak between 0.15–0.18 M NaCl. Inhibitor classification was determined by assaying inhibitory activity towards elastase, chymotrypsin and trypsin, as well as checking its molecular mass on analytical SDS/PAGE. The API fractions were subjected to chromatofocusing on a Mono P column, where apparently homogeneous fractions obtained from the Mono Q column showed considerable heterogeneity (Fig. 1). The active fractions could be divided into four subfractions for *M. domesticus* and *M. pahori* and into two subfractions in the case of *M. caroli*. These fractions do not resolve well on

a Mono P column but differ in migration rate during native PAGE (Fig. 2A). No molecular mass heterogeneity was demonstrated during SDS/PAGE and even the pooled active fractions from Mono P column stained as one sharp band of molecular mass about 55 kDa (Fig. 2B).

For all ten inhibitor subfractions their association rate constants with elastase and chymotrypsin were estimated. Among API subfractions belonging to a single species there were no significant differences in k_{on} values. The differences were detectable only between species and the following k_{on} values were determined for chymotrypsin: *M. domesticus*, $k_{on} = 1 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$; *M. caroli*, $k_{on} = 4 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$; *M. pahori*, $k_{on} = 2 \times 10^6 \text{ M}^{-1}\text{s}^{-1}$. Elastase reacts very fast with all mouse API's always giving values greater than $1 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, and for this reason the precise k_{on} estimation was not possible using our method [15].

Sequencing of the C-terminal peptides released after boiling of inhibitor-enzyme complex in the presence of SDS was finally used to determine which active isoforms are present in plasmas. The pooled API subfractions of each mouse species were separately incubated with equimolar amounts of elastase or chymotrypsin and, after complex formation, the residual enzyme activity was destroyed by addition of DFP. The samples were then boiled in SDS

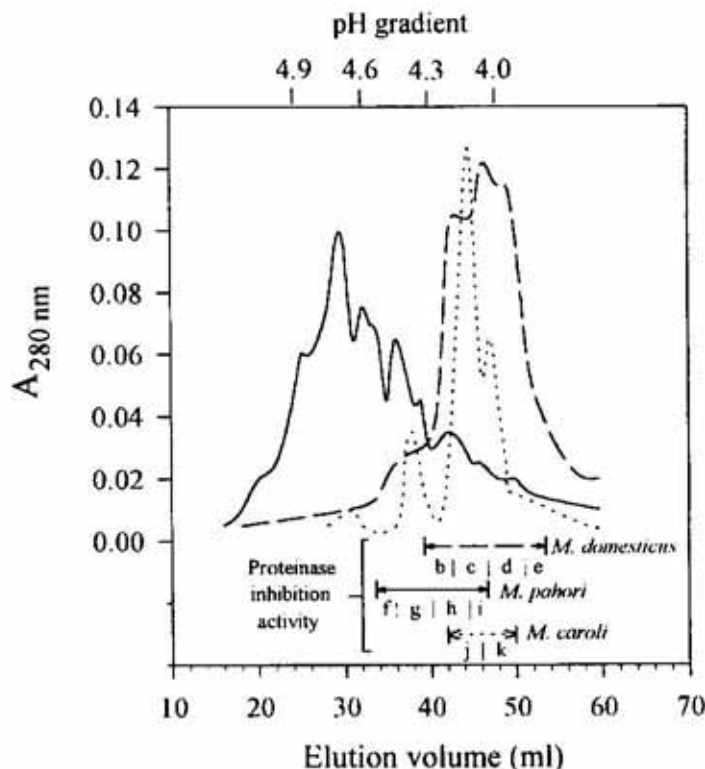


Fig. 1. Chromatofocusing of mouse alpha-1-proteinase inhibitor on Mono P column.

The figure contains three superimposed chromatograms of three mouse alpha-1-proteinase inhibitor preparations from 2 ml of plasma each. The ranges of inhibitory activity toward elastase, chymotrypsin and trypsin are indicated at the bottom as well as the division of active fractions into subfractions. For details see Materials and Methods section.

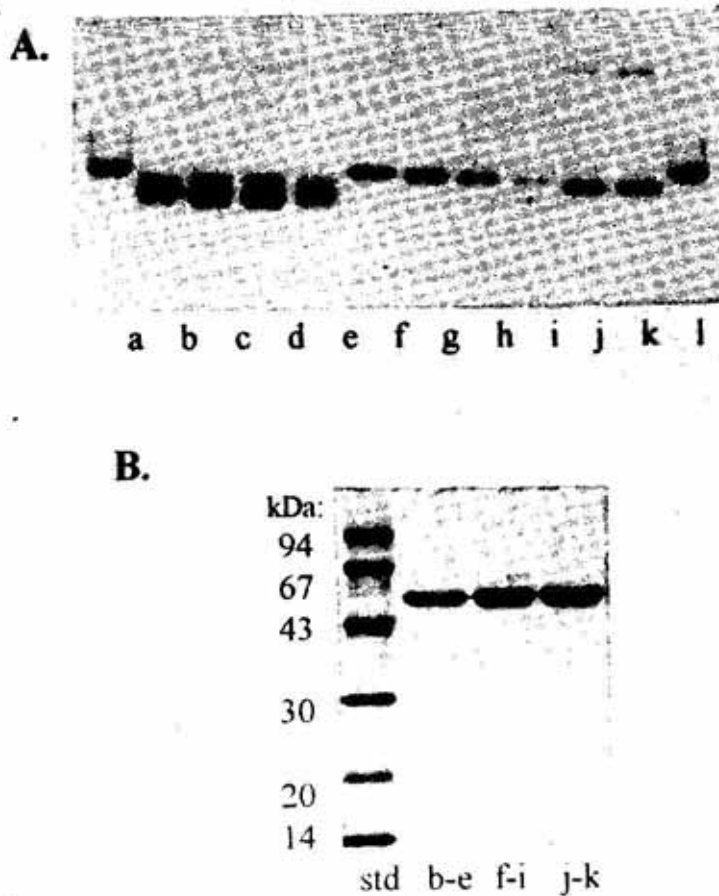


Fig. 2. The polyacrylamide gel electrophoresis of mouse alpha-1-proteinase inhibitor preparations.

A. Non-denaturing PAGE of active subfractions from Mono P column (see Fig. 1). Fractions a and l, human alpha-1-proteinase inhibitor (standard); fractions b to e, *M. domesticus*; fractions f to i, *M. pahori* and fractions j and k, *M. caroli*. B. SDS/PAGE of pooled, active fractions from Mono P column: b-e, *M. domesticus*; f-i, *M. pahori*; j-k, *M. caroli*.

sample buffer, subjected to SDS/PAGE and C-terminal peptides were blotted onto a PVDF membrane and sequenced. Only one sequence for *M. caroli* and *M. pahori* was obtained with either elastase or chymotrypsin; however, at least two sequences were obtained for *M. domesticus* (Table 1).

In summary, the mouse species *M. caroli* and *M. pahori*, having only one gene copy can transcribe and release one API form which inhibits elastase and chymotrypsin, similarly as it was found for human API. In the case of *M. domesticus*, having five API genes, two putatively are encoding methionine, two tyrosine, and one

Table 1
Alignment of reactive site sequence of C-terminal fragment of murine and human plasma alpha-1-proteinase inhibitors

	P ₇	P ₁ -P' ₁	P ₁₅
<i>M. domesticus</i> 1	V L Q M V P M	<u>S M P P I L R F D H P F L F I</u>	
<i>M. domesticus</i> 2	V F E A V P M	S M P P I L R F D H P F L F I	
<i>M. domesticus</i> 3	V [*] L L A V P Y	<u>S M P P I V R E D H P F L F I</u>	
<i>M. domesticus</i> 4	V L Q V A T Y	S M P P I V R F D H P F L F I	
<i>M. domesticus</i> 5	V L Q G G F L	S M P P I L M F N R P F L F I	
<i>M. pahori</i> **	V L G A V P M	<u>S M P P T V N F N H P F I F I</u>	
<i>M. caroli</i>	V L Q A V P M	<u>S M P P I L N F N K P F I F I</u>	
Human	V L E A I P M	S I P P E V K F N K P F V F L	

Amino-acids sequence from cDNA clone sequences [18] is shown in normal font. **Bold** and underlined fonts represent amino acids obtained after reaction with neutrophil elastase and chymotrypsin, respectively. *, Indicates position of a limited proteolysis by neutrophil elastase; **, personal communication (Dr F.G. Berger, University of South Carolina, Columbia, SC, U.S.A.).

leucine at the reactive center P₁ position (amino acid residue 353) [5]. The analysis of the reactive site fragments (Table 1) suggests the presence in plasma of at least two of those API isoforms but does not exclude the presence of all three isoforms. The almost identical molecular masses and other biochemical properties make the separation of mouse API isoforms very difficult. In our preliminary studies we have shown that the chromatofocusing on Mono P column is the only possible and most effective method. However, attention must be focused on the fact that this heterogeneity is more likely to be determined by variable glycosylation than by differences in amino-acid sequence. The lack of reasonable isolation procedures for API isoforms differing by only a few amino-acid residues is clearly frustrating. In addition, the one principal disadvantage of the method we have used in this study is the fact that only peptides cleaved from inhibitors in complex with proteinase can be sequenced and, therefore, the detection of any inactive API forms was impossible.

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