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CRYSTALLIZATION, PRELIMINARY X-RAY ANALYSIS AND PATTERSON SEARCH OF A NEW ASPARTIC PROTEASE ISOLATED FROM HUMAN URINE

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ABSTRACT: Aspartic protease (EC 3.4.23) make up a widely distributed class of enzymes in animals, plants, microbes and, viruses. In animals these enzymes perform diverse functions, which range from digestion of food proteins to very specific regulatory roles. In contrast the information about the well-characterized aspartic proteases, very little is known about the corresponding enzyme in urine. A new aspartic protease isolated from human urine has been crystallized and X-ray diffraction data collected to 2.45Å resolution using a synchrotron radiation source. Crystals belong to the space group $P2_12_12_1$. The cell parameters obtained were a=50.99, b=75.56 and c=89.90 Å. Preliminary analysis revealed the presence of one molecule in the asymmetric unit. The structure was determined using the molecular replacement technique and is currently being refined using simulated annealing and conjugate gradient protocols.

Key words: Aspartic protease, crystallography, drug design, synchrotron, X-ray analysis.

INTRODUCTION

Aspartic proteases are a group of proteolytic enzymes of the pepsin family that share the same catalytic mechanism and usually function in acid solution (1). These proteins are classified as the fourth major class of proteolytic enzymes, distinct from the serine, cysteine, and metallo-proteases (2).

They are broadly distributed in nature from retroviruses to humans. The functions of the enzymes are manifold from the nonspecific digestion of proteins to the highly specialized processing of proteinaceous substrates. The substrate-binding sites of aspartic proteases, being extended, are capable of interacting with up to seven amino acid residues of a substrate (3). Acid proteases are so called because they have an optimal catalytic activity at acid pH. They include mammalian digestive enzymes such as pepsin and cathepsin D, microbial proteases such as those

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produced by *Penicillium janthinellum* (penicillopepsin) (4,5), *Rhizopus chinensis* (rhizopuspepsin) (6), and *Endothia parasitica* (endothiapepsin) (6) and probably certain enzymes involved in mammalian control processes, for example renin, which acts on angiotensinogen (7). The acid proteases are characterized by the presence of two aspartic acid residues at the active site and most are inhibited by the microbial peptide analogue, pepstatin. They tend to cleave between hydrophobic amino acids but secondary interactions are important in the definition of their specificity (8). Extensive sequence homologies have been observed among the enzymes belonging to this family. Tertiary structures have been determined by X-ray crystallography for three microbial aspartic proteases, namely penicillopepsin (9), *Endothia parasitica* proteases (10), *Rhizopus chinensis* proteases (11), and the mammalian homologue, pig pepsin (12). All have broadly similar structures and this, together with the sequence homologies, would appear to indicate that all members of the family are likely to have similar three-dimensional structures. Therefore all should operate through a common catalytic mechanism for the hydrolysis of the substrates. However, substantial differences in substrate specificity, optimum pH and stability have been shown to exist among the various members of the family (13).

This communication describes the crystallization, preliminary X-ray analysis and Patterson search of an aspartic protease extracted from human urine. The investigation was made in order to gain further insight into the chemistry and functions of this protein.

MATERIAL AND METHODS

***Purification and Crystallization*:** The aspartic protease has been extracted from urine of healthy individuals using ion exchange chromatography (DEAE and Mono Q) and gel filtration (Superdex 10/75). It has a molecular weight of approximately 35 kDa, determined from size-exclusion chromatography and electrophoresis gel (SDS-PAGE). This aspartic protease has a single chain, two domains and maximum proteolytic activity in acidic pH values, as observed for other members of this family.

Lyophilized urinary aspartic protease was dissolved in deionized water (15 mg ml^{-1}) and centrifuged for 10 min at 18,000 rpm at 25°C. The clear supernatant was mixed 1:1 (v/v) with the reservoir solution that contained the appropriate buffer and precipitant. Crystals of urinary aspartic protease have been obtained in several different crystallization conditions, using the hanging drop vapor diffusion and sparse matrix methods (14). The best crystals were obtained after 1 week growth from drops in which 3 µl of enzyme solution was mixed with equal volume of 0.1 M Hepes buffer (pH 7.0) containing 2% polyethylene glycol 400 and 2.0 M ammonium sulfate. Crystal were mounted in capillary tubes of borosilicate glass for X-ray data collection.

***X-ray data collection and processing*:** X-ray diffraction data were firstly collected from a single urinary aspartic protease crystal at room temperature using a R-AXIS IV imaging plate system and graphite monochromated Cu Kα X-rays radiation generated by a Rigaku RU300 rotating

anode generator operated at 50kV and 100 mA at a crystal to detector distance of 150mm. 40 frames were collected using a oscillation range of 2.5 °. The exposure time per frame was 20 min. The X-ray diffraction data were processed to 2.8 Å resolution and scaled using the program PROCESS (15). A second X-ray diffraction data set were collect at a wavelength of 1.38 Å using the Synchrotron Radiation Source (Station PCr, Laboratório Nacional de Luz Síncrotron, LNLS, Campinas, Brazil) and a 30 cm MAR imaging plate detector (MAR Research) with an exposure time of 3 min per image at a crystal to detector distance of 175 mm. Using an oscillation range of 1.5 °, 65 images were collected and the raw X-ray diffraction data were processed to 2.45 Å resolution using the program DENZO (16) and scaled by the program SCALEPACK (16). Auto indexing procedures, combined with analysis of the X-ray diffraction pattern and averaging of equivalent intensities was used in the characterization of the Laue symmetry.

Molecular replacement: The X-ray data collected using synchrotron radiation were used in the molecular replacement. The crystal structure of the human urinary aspartic protease was determined by standard molecular replacement methods using the programs AMoRe (17) and X-PLOR (18). The atomic coordinates of ten different aspartic proteases deposited in the PDB (Brookhaven Protein Data Bank) were used as search models. All solvent and ligant molecules were removed from the search models and the temperature factors for all atoms were set to 20.00 $Å^2$. The search models which presented more than one subunit were modified, so that only one subunit was used in the molecular replacement. The atomic coordinates for all search models were translated so that their centre of gravity is at the origin, they were also rotated so that the principal axes of inertia of the search models are parallel to the orthogonal axes. The PDB accession numbers and identification of the search models are listed in Table 1. Initially self-rotation functions based on data in the resolution range 10-4.5 Å were calculated using a sampling step of 1° using the program AMoRe (17). Cross-rotation functions were calculated in the resolution range 10-4.5 Å using a sampling step of 2.5° for all 10 search models. These calculations were carried out with integration radius of 25 Å. The rotation which generated the highest correlation coefficient (CC) was applied to the search models and used in the subsequent translation function computations, based on data in the same resolution range.

The best solutions for each search model were selected based on the magnitude of the R_{factor} and correlation coefficient. The search model which generated the best solution were also used for the molecular replacement as implemented in the program X-PLOR (18), using the same resolution range and the same integration radius in order to confirm the solution obtained

Table 1. List of the aspartic protease used as search models for molecular replacement.

PDB Accession number	Protein
1eag	Aspartic protease from *Candida albicans* (*Candida* pepsin)
1lya	Cathepsin D from human liver
1zap	Aspartic protease from *Candida albicans*
1bbs	Human renin
1mpp	Pepsin (renin) from *Mucor pusillus*
2ren	Renin (recombinant human renin)
4apr	Aspartic protease from *Rhizopus chinensis*
1psn	Pepsin 3A from *Homo sapiens*
3app	Penicillopepsin from *Penicillium janthinellum*
1cms	Chymosin B from bovine (*Bos taurus*)

using the program AMoRe (17). Translation function for enantiomorphic space groups (P222, $P2_12_12$ and $P222_1$) has also been computated using the same resolution range and the best search model, in order to confirm the space group.

RESULTS AND DISCUSSION

The Figure 1 illustrates photomicrograph of urinary aspartic protease crystal suitable for X-ray diffraction experiments, with average dimensions of about 0.3 x 0.4 x 0.5 mm. The crystal has the orthorhombic space group $P2_12_12_1$ and the volume of the unit cell is 346 x 10^3 Å^3 compatible with one monomer in the asymmetric unit with V_m value of 2.17 Å^3/Da . Assuming a value of 0.74 cm^3 g^{-1} for the protein partial specific volume, the calculated solvent content in the crystal is 43.3% and the calculated crystal density 1.21 g cm^{-3}. The X-ray diffraction statistics for the two data sets are summarized in Table 2. Detailed data collection statistics for the Synchrotron data are given in Table 3.

Peak analysis of the self-rotation function did not reveal the presence of any significant local symmetry axis suggesting that a single subunit is contained in the asymmetric unit. This was in agreement with the estimated values of the crystal solvent content and V_m value (19).

The results of the molecular replacement using the 10 different search models are listed in Table 4. The correlation coefficients after translation function computation range from 14.4 to 67.8 % and the R_{factor}s range from 35.0 to 54.1 %. The search model which presented the best correlation coefficient and R_{factor} was the pepsin 3A from *Homo sapiens* (Accession number: 1psn). This search model was also submitted to molecular replacement using the program X-PLOR, and the solution obtained after the translation search was (α=23.6°, β=47.0°, γ=59.8°, x=0.314, y=0.029, z=0.443, R_{factor}=34.6 %) close to the one obtained by AMoRe (17).

Translation functions for enantiomorphic space groups (P222, $P222_1$, $P2_12_12$) has been computated using the coordinates of pepsin 3A as search model and the results are listed in Table 5. The correlation coefficients after translation function computation for the 3 enantiomorphic space groups range from 26.4 to 38.0 % and the R_{factor}s range from 46.4 to 51.1 %, which strongly indicates that the correct space group is $P2_12_12_1$.

The urinary aspartic protease is inhibited by the microbial peptide analogue, pepstatin. In order to obtain the structure of the binary complex between the urinary aspartic protease and pepstatin co-crystallization experiments have been initiated.

The origin of the urinary aspartic protease still unclear. The analysis of the molecular replacement solution indicates that the urinary aspartic protease structure shows high similarity with the structure of the pepsin. The partially refined model of the urinary aspartic protease is

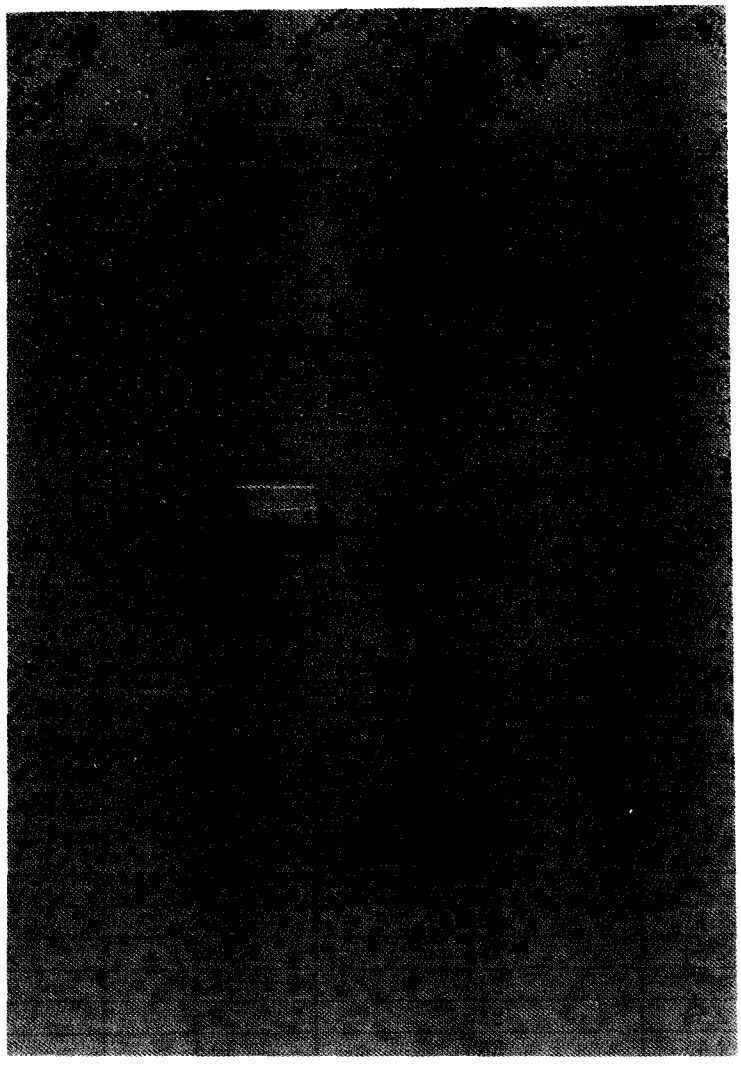

Figure 1: Photograph of the crystal of human urinary aspartic protease.

Table 2. Data collection statistics for both data sets.

	R-AXIS IV	LNLS
a (Å)	51.08	50.99
b (Å)	75.91	75.56
c (Å)	89.99	89.90
Number of Measurements with I>2 σ(I)	17686	24189
Number of Independent Reflections	10232	13134
R^*_{sym} (%)	10.4	7.7
Highest Resolution Shell (Å)	3.5 - 2.8	2.51 - 2.45
Completeness in the highest Resolution Shell (%)	79.3	99.1
R^*_{sym} in the Highest Resolution Shell (%)	18.5	30.3

*$R_{sym} = 100\ \Sigma\Sigma\ |I(h) - \langle I(h)\rangle| / \Sigma\ I(h)$ with I(h), observed intensity and $\langle I(h)\rangle$, mean intensity of reflection h over all measurement of I(h).

Table 3. Detailed data collection statistics for the synchrotron data.

Resolution Range		Number of independent reflections	R_{sym} (%)	Completeness (%)
13.00	5.86	916	4.2	94.4
5.86	4.73	888	6.1	98.1
4.73	4.15	895	6.1	99.0
4.15	3.78	894	7.0	99.8
3.78	3.52	892	7.7	99.9
3.52	3.31	881	8.3	99.7
3.31	3.15	864	9.8	99.5
3.15	3.02	862	12.1	99.4
3.02	2.90	880	15.1	99.5
2.90	2.80	861	17.4	99.8
2.80	2.71	865	20.2	99.2
2.71	2.64	886	22.4	99.0
2.64	2.57	836	26.4	98.8
2.57	2.51	846	28.5	98.9
2.51	2.45	868	30.3	99.1
All Reflections		13134	7.7	98.9

Table 4. Eulerian angles and fractional coordinates after translation function computation.

Protein	α (°)	β (°)	γ (°)	x	y	z	CC (%)	R^{*}_{factor} (%)
1eag	33.83	65.23	76.65	0.2231	0.1861	0.2065	14.4	54.1
1lya	156.00	46.61	121.10	0.3169	0.4727	0.0587	39.4	46.7
1zap	122.98	39.94	38.05	0.2905	0.2007	0.3434	14.4	55.2
1bbs	152.67	50.03	121.40	0.3250	0.4819	0.0588	26.5	50.8
1mpp	21.80	90.00	4.34	0.2000	0.1747	0.0514	16.2	53.5
2ren	30.54	40.01	235.18	0.3283	0.0154	0.4332	33.1	48.4
4apr	149.04	44.86	125.18	0.3176	0.4861	0.0618	22.1	52.5
1psn	24.44	46.13	59.44	0.3224	0.0247	0.4385	67.8	35.0
3app	146.53	46.99	307.78	0.3058	0.4739	0.0524	20.2	53.2
1cms	152.27	45.15	122.87	0.3209	0.4742	0.0610	39.3	48.9

*R_{factor} = 100 x $\Sigma|F_{obs} - F_{calc}|/\Sigma(F_{obs})$, the sums being taken over all reflections with $F/\sigma(F)>2$ cuttoff.

Table 5: Fractional coordinates after translation function computation for other possible enantiomorphic space groups.

Space Group	x	y	z	CC(%)	R_{factor}(%)
$P2_12_12$	0.0748	0.1244	0.1889	38.0	46.4
$P222_1$	0.0702	0.0254	0.3940	38.5	46.5
P222	0.0080	0.0045	0.0104	26.4	51.1

*R_{factor} = 100 x $\Sigma|F_{obs} - F_{calc}|/\Sigma(F_{obs})$, the sums being taken over all reflections with $F/\sigma(F)>2$ cuttoff.

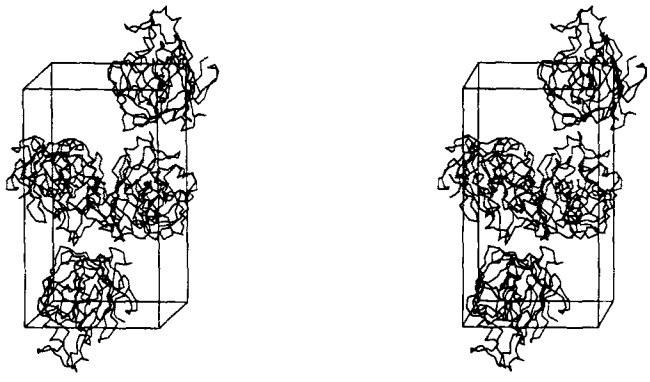

Figure 2: Crystal packing for the partially refined structure of the human urinary aspartic protease.

bilobal, consisting of two predominantly β-sheet lobes which, as observed in other aspartic protease, are related by pseudo 2-fold axis. Figure 2 presents the crystal packing for the partially refined structure of the urinary aspartic protease. Further refinement using the slow-cooling protocols will be performed using the program X-PLOR (18). The refined model of the urinary aspartic protease will be used for detailed comparison with other proteins of this family.

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