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High level expression and characterisation of Plasmepsin II, an aspartic proteinase from *Plasmodium falciparum*

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Abstract DNA encoding the last 48 residues of the propart and the whole mature sequence of Plasmepsin II was inserted into the T7 dependent vector pET 3a for expression in *E. coli*. The resultant product was insoluble but accumulated at ~20 mg/l of cell culture. Following solubilisation with urea, the zymogen was refolded and, after purification by ion-exchange chromatography, was autoactivated to generate mature Plasmepsin II. The ability of this enzyme to hydrolyse several chromogenic peptide substrates was examined; despite an overall identity of ~35% to human renin, Plasmepsin II was not inhibited significantly by renin inhibitors.

Key words: Aspartic proteinase; Plasmepsin II; *Plasmodium falciparum*

1. Introduction

During the blood borne stages of human infection by the malarial parasite *Plasmodium falciparum*, haemoglobin is digested in huge amounts as a source of nutrients to support metabolism. These reactions occur in a specialised, lysosome-like organelle, the digestive vacuole. Aspartic proteinase involvement in this processing has been established by demonstrating that the first step in haemoglobin degradation is inhibited by pepstatin [1]. To date, two aspartic proteinases have been isolated from parasite food vacuoles [2] and the genes encoding both enzymes have been cloned and sequenced [3,4]. These enzymes have now been systematically renamed Plasmepsin I (replacing previous names Aspartic haemoglobinase I [2] and Plasmodium aspartic proteinase G [4]), and Plasmepsin II (replacing Aspartic haemoglobinase II [2] and Plasmodium aspartic proteinase D [4]). The Plasmepsin proteinases (EC3.4.23.38) thus represent important new targets for antimalarial chemotherapy. Elucidation of structure/activity relationships for both enzymes would facilitate the development of potent, specific inhibitors for potential application as drugs. Since only trivial amounts of material can be isolated directly from the parasites, production of each enzyme as a recombinant protein is essential. To this end, this report describes the production of Plasmepsin II in *E. coli* in sufficiently high amounts to permit structural studies to be initiated together with initial characterisation of the recombinant enzyme.

2. Materials and methods

2.1. Cloning

The gene encoding the last 48 residues of the pro region and the whole of the mature Plasmepsin II was amplified from the pBluescript KS+ clone 0075M [4] in a polymerase chain reaction using synthetic primers 5′-CCGGAATTCGGATCCGAACATTTAACTATTGG-3′ and the M13 universal primer, 5′-GTAAAACGACGGCCAGT-3′. Following digestion of the amplified product with *Bam*HI, fragments were ligated into pET3a (AMS Biotechnology, Milton Keynes, Bucks., UK) that had been cut with *Bam*HI and phosphatase-treated. This vector permits high levels of expression of recombinant proteins as fusions with a 13 amino acid leader sequence derived from the N-terminus of the T7 gene 10 protein [5]. The sequence of a positive clone containing the Plasmepsin II insert in the correct orientation was determined in its entirety to ensure that no mutations had been introduced during the polymerase chain reaction.

2.2. Induction

E. coli BL21(DE3)pLysS cells transformed with the recombinant plasmid were grown to an A_{600} of 0.4 in LB medium supplemented with 150 μg/ml ampicillin, and were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.4 mM. Incubation was continued at 37°C for a further 2 h, at which time the cells were harvested by centrifugation (3,000 × *g* for 10 min) and resuspended in TN buffer (50 mM Tris-HCl, pH 7.2, 0.15 M NaCl). Lysozyme was added (final concentration = 10 μg/ml) and the cells were lysed by freezing/thawing.

2.3. Purification and refolding

Lysed cells (from 1 l of culture) were diluted to 200 ml in TN buffer and stirred overnight at 4°C. Insoluble material containing the recombinant protein was pelleted by centrifugation at 16,000 × *g* for 30 min. The pellet was resuspended in 200 ml buffer B (0.1 M Tris-HCl, pH 11.0, 50 mM β-mercaptoethanol) and stirred at 4°C for 4 h. Insoluble material was re-pelleted and washed with a further 200 ml buffer B. After centrifugation, the washed pellet was resuspended in 10 ml buffer C (6 M urea, 0.1 M Tris-HCl, pH 8.0, 1 mM glycine, 1 mM EDTA, 50 mM β-mercaptoethanol) and stirred overnight at 4°C to solubilise the recombinant material. Residual insoluble material was removed by centrifugation at 28,0000 × *g* for 2 h. The supernatant containing recombinant protein was rapidly diluted into 2 l buffer D (10 mM Tris-HCl, pH 8.5) and stirred at 25°C for a further 24 h to allow refolding of the recombinant protein. This solution was concentrated to a volume of 30 ml using a Filtron Ultrasette, 5 kDa cut-off tangential flow concentrator (Flowgen Instruments Ltd, Sittingbourne, Kent, UK) and the concentrate was loaded onto DEAE-cellulose Productiv PSC10-DE column (BPS Separations Ltd., Spennymoor, Co. Durham, UK) equilibrated in 0.1 M Tris-HCl buffer, pH 8.5. After extensive washing, the recombinant protein was eluted by a linear gradient (50 ml each) of 0–0.8 M NaCl in the same buffer.

2.4. Analytical measurements

SDS-PAGE was performed by the method of Laemmli [6] and gels were stained with Coomassie blue. Edman degradation was performed after blotting samples onto polyvinylidene difluoride (PVDF) as described previously [4]. Derivation of kinetic parameters (K_m, k_{cat}) for the hydrolysis of synthetic chromogenic substrates and K_i values for the interaction of naturally-occurring and synthetic inhibitors was carried out as described previously [7].

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3. Results and discussion

In common with all other known aspartic proteinases [8], Plasmepsin II is produced in the form of a precursor; but in contrast to other archetypal zymogens, the propart region of the Plasmodium proteinase(s) is much longer (>100 residues) [3,4].

Since precursors of mammalian and fungal aspartic proteinases with propart segments of only 45–50 residues have been expressed successfully in high yields [9,10], attempts were made to produce Plasmepsin II initially in the form of a precursor with a propart segment of approximately this length. The translated sequence at the N-terminus of the plasmid constructed was

Met-Ala-Ser-Met-Thr-Gly-Gly-Gln-Gln-Met-Gly-Arg-Gly-Ser-Glu-His-Leu-Thr-Ile-Gly~

in which the first 13 residues were derived from the plasmid and the authentic sequence of the Plasmodium protein begins with the sequence Ser-Glu-His-Leu-Thr-Ile-Gly~.

This construct thus omitted the first 76 residues of the full-length proPlasmepsin II described previously [4].

The truncated precursor which accumulated in an insoluble form in *E. coli* was solubilised, re-folded and concentrated as described in section 2, using a protocol adapted from those applied successfully to zymogens of other aspartic proteinases reported previously [9,10,11]. The resultant material obtained after DEAE-chromatography consisted primarily of a band that migrated on SDS-PAGE with an apparent mol.wt. of ~43K (Labelled pro; Lane 1, Fig. 1). Through 20 cycles, the sequence of this band determined from a PVDF blot, was found to be

Gly-Arg-Gly-Ser-Glu-His-Leu-Thr-Ile-Gly-Phe-Lys-Val-Glu-Asn-Ala-Hi s-Asp-Arg-Ile~

This corresponds to the authentic N-terminus of the (truncated) precursor protein, preceded by three residues of the gene 10 leader peptide (see above). A minor, accompanying band (labelled mature, Lane 1, Fig. 1) was also subjected to Edman degradation after blotting onto PVDF. The sequence elucidated was

Leu-Asn-Ser-Gly-Leu-Thr-Lys-Thr-Asn-Tyr-Leu-Gly-Ser-Ser-Asn-Asp-As n-Ile-Glu-Leu-Val-Asp-Phe-Gln-Asn~

Table 1
Kinetic constants for the cleavage of chromogenic substrates by recombinant Plasmepsin II

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1} \cdot s^{-1}$)
Ala-Leu-Glu-Arg-Thr-Phe*Nph-Ser-Phe-Pro-Thr	110	3.8	35
Lys-Pro-Ile-Val-Phe*Nph-Arg-Leu	10	1.6	160
Lys-Pro-Ile-Lys-Phe*Nph-Arg-Leu	n.d.	0.04	–

Measurements made in 0.1 M sodium citrate buffer, pH 4.7; n.d. = not determined.

which is coincident with the sequence predicted for recombinant, mature Plasmepsin II from the nucleotide sequence, as described previously [4].

Aliquots of this recombinant material were acidified to pH 4.7 and incubated at room temperature. Samples were removed at appropriate intervals and analysed by SDS-PAGE (Fig. 1) to monitor the autoactivation of the precursor to mature enzyme. This conversion was essentially complete after 40 min but was prevented completely by inclusion of pepstatin (final concentration of 100 nM) during the incubation (Lane 8, Fig. 1).

The N-terminal sequence of the resultant enzyme was also determined, after blotting onto PVDF, and was found to be

Leu-Asn-Ser-Gly-Leu-Thr-Lys-Thr-Asn-Tyr-Leu-Gly-Ser~

In this way, highly purified preparations of Plasmepsin II were obtained. The yield of purified, active enzyme was estimated to be ~20 mg/l of culture.

The recombinant enzyme thus produced is longer by 12 residues than its natural counterpart in the form that was analysed [2] after purification from isolated food vacuoles. It is not clear at present whether the latter truly exists within the food vacuole or whether it results artifactually because of N-terminal trimming during the lengthy procedures that had to be employed for purification of the food vacuolar enzyme [2]. Amongst others, an aminopeptidase [12] and a cysteine proteinase in the food vacuole [2] have been identified, which could contribute to such processing during purification of the natural enzyme. The recombinant protein was not exposed to such potential hazards. Furthermore, the N-terminal region of the *mature* forms of members of the aspartic proteinase family is known to be highly variable, both in content and in length [8].

As further confirmation of this, the mature recombinant

Table 2
Interaction of Plasmepsin II with various inhibitors

Inhibitor	IC_{50} (nM)
1. D-His-Pro-Phe-His-Leu ψ [CH_2-NH]-Leu-Val-Tyr	>10,000
2. Pro-His-Pro-Phe-His-Leu ψ [CH_2-NH]-Val-Ile-His-Lys	>4,000
3. Boc-His-Pro-Phe-His-Leu ψ [CH(OH)-CH_2]-Val-Ile-His	150
4. Boc-His-Pro-Phe-His——Sta——Leu-Phe-NH_2	1000
5. Pro-Thr-Glu-Phe ψ [CH_2-NH]-Phe-Arg-Glu	>4,000
6. Lac-Val——Sta——Ala-Sta	100
7. Ac-Val-Val——Sta——Ala-Sta	0.6*
8. Iva-Val-Val——Sta——Ala-Sta	0.1*

*Denotes K_i values which were calculated using high concentrations of enzyme; this was necessary due to the relatively low k_{cat} with the substrates available. Determined at pH 4.7.

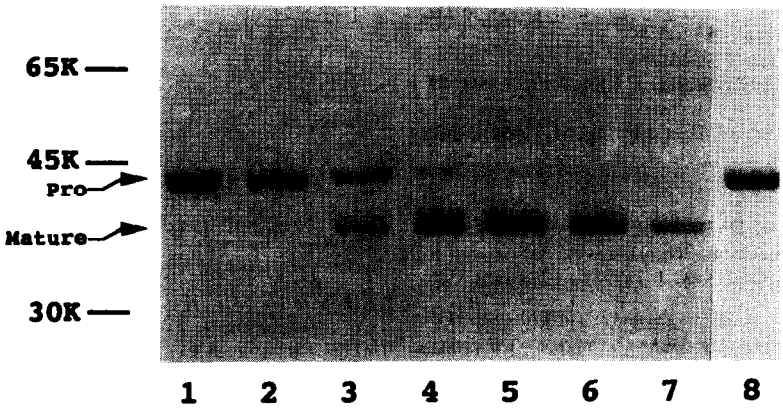

Fig. 1. Time course of the conversion of precursor to mature form of Plasmepsin II. Samples of precursor were acidified in 0.1 M sodium citrate buffer, pH 4.7, incubated at room temperature and aliquots were removed for analysis by SDS-PAGE at 0, 10, 20, 30, 40, 60, 120 min (lanes 1–7, respectively). A separate incubation performed in the presence of 100 nM isovaleryl-pepstatin is depicted in lane 8.

enzyme was examined for its activity. The initial bond cleaved within haemoglobin by the Plasmodium enzymes has been identified previously to be that between -Phe[33]*Leu[34] located in the alpha-chain [1,2]. A peptide based on this sequence was synthesised in which the Leu residue was replaced in the $P_{1'}$ position by the chromogenic reporter residue, *p*-nitrophenylalanine, as described previously [7]. The hydrolysis at pH 4.7 of the resultant peptide

Ala-Leu-Glu-Arg-Thr-Phe*Nph-Ser-Phe-Pro-Thr

by the recombinant Plasmepsin II was monitored spectrophotometrically and the kinetic parameters derived (Table 1) indicate that this substrate was effectively cleaved. The K_m value is directly comparable to the values derived previously for hydrolysis of equivalent peptides by a number of other aspartic proteinases (recombinant and naturally occurring), of human, animal and fungal origin [7]. However, the k_{cat} value obtained is at the low end of this range of values.

In order to ascertain whether Plasmepsin II was specific for Hb as a substrate, its activity was measured towards a second synthetic chromogenic peptide (Table 1), which is not derived from haemoglobin but which has been shown previously to be hydrolysed readily by the human enzymes, cathepsin D [13] and cathepsin E [14]. The K_m value measured for this substrate with Plasmepsin II was considerably better than that for the peptide derived from haemoglobin (Table 1). Indeed, the specificity constant (k_{cat}/K_m) determined for this second substrate was almost 5-fold better than that measured for the haemoglobin-derived sequence. However, when the Val residue in the P_2 position was substituted by a Lys, the resultant peptide was almost resistant to hydrolysis by Plasmepsin II (Table 1).

At the amino acid level, Plasmepsin II shows about 35% identity in sequence to human renin, cathepsin D and cathepsin E. In order to investigate this further, the susceptibility of Plasmepsin II to inhibition by a series of compounds, including some which are highly specific for renin, was examined (Table 2). The Plasmodium enzyme was not affected by inhibitors 1, 2 and 4 which are highly specific for renin, whilst compound 3, although certainly a potent renin inhibitor, has been shown previously [15] to be non-selective. Compound 6 is a water-soluble derivative of pepstatin, that inhibited Plasmepsin II with an IC_{50} of 100 nM, a value that is comparable to that derived previously for human cathepsin D [16]. As might be expected of an aspartic proteinase, Plasmepsin II was potently inhibited by the poorly water-soluble acetyl (compound 7) and isovaleryl (compound 8) pepstatins, which have been shown previously to have varying potencies towards aspartic proteinases of mammalian, fungal [17] and retroviral [18] origin.

Plasmepsin II is thus *not* a highly-specific aspartic proteinase like renin; since it is able to hydrolyse competently a substrate with a sequence unrelated to that of haemoglobin, it would appear to be a more degenerate enzyme, akin to cathepsin D or cathepsin E. Its inability to cleave a substrate with a P_2 Lys residue indicates behaviour comparable to that of cathepsin D [13].

The precursor and mature forms of Plasmepsin II were both produced in sufficiently high amounts to enable attempts at crystallisation now to be made with a view to elucidation of the 3-dimensional structure of the proteins. Such information, coupled with a mutagenesis programme, should enable evaluation of structure/function relationships in this important enzyme, together with the development of selective inhibitors that might have considerable therapeutic potential in combatting malaria.

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