Expression and characterization of a recombinant cysteine proteinase of *Leishmania mexicana*

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A major cysteine proteinase (CPB) of *Leishmania mexicana*, that is predominantly expressed in the form of the parasite that causes disease in mammals, has been overexpressed in *Escherichia coli* and purified from inclusion bodies to apparent homogeneity. The CPB enzyme, CPB2.8, was expressed as an inactive pro-form lacking the characteristic C-terminal extension (CPB2.8ΔCTE). Pro-region processing was initiated during protein refolding and proceeded through several intermediate stages. Maximum enzyme activity accompanied removal of the entire pro-region. This was facilitated by acidification. Purified mature enzyme gave a single band on SDS/PAGE and gelatin SDS/PAGE gels, co-migrated with native enzyme in *L. mexicana* lysates, and had the same N-terminal sequence as the native enzyme. The procedure yielded > 3.5 mg of active enzyme per litre of *E. coli* culture.

Key words: cathepsin L, pro-region processing, recombinant enzyme.

INTRODUCTION

Protozoal parasites of the genus *Leishmania* affect some 12 million people worldwide, causing visceral and cutaneous disease. Increasingly widespread drug resistance and the toxicity of available chemotherapy highlight the necessity for designing novel drugs against specific targets in the parasite [1]. One species, *L. mexicana*, expresses high levels of several classes of cysteine proteinases [2–4]; one group of which, CPB (denoting cysteine proteinase class B), has been shown to be a virulence factor [5]. This class of cysteine proteinase is expressed from a single tandem array comprising 19 gene copies with individual genes encoding subtly different isoenzymes [6]. These CPBs exhibit similarities to cathepsin L variants from different mammals which are involved in protein degradation and implicated with cancer metastasis and arthritis [7–9].

L. mexicana mutants lacking CPB enzymes have greatly reduced infectivity, compared with wild-type parasites, towards macrophages *in vitro* and Balb/c mice [5]. Re-expression of a single isoform from the *CPB* array (CPB2.8, an isoform encoded by a gene internal to the array; 2.8 refers to the gene size) restored infectivity towards macrophages to wild-type levels, suggesting that it is a virulence factor. Furthermore, inhibitors of the CPB isoenzymes reduce the infectivity of *Leishmania*, both *in vitro* towards macrophages and also to mice [5,10].

L. mexicana CPB isoenzymes are expressed as inactive zymogens comprising an 18 amino acid pre-region, that is thought to be rapidly removed by a signal peptidase upon transfer into the endoplasmic reticulum, a 106 amino acid pro-region, a 219 amino acid mature domain that includes the active site, and a C-terminal domain of either 16 or 100 amino acids [4]. The long C-terminal extension is characteristic of this class of cysteine proteinases of trypanosomatids and is not found with mammalian cysteine proteinases. Its role remains unknown, although deletion of this domain does not prevent *in vitro* processing of recombinant enzyme or abolish activity of mature enzyme [11,12]. Conversion of zymogen into mature, active CPB requires processing of the pro-region and possibly part of the C-terminal domain and results in mature enzymes of approximate M_r 23300 [3]. Little is known of zymogen processing in *Leishmania* parasites, whereas this process has been studied with the related mammalian cathepsins L and B [13–15], papain [16,17] and the major cysteine proteinase, cruzain, of *Trypanosoma cruzi*, another parasitic trypanosomatid [11,18]. Evidence supports a role for the pro-region in enzyme inhibition [19–23], whereby the pro-region peptide lies along the substrate-binding cleft of the mature enzyme, but in the reverse orientation to substrates. The pro-region is also thought to be important for enzyme stability, for correct folding, and in the targeting of the enzyme from the endoplasmic reticulum and Golgi to lysosomes [24–28].

The importance of leishmanial cysteine proteinases in the host–parasite interaction [2], and particularly immune evasion [29], highlights their potential both as drug targets and as vaccine candidates. Large-scale production of recombinant enzyme is a key factor for both approaches, in that it enables further characterization of the enzyme's pivotal role in immune evasion and also facilitates high-throughput screening using substrate and inhibitor libraries. In addition, detailed characterization of zymogen processing and activation is made possible; current evidence suggests that these events may differ significantly in these lower eukaryotes from the processes that occur in mammals. To this end, a novel procedure has been developed for the purification and activation of leishmanial CPB isoenzymes overexpressed in *E. coli*, and the steps in the processing of the pro-form to the fully active mature enzyme have been determined.

Abbreviations used: CPB, cysteine proteinase class B; CPB2.8, CPB isoform 2.8; CPB2.8ΔCTE, CPB2.8 lacking the C-terminal extension; BzPFRNan, *N*-benzoyl-Pro-Phe-Arg *p*-nitroanilide hydrochloride; DTT, dithiothreitol; E-64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

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MATERIALS & METHODS

Expression of *L. mexicana* cysteine proteinase isoenzyme CPB2.8 in *E. coli*

The gene for CPB2.8, derived from a *L. mexicana* genomic library screened with ^{32}P-labelled CPB cDNA clone pSWB1a [30], was cloned into the *Sal*I site of pBluescript SK II, as described previously [6]. Primers were designed for the amplification by PCR of a truncated version of *CPB2.8* lacking the C-terminal extension (*CPB2.8ΔCTE*). Restriction sites (underlined) were added to the 5′ ends for ligation with expression vector pQE-30 (Qiagen, Crawley, W. Sussex, U.K.). Primer 1, JH9601 5′-GGATCCGCCTGCGCACCTGCGCGCGCGA-3′; primer 2, JH9602 5′-AAGCTTCTACCGCACATGCGCGGACACGG-3′.

Reactions used 1 U of the proof-reading high-fidelity (VENT) DNA polymerase (NEB, Hitchen, Herts, U.K.) for 1 cycle at 94 °C for 4 min, 15 cycles at 94 °C (denaturing) for 30 s, 55 °C (annealing) for 30 s, 72 °C (extension) for 2 min, and 1 cycle at 72 °C for 7 min. PCR products were incubated at 72 °C for 10 min with 2.5 U of *Taq* polymerase (Advanced Biotechnologies Ltd, Epsom, Surrey, U.K.) to add 3′ A-overhangs, and cloned into pTAG vector (R&D Systems). *CPB2.8ΔCTE* was excised from this vector by digestion with *Bam*HI and *Hin*dIII, gel-purified and sub-cloned into pQE-30 vector (Qiagen) previously digested with the same restriction enzymes, to give plasmid pGL180. pGL180 encodes an N-terminally His_6-tagged proform of CPB2.8 lacking the C-terminal extension (His_6-CPB2.8ΔCTE). pGL180 was sequenced across the insert region, using automated sequencing (AmpliTaq Ready Reaction Mix; Perkin–Elmer), to confirm that no mutations had been introduced during PCR. *E. coli* M15pREP4 expression strain was transformed with plasmid pGL180 and grown at 37 °C in 200 ml of Luria-Bertani media supplemented with 100 $\mu g \cdot ml^{-1}$ ampicillin and 25 $\mu g \cdot ml^{-1}$ kanamycin, seeded (1:50) from an overnight culture. At a D_{600} of 0.6–0.8, the culture was induced with 0.5 mM isopropyl β-D-thiogalactoside and grown for a further 4.5 h at 37 °C. Cells were pelleted at 4000 ***g*** for 10 min, and frozen overnight at −20 °C.

Purification and activation of *L. mexicana* cysteine proteinase CPB2.8 from *E. coli*

The method of Kuhelj et al. [31] was modified as follows: AnalaR-grade urea (Merck) was purified further by extraction over Amberlite MB-1/IRA-400 beads (Sigma) for 2 h at room temperature, filtered and used immediately. Initial steps in enzyme purification were carried out at 4 °C. Following solubilization of inclusion bodies in 8 M urea, 0.1 M Tris/HCl (pH 8.0), and 10 mM dithiothreitol (DTT), and sedimentation of particulate material at 6000 ***g*** for 10 min, the protein concentration of the soluble phase was reduced to 0.1 mg/ml by dilution in 8 M urea, 0.1 M Tris/HCl (pH 8.0), and 5 mM EDTA. The sample was dialysed overnight at 4 °C against 0.1 M Tris/HCl (pH 7.0), 5 mM EDTA, 5 mM cysteine, then for 2 h at 4 °C against 20 mM Tris/HCl (pH 7.0), 5 mM EDTA, and clarified at 6000 ***g*** for 10 min. Maximum recovery of soluble enzyme was dependent upon low (0.1–0.5 mg/ml) protein concentrations during refolding. The optimum pH for solubilization was pH 8.0 and for refolding pH 7.0. The resulting supernatant was subjected to anion-exchange chromatography using a 1 ml Mono Q (Amersham Pharmacia Biotech) column equilibrated in 20 mM Tris/HCl (pH 7.0), 5 mM EDTA, 0.01 % (v/v) Triton X-100 with elution at 1 ml/min with a 40 ml 0–100 % gradient of 1 M NaCl in 20 mM Tris/HCl, pH 7.0, 5 mM EDTA, 0.01 % (v/v) Triton X-100. Enzyme activity eluted over 350–550 mM NaCl, was pooled and activated by acidification, using a modification of the protocol devised for cruzain (Professor J. McKerrow, personal communication). This comprised incubation of enzyme (0.1–0.5 mg/ml) at 37 °C in the presence of 0.1 M sodium acetate buffer (pH 5.5), 0.9 M NaCl, 2 mM EDTA, and 10 mM DTT until full conversion was observed (approx. 2–4 h). Activation was monitored by SDS/PAGE, gelatin SDS/PAGE, and by assay using substrate *N*-benzoyl-Pro-Phe-Arg *p*-nitroanilide hydrochloride (BzPFRNan, Sigma). Pro-region peptides released during the activation process were removed by gel filtration at room temperature using a 50 ml Sephadex G-50 column (Amersham Pharmacia Biotech) equilibrated in 0.1 M sodium acetate (pH 5.5), 2 mM EDTA, 10 mM DTT, 0.45 M NaCl, and 0.01 % (v/v) Triton X-100 at a flow rate of 0.75 ml/min over 55 ml. Eluted CPB2.8ΔCTE was concentrated using Centricon-10 (Amicon). CPB2.8ΔCTE was also purified from solubilized *E. coli* cell lysates using nickel-agarose chromatography (Qiagen), but fully active enzyme could not be obtained in this way.

Protein quantification, gel electrophoresis, enzyme assays and Western blotting

Protein concentrations were determined using the Bio-Rad and Pierce BCA protein assays. Denaturing gel electrophoresis was according to the standard method of Laemmli [32]. N-terminal amino acid sequencing used Fluka-grade acrylamide gels cross-linked with piperazine diacrylamide (Bio-Rad). Gels were pre-run at 6 mA for 1 h with 50 μM reduced glutathione in the upper reservoir buffer, which was replaced with 0.1 mM sodium thioglycolate for the sample run. Proteins were transferred onto Problott transfer membrane (Applied Biosystems, Foster City, CA, U.S.A.) [33]. For M_r determinations, a Benchmark protein ladder (Life Technologies) and Mark 12 MW standard (Novex, Frankfurt, Germany) were used. Gelatin SDS/PAGE was as described in [34,35]. Activity towards BzPFRNan was assayed as described in [33], or continuously at 37 °C for 30 s at 410 nm in 400 μl of 0.1 M sodium phosphate (pH 6.0), 10 mM DTT, and 250 μM substrate, following pre-incubation of the buffer for 3 min at 37 °C. Kinetic analyses used 1–2 μg of enzyme and were in triplicate, using 12 individual substrate concentrations from $K_m \times 0.25$ to $K_m \times 10$. Active-site titration using *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E-64) followed [36]. Following SDS/PAGE, proteins were transferred to Hybond ECL® nitrocellulose (Amersham Pharmacia Biotech) for 45 min at 4 °C and 100 V in 20 mM Tris, 150 mM glycine, 20 % (v/v) methanol. The membrane was blocked for 4–6 h at room temperature in 20 mM Tris/HCl (pH 7.5), 15 mM NaCl, 5 % (w/v) powdered milk, 0.2 % (v/v) Tween 20, and probed overnight at 4 °C with a 1 in 2000 dilution of anti-CPB serum in 20 mM Tris/HCl (pH 7.5), 15 mM NaCl, 1 % (w/v) powdered milk, 0.1 % (v/v) Tween 20. All subsequent steps were at room temperature. Membranes were washed for 2 h with four changes of wash buffer [20 mM Tris/HCl (pH 7.5), 15 mM NaCl, 1 % (w/v) powdered milk] and probed for 2 h with a 1 in 2000 dilution of donkey anti-rabbit horseradish peroxidase-linked antiserum (Scottish Antibody Production Unit, Carluke, Lanarkshire, U.K.), in 20 mM Tris/HCl (pH 7.5), 150 mM NaCl, 1 % (w/v) powdered milk. Unbound secondary antibody was removed by washing with wash buffer for 1.5 h followed by a 30 min wash in 20 mM Tris/HCl, pH 7.5, 150 mM NaCl. Detection used a chemiluminescent substrate (Pierce). Polyclonal antiserum raised in New Zealand White rabbits was against His_6-Pro-CPB2.8ΔCTE purified in this study by nickel-agarose affinity chromatography. A 300 μg portion of protein was homogenized

with Freund's complete adjuvant for the first inoculation, and 200 μg with Freund's incomplete adjuvant for three subsequent boosts. Antiserum was collected 5 days after the final boost.

RESULTS

Expression, purification and refolding of CPB2.8ΔCTE in *E. coli*

Expression of CPB2.8ΔCTE in *E. coli* resulted in an abundant protein of approx. M_r 38000 (Figure 1A, lane 2). This corresponds well with the predicted M_r of 37100 for the zymogen form of CPB2.8ΔCTE with an additional 930 M_r contributed by the His_6-tag. 90% of the expressed protein was associated with inclusion bodies and 10% with the soluble phase (Figure 1B, lanes 1–3). CPB2.8ΔCTE was expressed as a His_6-tag fusion for efficient purification using nickel-agarose resin. $(His)_6$-CPB2.8ΔCTE was obtained using this method (Figure 1C) and employed in the production of specific antiserum. However, the preparation had very low enzyme activity and all attempts to convert the pro-form to mature, fully active enzyme were unsuccessful. The alternative method for enzyme purification and activation, based on [31], was highly successful. Triton X-100 and 2 M urea-buffered extraction of the inclusion body phase resulted in a significant increase in purity (Figure 1B, lanes

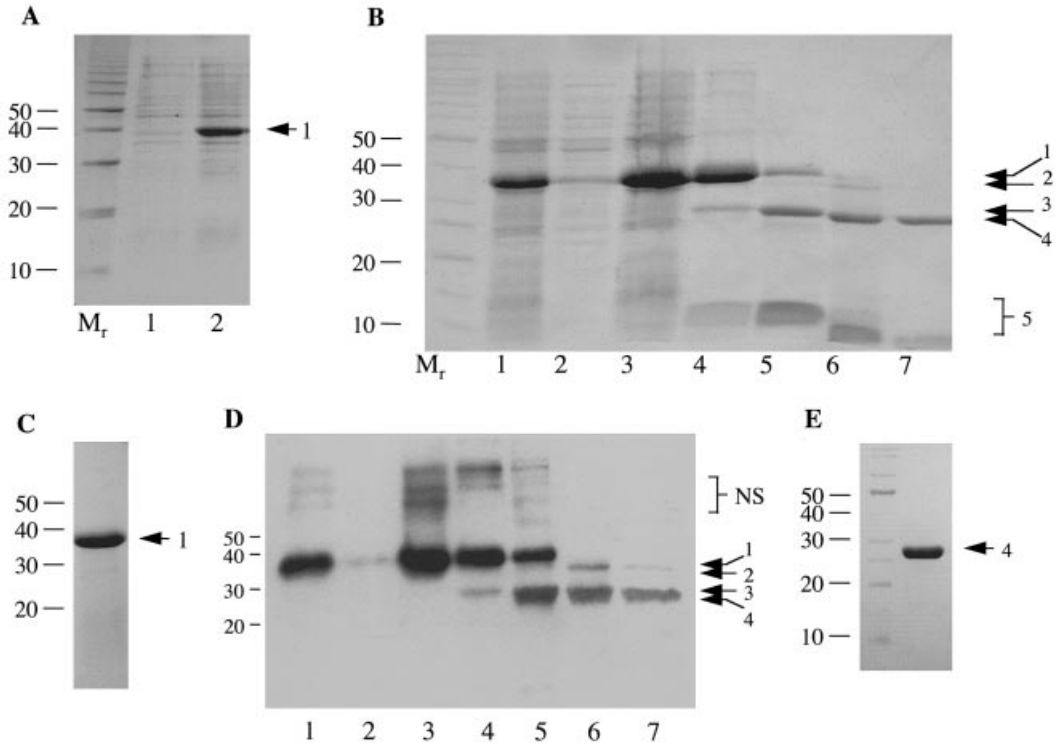

Figure 1 SDS/PAGE and Western blot analysis of the purification of CPB2.8ΔCTE from *E. coli*

(**A**) Expression of CPB2.8ΔCTE. SDS/PAGE gel stained with Coomassie Blue. Lane M_r, protein standards; lane 1, *E. coli* lysate prior to isopropyl β-D-thiogalactoside induction; lane 2, *E. coli* lysate after induction. Arrow indicates His_6-Pro-CPB2.8ΔCTE. (**B**) Purification from inclusion bodies and activation of CPB2.8ΔCTE. SDS/PAGE gel stained with Coomassie Blue. Lane M_r, protein standards; lane 1, *E. coli* whole cell lysate; lane 2, soluble fraction of *E. coli* lysate; lane 3, inclusion body fraction; lane 4, inclusion body fraction washed and solubilized in 8 M urea; lane 5, post-dialysis; lane 6, pooled Mono Q fractions; lane 7, post-activation. Arrows indicate the major, detectable forms of CPB2.8ΔCTE: 1, His_6-Pro-CPB2.8ΔCTE; 2, 34000-M_r intermediate; 3, 27000-M_r intermediate; 4, mature enzyme; 5, small peptides. (**C**) His_6-Pro-CPB2.8ΔCTE purified by nickel-agarose resin. SDS/PAGE analysis, Coomassie Blue staining, the protein is indicated by the arrow. (**D**) Western blot analysis of the same samples detailed in (**B**), using polyclonal antiserum raised against His_6-Pro-CPB2.8ΔCTE. NS, putative aggregated forms of CPB2.8ΔCTE. (**E**) Silver-stained SDS/PAGE gel of CPB2.8ΔCTE eluted from the Sephadex G50 column. $10^{-3} \times M_r$ is indicated on the left side of each panel.

Table 1 Purification summary

Purification step	Total volume (ml)	Total protein (mg)	Recovery* (%)	Activity† (nmoles · min^{-1} · ml^{-1})	Specific activity (nmoles · min^{-1} · mg^{-1})	Purification factor
Post-dialysis	105	37.3	100	4.2	11.8	1
Post-Mono Q	30	12.1	32.4	14.6	36.2	3
Post-acidification	33	6.2	16.6	353	1878	159
Post-Sephadex G50	9.2	2.5	6.7	2219	8166	692

* Based on total protein.
† Using continuous assay with BzPFRNan as substrate.

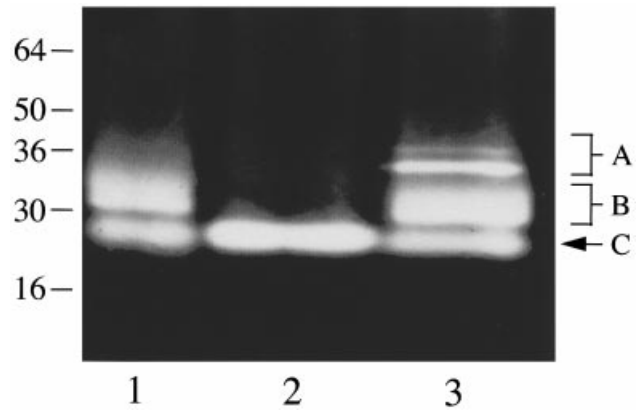

Figure 2 Gelatin-SDS/PAGE analysis of CPB2.8ΔCTE during purification

Lane 1, pooled Mono Q fractions before activation; lane 2, post-activation (see lane 7, Figure 1B); lane 3, whole cell lysate of *L. mexicana* CPB-null mutant expressing full length CPB2.8 via an episome [5,6]. Three groups of activities were apparent: A and B, processing intermediates; C, mature enzyme. $10^{-3} \times M_r$ is indicated on the left side of the Figure.

3 and 4). At this stage, the majority of CPB2.8ΔCTE was still in the pro-mature form, as judged by its relative mobility on SDS/PAGE (Figure 1B, lane 4). During dialysis, approx. 60% of the protein converted to two higher mobility forms, 27000 M_r and 15000 M_r (Figure 1B, lane 5). Both the 38000 and 27000 M_r species reacted with antiserum raised against CPB (Figure 1D, lane 5); however, the sample exhibited only low activity towards BzPFRNan (Table 1).

Subsequent to anion-exchange chromatography, the sample contained four main protein bands of 38000 M_r, 34000 M_r, 27000 M_r, and < 10000 M_r (Figure 1B, lane 6), resulting from the conversion of the 38000-M_r form to 34000 M_r and further proteolysis of the 15000-M_r band. The 34000-M_r form was also detected by Western blotting (Figure 1D). Two bands of activity were observed on gelatin gels (Figure 2, lane 1). The upper band appeared on occasions to comprise of two closely migrating activities. The specific activity of this sample towards BzPFRNan was 36.2 nmoles/min per mg protein (Table 1), reflecting enhanced purification and some activation from the Mono Q step. The Mono Q step contributed to the continued purification of CPB2.8ΔCTE, but it also appeared to be vital for complete conversion to active, mature enzyme. Attempts to activate CPB2.8ΔCTE prior to resolution on Mono Q were largely unsuccessful.

Activation of CPB2.8ΔCTE

The efficacy of the activation protocol developed for the conversion of the 38000-M_r and 34000-M_r species to the M_r 26000 mature form was confirmed by SDS/PAGE (Figure 1B, lane 7), Western blotting (Figure 1D, lane 7), gelatin SDS/PAGE (Figure 2, lane 2) and by a significant increase in activity towards BzPFRNan (Table 1). The activation was accompanied by further proteolysis of the peptides migrating at the gel front (Figure 1B, lane 7). Activation was optimal at low protein concentrations (< 0.3 mg/ml) and elevated temperatures (i.e. 37 °C) in the presence of reducing conditions and high ionic strength, and was also dependent upon acidic conditions. Complete conversion occurred within 2–4 h at 37 °C and pH 5.0–5.5, whereas at pH 7.0 none was observed within 8 h. At pH 4.0, activation proceeded rapidly, resulting in a high activity by 10 min, but the activity rapidly declined thereafter (results not shown), probably due to rapid auto-proteolysis at this low pH [37].

N-terminal amino acid analysis of the various protein bands detected using SDS/PAGE confirmed their identities and gave insight into the processing events that occurred during production of the mature recombinant enzyme (Figure 3). The N-terminal sequences showed that the 38000-M_r species was the pro-mature protein ($^{-117}$MRGSHHHHHH), the 34000-M_r species was an intermediate with part of the pro-region removed ($^{-94}$TPAAALFEEF), the 27000-M_r species (pre-Mono Q) was an intermediate with seven amino acids residues of the pro-region remaining ($^{-8}$KARADLSAVP), and the activated enzyme (approx. M_r 26000) had the same N-terminus as native CPB enzyme ($^{-1}$AVPDAVDWRE). The M_r of 26000 estimated using SDS/PAGE was higher than the predicted 23300-M_r for mature

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                                    <--helix 1-->      <-------------helix 2-
Cat L                            TLTFDHSLEAQWTKWKAMHNRLYGMN-EEGWRRAVWEKNMKMIELH
T.cruzi                          SLHAEETLTSQFAEFKQKHGRVYESAAEEAFRLSVFRENLFLARLH
T.brucei                       GSLHVEESLEMRFAAFKKKYGKVYKDAKEEAFRFRAFEENMEQAKIQ
L.mex 2.8 MRGSHHHHHHGSACAPARAIHVGTPAAALFEEFKRTYGRAYETLAEEQQRLANFERNLELMREH
          ↑                        ⇑ ↑   ⇑
          1                          2

                  β-sheet 1                          β-sheet 2
          ------>   <-->          <helix 3>            <-->
Cat L     NQEYREGKHSFTMAMNAFGDMTSEEFRQ-VMNGFQN--RKPRKGKVFQEPLFYE---APRSVDW
T.cruzi   A--AANPHATF--GVTPFSDLTREEFRSRYHNGAAHFAAAQERARVRVKVEVVG---APAAVDW
T.brucei  A--AANPYATF--GVTPFSDMTREEFRARYRNGASYFAAAQKRLPKTVNVTTGR---APAAVDW
L.mex 2.8 Q--ARNPHAQF--GITKFFDLSEAEFAARYLNGAAYFAAAKRHAAQHYRKARADLSAVPDAVDW
                                    *****                 ↑       ↑⇑
                                                          3       4
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Figure 3 Pro-region sequence alignment

The deduced amino acid sequences from human cathepsin L [45] (Cat L); *T. cruzi* recombinant enzyme cruzain [18] (T. cruzi); and *T. brucei* cysteine proteinase [44] (T. brucei) pro-regions are compared to that derived for CPB2.8ΔCTE [5] (L. mex 2.8.). The amino acid sequence surrounding the His_6-tag is given as this deviates from the gene sequence of the native gene [6]. Residues identical across all four species are shown in bold. The sites of pro-region processing are shown by arrows (numbered ↑ for *L. mexicana* CPB2.8, this paper; ⇑ for cruzain [11]). The positions of secondary structure for cathepsin L are also shown [21]. Asterisks indicate the YHNGA motif. Spaces are included to allow maximum alignment.

Table 2 Kinetic parameters for recombinant enzymes using BzPFRNan

Enzyme	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1} \cdot M^{-1}$)
CPB2.8ΔCTE	83	17.8†	2.2×10^5
Cruzain*	89	3.2	3.6×10^4

* Data from [18].
† Assuming an M_r of 23300; functional molarity determined by active-site titration using E-64.

CPB2.8ΔCTE, although the sample co-migrated on gelatin SDS/PAGE gels (Figure 2) and Western blots (results not shown) with the native enzyme from *L. mexicana* promastigotes.

The final step in the purification of CPB2.8ΔCTE involved the removal by gel filtration of small peptides (presumably derived from the pro-region). This resulted in a further increase in specific activity (Table 1). Silver- and Coomassie-stained SDS/PAGE revealed a single band of approx. M_r 26000, indicative of a homogenous preparation (Figure 1E).

Characterization of CPB2.8ΔCTE activity

Leupeptin, antipain and E-64 were effective inhibitors of the recombinant mature CPB2.8ΔCTE, whereas inhibitors of aspartic proteinases (pepstatin) and serine proteinases (PMSF) had no effect (results not shown). Active site titration using E-64 and BzPFRNan indicated that approx. 30% of the purified recombinant protein (determined using the Bio-Rad protein assay) was active. Thus the procedure yielded 3.8 mg of functionally active enzyme per litre of *E. coli* culture. The kinetic parameters for the recombinant enzyme are detailed in Table 2. The k_{cat} and k_{cat}/K_m values were similar to those reported for recombinant mammalian cathepsin B [31] and cruzain [18] purified from inclusion bodies (Table 2).

DISCUSSION

A major cysteine proteinase of *L. mexicana*, CPB2.8, has been successfully overexpressed in *E. coli* and more than 3.5 mg of highly pure, active enzyme routinely purified from 1 litre of *E. coli* culture using a protocol developed in this study. The proportion of active enzyme recovered (approx. 30%) reflects the relative difficulty in successfully refolding and re-naturing an insoluble disulphide-containing protein [38]. However, as the colour development may vary between the protein standards used (BSA and bovine gamma globulin) and CPB2.8, this may represent an over- or under-estimation of the yield. To facilitate the refolding of the recombinant CPB, the C-terminal extension was omitted, which reduced the cysteine content of the protein by approx. 50%. Previous attempts to express a full length CPB isoenzyme resulted in inactive enzyme, probably due to misfolding [39]. Evidence suggests that the C-terminal domain is not essential for enzyme activity of the related cysteine proteinases of trypanosomes [11,12] or CPB1 of *L. mexicana*, which has a truncated C-terminal extension of 15 amino acids but is active *in vivo* [6].

Recombinant, mature CPB2.8 was over 20-fold more active than a mixture of native CPB enzymes purified from the parasite (which had a specific activity of 360 nmoles/min per mg protein towards the same substrate [34]). Kinetic analyses of CPB2.8ΔCTE and cruzain of *T. cruzi* [18] revealed similar K_m, k_{cat} and k_{cat}/K_m values, confirming the recovery of significant enzyme activity following refolding from inclusion bodies.

Production of the mature form of the recombinant CPB involved several hydrolytic events that resulted in removal of the full pro-domain. The sites of the cleavages differed from those reported for cruzain of *T. cruzi* (Figure 3). Comparison of the pro-region amino acid sequences of CPB2.8ΔCTE, cathepsin L, cruzain and a similar cysteine proteinase of *T. brucei* (Figure 3), and taking into account the known three-dimensional structures of cathepsin L and cruzain [20,40], reveals that the sites of CPB2.8ΔCTE processing appear to be situated in accessible regions between the secondary structure [21]; at the N-terminus of helix 1 (site 2) and the C-terminus of β-sheet 2 (site 3). Notably, cruzain is also processed at two sites near helix 1 [11].

Recently, the inhibitory activity of the pro-region of two trypanosome homologues of CPB was found to reside in a YHNGA motif [23]. CPB2.8ΔCTE exhibits strong homology with the trypanosome enzymes in this region, so it is likely that this sequence will have similar inhibitory properties. The 34000-M_r processing intermediate retains the YHNGA motif, which correlates with this form's lack of enzyme activity towards BzPFRNan. In contrast, both the mature enzyme and the 27000-M_r intermediate have lost this sequence. Nevertheless, the 27000-M_r intermediate exhibits only low proteinase activity. It is unlikely that the remaining seven residues of the pro-region are inhibiting the active site, as structural studies in related cysteine proteinases indicated that there could be no involvement in active site inactivation by the extreme C-terminus of the pro-region [19,20,41]. It is more likely that clipped pro-region peptides remained attached to this form before electrophoresis and so inhibited it. Isolated pro-peptides can inhibit mature enzyme and such binding would be likely to be strong at the neutral pH and low ionic strength of the dialysis buffers [19,41–43]. These associations are disrupted during SDS/PAGE, but analysis by native PAGE revealed several slow mobility protein species, none of which co-migrated with fully activated CPB2.8ΔCTE (results not shown), consistent with non-covalent interaction between processing intermediates and pro-region peptides. The benefits of the Mono Q step probably resulted from it being a positively charged resin, which could compete with, and thereby remove, some positively charged pro-region peptides associating with the mature enzyme. Spontaneous activation of human procathepsin L also followed purification on Mono Q resin or incubation with negatively charged dextran sulphate, which was thought to induce a small conformational change in the positively charged pro-region [37]. Stimulation in enzyme activity during the acidification step would result from the dissociation of such clipped pro-region peptides.

The production of recombinant forms of individual CPB isoenzymes from the *CPB* array makes possible a range of investigations of this important class of parasite enzymes not previously possible owing to difficulties in purification and the presence of multiple isoforms in the parasite. Structural and immunological studies using the recombinant enzyme are now underway.

S.J.S. is funded by a European Union International Cooperation Programme grant, ERB3514PL961411, K.G.J.P. is funded by a Medical Research Council studentship, and J.D.H. is funded by a University of Glasgow scholarship. J.C.M. is a Medical Research Council Senior Fellow. We thank James H. McKerrow for suggestions on the activation protocols. N-terminal amino acid sequence analysis was carried out by Dr. B. Dunbar at the Proteomics Unit, Department of Molecular & Cell Biology, University of Aberdeen.

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Received 30 April 1999/13 December 1999; accepted 25 January 2000