Purification and Characterization of an Immunogenic Aminopeptidase of Brucella melitensis

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An immunogenic aminopeptidase was purified from Brucella melitensis strain VTRM1. The purification procedure consisted of ammonium sulfate fractionation and three chromatographic steps. This procedure resulted in a yield of 29% and a 144-fold increase in specific activity. The aminopeptidase appeared to be a monomeric enzyme with a molecular mass of 96 kDa and an isoelectric point of 4.8. Its activity was optimal at pH 7.0 at 40°C. The enzyme was strongly inhibited by EDTA, 1,10-phenanthroline, and divalent cations (Zn2+ and Hg2+), suggesting that this protein was a metalloaminopeptidase. The enzyme showed preference for alanine at the N termini of aminoacyl derivatives. The \( K_m \) values for L-alanine-\( p \)-nitroanilide (Ala-\( p \)-NA) and Lys-\( p \)-NA were 0.35 and 0.18 \( \mu \)M, respectively. The N-terminal sequence of aminopeptidase was used for a homologous search in the genomes of \( B. \) melitensis 16M and \( B. \) suis 1330. The analysis revealed an exact match of the probe sequence (36 bp) with an open reading frame of 2,652 bp encoding a protein predicted to be alanine aminopeptidase (aminopeptidase N). Collectively, these data suggest designation of the \( B. \) melitensis enzyme as an aminopeptidase N. The aminopeptidase was recognized by sera from patients with acute and chronic brucellosis, suggesting that the enzyme may have important diagnostic implications.

Brucellosis is a major problem in the Mediterranean region and parts of Asia, Africa, and Latin America, where it remains endemic, generating severe economic losses (4). Bovine and caprine brucellosis caused by \( B. \) abortus and \( B. \) melitensis, respectively, are characterized by late-term abortions in pregnant females (4). In Mexico, brucellosis, mainly produced by \( B. \) melitensis, is one of the most important zoonoses causing severe morbidity in humans. The infection in humans is commonly acquired by drinking unpasteurized milk or eating milk by-products derived from infected goats or cows (15). The disease is characterized by recurrent high fever, headache, cachexia, lethargy, arthritis, and splenomegaly. The genus \( B. \) melitensis consists of 6 recognized species classified as facultative intracellular pathogens. They are able to invade macrophages, adapt to the acidic environment, and multiply in the vacuolar compartments (27). Survival inside host cells allows the bacteria to evade the host’s protective humoral immune mechanisms, such as those mediated by specific antibodies and complement. The macrophage subjects the bacteria to a harsh intracellular environment characterized by reactive oxygen intermediates, low pH, and decreased iron availability. Ironically, it has been shown that this environment, especially its acidity, activates \( B. \) melitensis genes coding for products essential for intracellular survival in this niche (28).

The induction of \( B. \) melitensis heat shock proteins has been involved in adaptive responses to adverse environmental conditions (28). Some of these proteins such as \( B. \) melitensis DnaK (11), HtrA (7), and Lon have been studied in detail (29). The Lon protein is an ATP-dependent protease (19), and in \( B. \) abortus, it functions as a stress response protease required during the initial stages of infection in the mouse model (29).

Intracellular proteolytic degradation is important in bacteria for the elimination of damaged proteins, modulation of protein levels, and maintenance of amino acid pools. Proteolytic enzymes represent one of the best-studied and understood classes of proteins. For many years it was accepted that proteolytic enzymes functioned primarily in the acquisition of nutrients for growth and proliferation through the degradation of host tissues (3). However, recent observations indicated that pathogen-derived proteolytic enzymes can also play important roles in the regulation of critical host processes in order for the invading microbes to survive in a hostile host environment (16).

Aminopeptidases (APEs) of many bacteria have been studied with some detail both structurally and enzymatically (2, 17, 31). On the other hand, it has been observed that interleukin-2 and gamma interferon are rapidly inactivated and degraded by proteases from \( P. \) aeruginosa and \( L. \) pneumophila (10, 24). Some specific proteases have been shown to be related to severe periodontitis and cardiovascular disease (21). It has been suggested that extracellular enzymes of \( A. \) spp. play an important role in invasiveness and in establishment of the infection (14). Limited studies have been carried out with \( B. \) melitensis along these lines.

In this study, we describe the purification and characterization of an immunogenic APE obtained from \( B. \) melitensis. The
properties of the purified enzyme are discussed in relation to those of previously characterized APEs. To our knowledge, this is the first report of the purification of an APE from \textit{B. melitensis}. 

\section*{MATERIALS AND METHODS}

\textbf{Bacterial strains and growth conditions.} \textit{B. melitensis} 16M was kindly donated by Central Veterinary Laboratory (New Haw, Weybridge, United Kingdom), and the \textit{B. melitensis} rough mutant VTRM1 was obtained in 1995 from the \textit{Brucella} culture collection at Virginia Tech (S. M. Boyle). Strain VTRM1 was obtained by inserting a Tn5 element in the \textit{wcoA} gene of strain 16M (35). The strains were grown on Trypticase soy agar (Difco) supplemented with 0.5\% (wt/vol) yeast extract (Difco) (TSA-Y) or Trypticase soy broth (Difco) supplemented with 0.5\% (wt/vol) yeast extract (Difco) (TSA-B) at 37°C for 30 h.

\textbf{Preparation of cell extract.} The growth of two 500-ml (TSA-B) batch cultures of \textit{B. melitensis} 16M and \textit{B. melitensis} VTRM1 was monitored every 2 h to establish a growth curve, and enzyme activity detection was performed at 6-h intervals. Cultures at different growth stages were subjected to the following fractionation procedure. The cells were harvested by centrifugation (10,000 \times g, 30 min) at 4°C, washed twice with 10 mM Tris-HCl (pH 7.0), and resuspended in the same buffer. Glass beads (0.1-mm diameter) were added, and the suspension was stirred with a magnetic stirrer for 5 h at 4°C. Unbroken cells were removed by centrifugation (10,000 \times g, 30 min), and the opalescent supernatant was subjected to ultracentrifugation (100,000 \times g, 120 min), yielding a pellet (PE) containing insoluble bacterial components and a supernatant that was considered a membrane-free bacterial extract (MFBE). All fractions, cell-free culture medium (CM), PE, and MFBE, were assayed for APE activity with \textit{l-lysine-\textit{p}}-nitroanilide (Lys-\textit{p}NA) as the substrate.

\textbf{Enzyme purification.} As both strains exhibited APE activity, the purification was performed with the \textit{B. melitensis} rough mutant VTRM1 strain to eliminate smooth lipopolysaccharide contamination. The strain was grown on TSA-Y plates at 37°C for 30 h. Bacterial cells were resuspended in Tris-HCl (pH 7.0), and the extract was obtained as described above. Solid ammonium sulfate was used to precipitate proteins between 40 and 70% saturation at 4°C; the insoluble proteins were collected by centrifugation (15,000 \times g, 30 min), dissolved in 10 mM imidazole buffer (pH 7.0), and extensively dialyzed against the same buffer (overnight at 4°C). The dialyzed fraction was clarified by centrifugation (15,000 \times g, 30 min) and then was filtered through a 0.22-\textmu m-pore-size membrane. The sample was applied onto an XK50 column packed with high-performance Q protein liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden). Gel filtration. APE activity was assayed by measuring the hydrolysis of Lys-\textit{p}NA (Sigma, St. Louis, Mo.). The incubation mixture consisted of 30 \mu l of substrate (10 mM), 250 \mu l of 100 mM Tris-HCl buffer (pH 7.0), 120 \mu l of water, and 100 \mu l of enzymatic samples. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 500 \mu l of ZnSO4 (5\%) and 100 \mu l of BaCl2 (7.5\%). The mixture was centrifuged (10,000 \times g for 10 min), and absorbance of the released p-nitroaniline was determined at 405 nm in the clear supernatant. One unit of APE activity was defined as the amount of enzyme that liberates 1 \textmu mol of p-nitroaniline per minute at 37°C. The molar absorption coefficient for 4-nitroaniline at 405 nm is \epsilon_{405 \text{nm}} = 9,900 M^{-1} cm^{-1}, and this value was used for the calculation of enzyme activity.

\textbf{Enzyme activity assay.} Protein concentrations were determined with a Bradford reagent kit (Sigma) by following the manufacturer’s protocol. Gel electrophoresis. The sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) system developed by Laemmli (13) was used to monitor enzyme purification and estimate the enzyme’s molecular mass. Gels were stained with Coomassie blue G250. A wide molecular marker kit (20,000 to 14,000 kDa; Sigma) was used as a standard.

\textbf{Amino acid sequencing.} For amino-terminal sequence analysis, the protein band resolved by SDS-PAGE was electrotransferred to a polyvinylidene difluoride membrane (18) and the N-terminal sequence was determined by automated Edman degradation on an Applied Biosystems Procise model 494 protein sequence (Biomolecular Research Facility, University of Virginia Health System). The amino acid sequence obtained was compared with available sequences at the website of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) by using the PSI-BLAST program (1). Hydrophobic regions and motif sequences were analyzed with the Prosite program (9).

\textbf{Enzyme characterization.} (i) Molecular mass measurement. The relative molecular mass was estimated by fast protein liquid chromatography gel filtration and SDS-PAGE. Gel filtration (mean of four determinations) was executed on a Superose 12 HR 10/30 column (Amersham Pharmacia Biotech) calibrated with the following standard markers (molecular masses are given in parentheses): thyroglobulin (670 kDa), gamma globulin (158 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), and vitamin B12 (1.3 kDa) (kit from Bio-Rad). Equilibrium and elution (0.2 ml/min) were performed with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl.

(ii) Determination of pH. The isoelectric point was determined by chromatofocusing on a MonoP column equilibrated with 25 mM imidazole (pH 7.4) by using a pH gradient from 7.0 to 4.0 (Polybuffer 74; Amersham Pharmacia Biotech).

(iii) Effect of temperature and pH dependence on APE. The effect of pH on APE activity was examined by using McIlvaine buffers at pH values ranging from 3.0 to 7.0, 100 mM Tris-HCl at pH values from 7.0 to 9.0, and 100 mM carbonate-bicarbonate buffer at pH values from 9.0 to 11.0. The pH stability of the APE was tested by incubation of the purified enzyme for 12 h at 4°C in each buffer followed by the standard enzyme activity assay. The optimal temperature for enzyme activity was determined in 100 mM Tris-HCl buffer (pH 7.0) in the range of 4 to 70°C. The temperature stability was tested by incubation of the enzyme at 4, 10, 20, 30, 40, 50, 60, and 70°C for 30 min in 50 mM Tris-HCl buffer, pH 7.0, followed by the standard enzyme activity assay. The protein was subjected to several cycles of freezing and thawing, and the activity was measured every month. In every case, activity was expressed as a percentage of the activity obtained at either the optimal pH or the optimal temperature.

(iv) Determination of kinetic parameters. Kinetics constants of the purified enzyme were estimated for Lys-\textit{p}NA and Ala-\textit{p}NA, with substrate concentrations ranging from 0.01 to 1.0 mM. Activity was measured under the same conditions as described above and expressed as the mean of three different determinations. The \textit{k}_{\text{cat}} and \textit{V}_{\text{max}} were obtained from a Michaelis-Menten plot.

(v) Effect of protease inhibitors and metal cations. The protease inhibitors bestatin (250 \mu M), pepstatin (250 \mu M), leupeptin (50 \mu M), pefabloc (5 \mu M), 1,10-phenanthroline (10 \mu M), EDTA (2.5 mM), phenylmethylsulfon fluoride (PMSF) (5 \mu M), and trans-epoxysuccinyl-l-leucylamido-(4-guanidino) butane (E64) (50 \mu M) and metal cations ZnCl2 (1 mM), CoCl2 (1 mM), CaCl2 (1 mM), HgCl2 (1 mM), and MgCl2 (1 mM) were added to the enzyme solution. The purified enzyme was incubated in presence of each inhibitor for 30 min followed by the standard enzyme assay with Lys-\textit{p}NA as the substrate. Activity was expressed as a percentage of the activity obtained in the absence of the added inhibitor.

\textbf{Substrate specificity.} The relative activities of the APE from \textit{B. melitensis} against several aminocyclo-PNA (Glup-PNA, Ala-PNA, Leup-PNA, Gly-PNA, Met-PNA, Lys-PNA, and Proy-PNA) and dipeptidyl-PNA (Ala-Pro-PNA, Gly-Phe-PNA, Ala-Ala-PNA, and Ala-Phe-PNA) (Bachem) substrates were determined by the standard activity assay. Relative activity was expressed as a percentage of the rate of hydrolysis of Ala-PNA which was assigned a value of 100%.

Western blotting. Immunoblotting was carried out by using the method of Towbin et al. (33). A prestained molecular marker kit (Gibco BRL) was used as a standard.

\textbf{Human sera.} Sera from 20 patients with brucellosis at different stages of the disease belonging to our serum collection were used. The samples were selected according to the following criteria. Ten had acute brucellosis as determined by the clinical picture of brucellosis: an agglutination titer equal to or higher than 1:80 and positive 2-mercaptoethanol agglutination. Also, sera from 50 volunteers, without any previous history of brucellosis, and negative to standard and 2-mercaptoethanol agglutination tests were included.
Preparation of anti-APE serum. Two mice were injected intraperitoneally with 20 μg of purified enzyme emulsified with complete Freund adjuvant and reinjected with 20 μg of mixed with incomplete Freund adjuvant 3 weeks later. The mice were bled before the first injection (preimmune serum) and 1 week after the booster (anti-APE serum) to obtain immune serum.

RESULTS

Purification of APE activity. Preliminary experiments indicated the presence of an intracellular Lys-pNA activity in MFBE from both B. melitensis 16M and B. melitensis rough mutant VTRM1. Results of enzymatic activity detection in different fractions of the B. melitensis rough mutant VTRM1 indicated that the majority of this enzyme was found in the MFBE while some remaining activity was found associated with PE; no activity was found in CM alone (Fig. 1). Similar results were obtained with B. melitensis 16M strain (data not shown). Detection of free (MFBE) and cell-associated (PE) APE activity did not occur until the early stationary phase. A substantial increase in activity occurred after 30 h of incubation (Fig. 1). The ammonium sulfate precipitation (40 to 70%) and the subsequent chromatography steps (Fig. 2) resulted in the final isolation of a pure enzyme. The APE was purified 144-fold with a recovery of 29% of the enzyme activity (Table 1).

Molecular mass. SDS-PAGE analysis of the purified enzyme revealed the presence of a single protein band with an apparent molecular mass of 96,000 Da (designated APE97) (Fig. 3). The molecular mass of the native enzyme obtained by gel filtration was 97,000 Da. Similarities in both results suggested that the purified enzyme was a monomer.

Isoelectric point determination. The isoelectric point was estimated to be 4.8 by isoelectric chromatofocusing.

Effects of pH and temperature. Using the APE activity assay with Lys-pNA as a substrate, it was found that the purified enzyme showed an optimal activity at pH 7.0. The enzyme was stable in the pH range of 6.0 to 8.5. APE97 had no activity at a pH below 6.0 or over 9.0. The activity of the purified enzyme was optimal at 40°C. Enzyme activity decreased significantly at temperatures over 50°C, although around 15% of the activity was observed at 60°C. The thermal stability results showed that the enzyme was inactivated >95% after incubation for 30 min at 50°C. We also determined that APE97 was resistant to several cycles of freezing and thawing. The enzyme showed no appreciable loss of activity when kept frozen at −70°C for at least 6 months, whereas it retained approximately 50% of its original activity when kept at −20°C over the same period of time.

Effect of inhibitors on enzyme activity. The influences of several agents on the activity of APE97 are summarized in Table 2. The activity of APE97 was almost completely inhibited in the presence of the chelating agents 1,10-phenanthroline (10 μM) and EDTA (2.5 mM). Bestatin, a typical inhibitor of exopeptidases, inhibited more than 50% the activity. APE97 was insensitive to serine-protease inhibitors such as leupeptin, pefabloc, and PMSF. E64, an inhibitor of cysteine proteinases
and the aspartate-specific inhibitor pepstatin, had no effect on the activity of APE_{97}. The presence of Zn^{2+} and Hg^{2+} caused complete inhibition at 1.0 mM while slight effects (10 to 15% inhibition) were observed in the presence of Ca^{2+}, Mg^{2+}, and Co^{2+} (Table 2). Concentrations of ammonium sulfate, used in the purification procedure, had no inhibitory effect on APE_{97} activity.

**Substrate specificity.** The specificity of the enzyme towards the N-terminal residue was tested with several aminoacyl-pNA and dipeptidyl-pNA substrates. APE_{97} was able to release the hydrophobic amino acids Ala (100%), Leu (25.5%), and Met (18.5%), the uncharged amino acid Gly (13.5%), and the hydrophilic amino acid Lys (50%). Ala-pNA was most actively hydrolyzed, whereas Lys-pNA, Leu-pNA, Met-pNA, and Gly-pNA were hydrolyzed at lower rates. The acid derivative Glu-pNA showed no detectable reactivity with APE_{97}. Weak activity was detected against Ala-Pro-pNA (1.5%), and the other dipeptidyl substrates were not hydrolyzed.

**Kinetics parameters.** The $K_m$ and $V_{max}$ were 0.35 mM and 45 $\mu$mol min$^{-1}$ mg of protein$^{-1}$, respectively, for Ala-pNA and 0.18 mM and 6.1 $\mu$mol min$^{-1}$ mg of protein$^{-1}$, respectively, for Lys-pNA.

### Table 1. Purification of APE_{97} from B. melitensis

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Amt of protein (mg)</th>
<th>Total activity (U)</th>
<th>Sp act (U/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>901.0</td>
<td>998.6</td>
<td>1.1</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Ammonium sulfate (40–70%)</td>
<td>542.1</td>
<td>773.3</td>
<td>1.4</td>
<td>77.0</td>
<td>1.3</td>
</tr>
<tr>
<td>High-performance Q Sepharose</td>
<td>40.0</td>
<td>502.0</td>
<td>12.5</td>
<td>50.0</td>
<td>11.3</td>
</tr>
<tr>
<td>Phenyl Sepharose</td>
<td>4.2</td>
<td>411.2</td>
<td>98.0</td>
<td>41.1</td>
<td>89.0</td>
</tr>
<tr>
<td>Superose 12HR</td>
<td>1.8</td>
<td>286.8</td>
<td>159.0</td>
<td>29.0</td>
<td>144.5</td>
</tr>
</tbody>
</table>

**FIG. 3.** SDS-PAGE of purified APE. Electrophoresis was performed on an SDS-polyacrylamide gel (10%). Lane 1, molecular mass markers; lane 2, purified APE (5 $\mu$g). The molecular mass markers were as follows: myosin, 205 kDa; beta-galactosidase, 116 kDa; phosphorylase b, 97 kDa; fructose-6-phosphate kinase, 84 kDa; albumin, 66 kDa; glutamic dehydrogenase, 55 kDa; ovalbumin, 45 kDa; glyceraldehyde-3-phosphate dehydrogenase, 36 kDa; carbonic anhydrase, 30 kDa; trypsinogen, 24 kDa; trypsin inhibitor, 20 kDa; alfa-lactalbumin, 14 kDa; aprotinin, 6.5 kDa.

### Table 2. Effect of proteinase inhibitors and divalent cations on the activity of B. melitensis APE_{97}

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Conc</th>
<th>Relative activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>None</td>
<td>100</td>
</tr>
<tr>
<td>Zn^{2+}</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>Co^{2+}</td>
<td>1 mM</td>
<td>93</td>
</tr>
<tr>
<td>Mg^{2+}</td>
<td>1 mM</td>
<td>86</td>
</tr>
<tr>
<td>Hg^{2+}</td>
<td>1 mM</td>
<td>0</td>
</tr>
<tr>
<td>EDTA</td>
<td>2.5 mM</td>
<td>0</td>
</tr>
<tr>
<td>Pefabloc</td>
<td>5 $\mu$M</td>
<td>60</td>
</tr>
<tr>
<td>Bestatin</td>
<td>250 $\mu$M</td>
<td>45</td>
</tr>
<tr>
<td>Pepstatin</td>
<td>250 $\mu$M</td>
<td>90</td>
</tr>
<tr>
<td>Leupeptin</td>
<td>50 $\mu$M</td>
<td>100</td>
</tr>
<tr>
<td>PMSF</td>
<td>5 $\mu$M</td>
<td>94</td>
</tr>
<tr>
<td>E-64</td>
<td>50 $\mu$M</td>
<td>90</td>
</tr>
<tr>
<td>1,10-Phenanthroline</td>
<td>10 $\mu$M</td>
<td>20</td>
</tr>
</tbody>
</table>

**N-terminal amino acid sequencing.** The N-terminal sequence was determined to be MRTEHTFRLE. This sequence was then used as a probe to search for the complete sequence of the APE gene in the genome bank of B. melitensis 16M (5). The results revealed an exact match of this sequence with a potential open reading frame of 2,652 bp (GenBank accession number NP_540241). This gene codes for a single protein consisting of 884 amino acid residues and predicted to be an alanyl APE (PepN). The apparent molecular mass determined for APE_{97}, based on its migration position in the SDS gel, was close to the theoretical value of 98,048 Da calculated for the pepN gene product based on its amino acid composition.

**Sequence comparison.** The comparison obtained by using the Psi-BLAST program showed that the deduced primary structure of the purified APE from B. melitensis was extremely similar (99%) to the putative APE N from B. suis 1330 (GenBank accession number NP_687531). Lower identities were observed with the putative APE N from Mesorhizobium loti (63%; GenBank accession number NP_107963), a putative APE N from Sinorhizobium meliloti (59%; GenBank accession number NP_385132), and a putative APE N from Agrobacterium tumefaciens (58%; GenBank accession number NP_354008).

**Reactivity of human sera.** To establish the ability of this protein to induce a specific antibody response in humans, Western blot analysis was performed. Using human sera from patients with acute and chronic brucellosis and sera from healthy individuals, it was demonstrated that patients with brucellosis responded to the protein while healthy individuals were seronegative (Fig. 4). We used a mouse anti-APE serum as a positive control.

**DISCUSSION**

The role of proteases from several bacteria has been the focus of many studies in bacterial pathogenesis (10, 21, 24). APEs belong to the MA1 family, which forms part of the MA clan of metalloproteases (3). APEs occur in a wide variety of microbial species, including bacteria and fungi, act on a free N-terminal amino acid of a polypeptide chain, and release a single amino acid residue (3). The APEs of *Escherichia coli* as...
The peak enzyme production occurred at the early stationary growth phase (30 h). The APE activity was found predominantly in a bacterial cell extract, although some residual activity was found associated with Brucella membranes (PE) but was considered a product of contamination. Immunogold labeling of Lactococcus lactis cells with antibodies against APE N strongly supported an intracellular location (34); this seems to be the case for APE$_{97}$ from Brucella. Purification of B. melitensis APE from strain VTRM1 was achieved by ammonium sulfate fractionation and three consecutive chromatographic steps. The enzymatic activity remained stable during the purification process and was not inactivated by ammonium sulfate. The native enzyme obtained by gel filtration chromatography showed a peak activity with an apparent molecular mass of 97,000 Da. Results from the SDS-PAGE gel revealed a single protein band with an apparent molecular mass of 96,000 Da and implied that the purified enzyme was a monomer.

APE$_{97}$ shared several features in common with APEs purified from other bacteria. The molecular mass of the purified enzyme was similar to those described for Streptococcus salivarius (98 kDa) (22), L. lactis (95 kDa) (8), and E. coli K-12 (87 kDa) (20). APE$_{97}$ exhibited a pI of 4.8, which is similar to the pI of 4.6 reported for the APE N of E. coli K-12 (20) and to the pI of 4.5 reported for the APE N of L. lactis (8). In order to further characterize the enzymatic activity, various inhibitors were tested. The activity of APE$_{97}$ was almost completely inhibited in the presence of chelators, 1,10-phenanthroline, and EDTA. APE$_{97}$ was insensitive to serine-protease inhibitors such as leupeptin, pefabloc, and PMSF. E64, an inhibitor of cysteine proteinases and the aspartate-specific inhibitor pepstatin, had no effect on the activity of APE$_{97}$. Zn$^{2+}$ and Hg$^{2+}$ caused complete inhibition of APE$_{97}$. Inhibition by the metal ion of APE$_{97}$ has been reported for S. salivarius (22) and L. lactis (8). Altogether, these results strongly suggest that APE$_{97}$ is a metallo-APE.

APE$_{97}$ showed optimal activity at 40°C, a temperature similar to that found for other bacterial APE N’s (PepN) (8, 22, 36). APE$_{97}$ showed narrow pH requirements, as the enzyme was stable at a pH range of 6.0 to 8.0, with maximal activity at pH 7.0. This is consistent with bacterial PepN, which in general has a pH optimum between 6.5 and 7.5 (3). Bacterial PepN is capable of hydrolyzing a broad range of peptides, removing the N-terminal amino acid. APE$_{97}$ released a single amino acid and was able to cleave a variety of N-terminal amino acids from pNA derivatives. In decreasing order of preference, they were Ala, Lys, Leu, and Met; the enzyme was not able to liberate either Glu or dipeptidyl substrates. The profile of these relative activities within a range of pNA derivatives was therefore similar to those reported for the APE N from E. coli K-12, S. salivarius, and L. lactis (8, 22, 36). In general, APE$_{97}$ showed a strong preference for bonds involving the hydrophobic amino acid Ala and hydrophilic Lys N-terminal aminoacyl residues, a relatively high activity toward hydrophobic amino acids Leu and Met, and very little activity toward glutamyl residues.

The N-terminal sequence obtained from the APE served as a probe to search for the complete DNA sequence of the APE gene in the genome bank of B. melitensis 16M (5). The results revealed an exact match of this sequence with a potential open reading frame of 2,652 bp. The identified gene codifies a single protein consisting of 884 amino acid residues, which is predicted to be an alanyl APE. This homology, in addition to our experimental results on molecular mass and biochemical and enzymatic properties, appears sufficient to warrant designation of APE$_{97}$ as an APE N.

A high similarity between the APE$_{97}$ amino acid sequence and the putative PepN APE from B. suis 1330 was found. Lower similarities were observed with the putative PepN APE from M. loti, S. meliloti, and A. tumefaciens, all of which are phylogenetically related to Brucella (25).

The databanks currently assign the sequences of bacterial alanyl APE to EC 3.4.11.2, for which the recommended name is membrane alanine APE. However, this appears inappropriate, since the bacterial sequences contain no transmembrane or membrane-associated helices or any hydrophobic segments likely to be part of a signal peptide. In contrast, many of the mammalian APE N’s, which are thought to play important roles in the hydrolysis of peptides, are membrane-associated glycoproteins (3). We did not identify hydrophobic segments in the sequence of the putative APE N from B. melitensis M16; on the other hand, it contains the characteristic catalytic site of metallopeptidases in which the zinc ligands are the two histidines in the His-Glu-Xaa-Xaa-His (HEXXH) motif (3).

Bacterial PepN plays an essential role in bacterial metabolism and catabolism, since it may be involved in nitrogen supply and in the degradation of intracellular peptides generated by protein breakdown during normal growth as well as in response to nutrient starvation (3). The functions of five intracellular peptidases, PepN, PepC, PepX, PepT, and PepO, involved in the degradation of oligopeptides in L. lactis have been investigated. Mutations in all of these genes led to slower growth rates in milk relative to the wild-type strain (23). Further...
thermore, a single mutation of PepN leads to a significant decrease in the growth rate, thus PepN seems to play a more prominent role than do the other proteases (23). Some studies have revealed that environmental stress (e.g., heat, oxidative, and acid shock) rapidly modified gene expression and the corresponding synthesis of proteins in *Brucella*; these proteins may be related to resistance to live intramacrophagic *brucellae* (28, 32). In prokaryotes, most ATP-dependent ClpP proteases are involved in protein catabolism under both optimal and stress conditions (26). As heat shock leads to severe downregulation of ClpP expression in *B. melitensis*, it is possible that alternative proteases may compensate for its absence. In this context, the role of APE$_p$, and other intracellular enzymes may be involved in the degradation (turnover) and the de novo synthesis of proteins under stress conditions in brucellae. A phenotypic evaluation of an isogenic *B. melitensis* pepN mutant could shed some light on the mouse infection process and its possible participation in eliciting protective immunity.

The use of metalloproteases in the diagnosis of infectious diseases has been reported, as in the case for systemic candidiasis (6). In the last few years, several immunoreactive *Brucella* proteins have been identified (12, 30). Nevertheless, there are no reports regarding the antigenic properties of any APE in *Brucella*. In order to assess whether the APE$_p$ of *B. melitensis* is immunoreactive, we determined whether patient serum reacted with the protein. Sera from patients with acute and cross-reactions by enzyme-linked immunosorbent assay.

In summary, this study describes for the first time the presence of an APE in *B. melitensis* and suggests that such enzyme can be used for diagnostic purposes. At present, the cloning of this 97-kDa APE gene is under way and will permit large-scale production and purification of this protein. Such preparations will enable us to further analyze the peptidase’s characteristics as well as to better define the value of this protein in *Brucella* immunity and diagnosis.

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