

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 mM, respectively. The N-terminal sequence of aminopeptidase was used for a homologous search in the genomes of *B. melitensis* 16M and *Brucella 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 alanyl 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 *Brucella abortus* and *Brucella 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 *Brucella* 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 *Brucella* genes coding for products essential for intracellular survival in this niche (28).

The induction of *Brucella* heat shock proteins has been in-

involved in adaptive responses to adverse environmental conditions (28). Some of these proteins such as *Brucella* 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 proteinases from *Pseudomonas aeruginosa* and *Legionella pneumophila* (10, 24). Some specific proteinases have been shown to be related to severe periodontitis and cardiovascular disease (21). It has been suggested that extracellular enzymes of *Aeromonas* spp. play an important role in invasiveness and in establishment of the infection (14). Limited studies have been carried out with *Brucella* along these lines.

In this study, we describe the purification and characterization of an immunogenic APE obtained from *B. melitensis*. The

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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 *B. melitensis*.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *B. melitensis* 16M was kindly donated by Central Veterinary Laboratory (New Haw, Weybridge, United Kingdom), and the *B. melitensis* rough mutant VTRM1 was obtained in 1995 from the *Brucella* culture collection at Virginia Tech (S. M. Boyle). Strain VTRM1 was obtained by inserting a Tn5 element in the *wboA* 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.

Preparation of cell extract. The growth of two 500-ml (TSA-B) batch cultures of *B. melitensis* 16M and *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 *L*-lysine-*p*-nitroanilide (Lys-*p*NA) as the substrate.

Enzyme purification. As both strains exhibited APE activity, the purification was performed with the *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- μ m-pore-size membrane. The sample was applied onto an XK50 column packed with high-performance Q Sepharose (60 ml) equilibrated with 10 mM imidazole buffer (pH 7.0). A linear gradient of 0 to 1 M NaCl was applied at a flow rate of 120 ml/h, and 15-ml fractions were collected and assayed for APE activity. Fractions with enzymatic activity were pooled and saturated with 1.5 M ammonium sulfate. The sample was then loaded onto a phenyl-Superose HR 5/5 column previously equilibrated with 100 mM sodium phosphate and 1.5 M ammonium sulfate (pH 7.0). The column was eluted with a descending gradient from 1.5 to 0 M ammonium sulfate at a flow rate of 30 ml/h. The fractions with enzymatic activity were pooled and concentrated in a Centricon 50 tube (Amicon). The sample was loaded onto a Superose 12 HR 10/30 column previously equilibrated with 50 mM phosphate buffer (pH 7.0) containing 150 mM NaCl and eluted at a flow rate of 12 ml/h. The fractions with APE activity obtained by gel filtration chromatography were used for characterization. All purification steps were performed at 4°C by using a fast protein liquid chromatography system (Amersham Pharmacia Biotech, Uppsala, Sweden).

Enzyme assays. APE activity was assayed by measuring the hydrolysis of Lys-*p*NA (Sigma, St. Louis, Mo.). The incubation mixture consisted of 30 μ l of substrate (10 mM), 250 μ l of 100 mM Tris-HCl buffer (pH 7.0), 120 μ l of water, and 100 μ l of enzymatic samples. After incubation at 37°C for 20 min, the reaction was stopped by the addition of 500 μ l of ZnSO₄ (5%) and 100 μ 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 μ mol of *p*-nitroaniline per minute at 37°C. The molar absorption coefficient for 4-nitroaniline at 405 nm is $\epsilon_{405 \text{ nm}} = 9,900 \text{ M}^{-1} \text{ cm}^{-1}$, and this value was used for the calculation of enzyme activity.

Protein determination. 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 (200,000 to 14,000 kDa; Sigma) was used as a standard.

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 sequencer (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).

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 pI. 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-*p*Na and Ala-*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 K_m and V_{max} were obtained from a Michaelis-Menten plot.

(v) Effect of protease inhibitors and metal cations. The protease inhibitors bestatin (250 μ M), pepstatin (250 μ M), leupeptin (50 μ M), pepabloc (5 μ M), 1,10-phenanthroline (10 μ M), EDTA (2.5 mM), phenylmethylsulfonyl fluoride (PMSF) (5 μ M), and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino) butane (E64) (50 μ 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-*p*NA as the substrate. Activity was expressed as a percentage of the activity obtained in the absence of the added inhibitor.

Substrate specificity. The relative activities of the APE from *B. melitensis* against several aminoacyl-*p*NA (Glu-*p*NA, Ala-*p*NA, Leu-*p*NA, Gly-*p*NA, Met-*p*NA, Lys-*p*NA, and Pro-*p*NA) and dipeptidyl-*p*NA (Ala-Pro-*p*NA, Gly-Phe-*p*NA, Gly-Pro-*p*NA, Ala-Ala-*p*NA, and Ala-Phe-*p*NA) (Bachem) substrates were determined by the standard activity assay. Relative activity was expressed as a percentage of the rate of hydrolysis of Ala-*p*NA 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.

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, half of the patients had positive blood cultures. The other 10 patients were considered to have chronic brucellosis, as determined by a history of persisting symptoms or relapses and persisting agglutination titers for more than 1 year. Also, sera from 50 volunteers, without antecedents of brucellosis, and negative to standard and 2-mercaptoethanol agglutination tests were included.

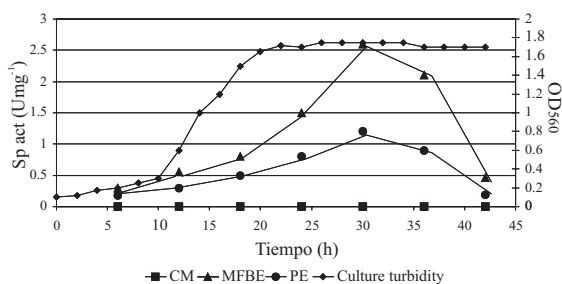


FIG. 1. *B. melitensis* strain VTRM1 growth and activity curve. Culture turbidity was measured at 560 nm (\blacklozenge). Determination of APE activity was measured every 6 h in CM (\blacksquare), MFBE (\blacktriangle), and PE (\bullet).

Preparation of anti-APE serum. Two mice were injected intraperitoneally with 20 μ g of purified enzyme emulsified with complete Freund adjuvant and reinjected with 20 μ g 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-*p*NA 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-*p*NA 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

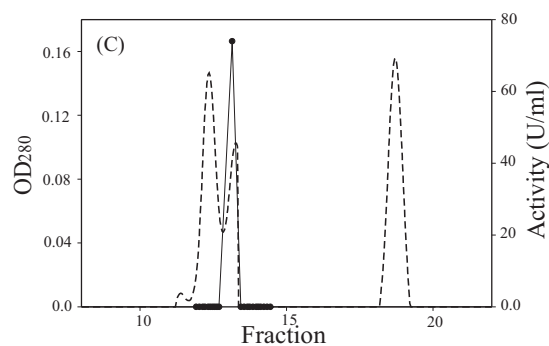
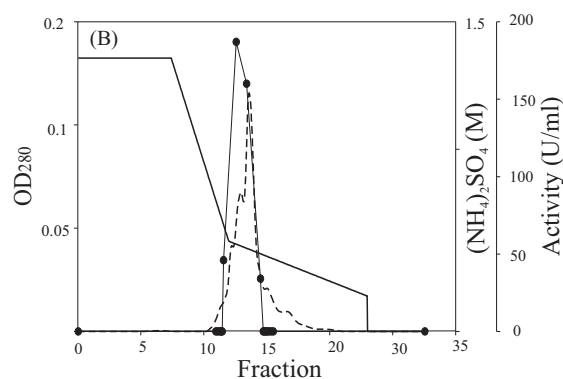
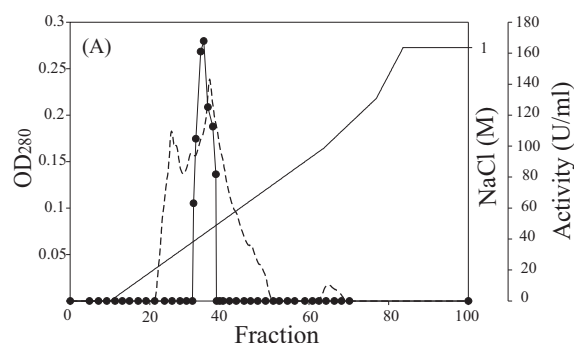


FIG. 2. Chromatograms of the purification of *B. melitensis* APE. Proteins were detected by measuring the absorbance at 280 nm (optical density at 280 nm [OD₂₈₀]) (dotted line); APE activity was assayed with Lys-*p*NA at pH 7.0 (solid circles). (A) High-performance Q Sepharose chromatography with NaCl gradient (solid line); (B) phenyl-Superose chromatography with (NH₄)₂SO₄ gradient (solid line); (C) Superose chromatography.

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 μ M) and EDTA (2.5 mM). Bestatine, a typical inhibitor of exopeptidases, inhibited more than 50% the activity. APE₉₇ was insensitive to serine-protease inhibitors such as leupeptin, pepabloc, and PMSF. E64, an inhibitor of cysteine proteinases

TABLE 1. Purification of APE₉₇ from *B. melitensis*

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

and the aspartate-specific inhibitor pepstatin, had no effect on the activity of APE₉₇. The presence of Zn²⁺ and Hg²⁺ caused complete inhibition at 1.0 mM while slight effects (10 to 15% inhibition) were observed in the presence of Ca²⁺, Mg²⁺, and Co²⁺ (Table 2). Concentrations of ammonium sulfate, used in the purification procedure, had no inhibitory effect on APE₉₇ activity.

Substrate specificity. The specificity of the enzyme towards the N-terminal residue was tested with several aminoacyl-*p*-NA and dipeptidyl-*p*-NA substrates. APE₉₇ 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-*p*-NA was most actively hydrolyzed, whereas Lys-*p*-NA, Leu-*p*-NA, Met-*p*-NA, and Gly-*p*-NA were hydrolyzed at lower rates. The acid derivative Glu-*p*-NA showed no detectable reactivity with APE₉₇. Weak activity was detected against Ala-Pro-*p*-NA (1.5%), and the other dipeptidyl substrates were not hydrolyzed.

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

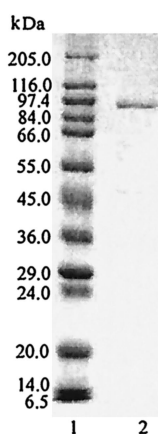


FIG. 3. SDS-PAGE of purified APE. Electrophoresis was performed on an SDS-polyacrylamide gel (10%). Lane 1, molecular mass markers; lane 2, purified APE (5 μg). The molecular mass markers were as follows: myosin, 205 kDa; beta-galactosidase, 116 kDa; phosphorylase *b*, 97 kDa; fructose-6-phosphate kinase, 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* APE₉₇

Inhibitor	Concn	Relative activity (%)
None	None	100
Zn ²⁺	1 mM	0
Co ²⁺	1 mM	93
Ca ²⁺	1 mM	86
Hg ²⁺	1 mM	0
Mg ²⁺	1 mM	96
EDTA	2.5 mM	0
Pefabloc	5 μM	60
Bestatin	250 μM	45
Pepstatin	250 μM	90
Leupeptin	50 μM	100
PMSF	5 μM	94
E-64	50 μM	90
1,10-Phenanthroline	10 μM	20

N-terminal amino acid sequencing. The N-terminal sequence was determined to be MRTETGHTFRLE. 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 APE₉₇, 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_697631). 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 M1 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

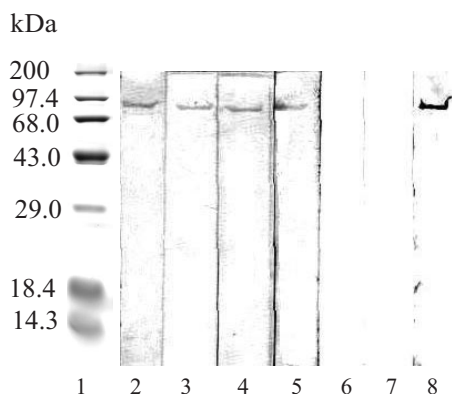


FIG. 4. Immunoreaction of serum to purified APE. Lane 1, molecular mass markers; lanes 2 and 3, sera from patients with acute brucellosis; lanes 4 and 5, sera from patients with chronic brucellosis; lanes 6 and 7, sera from healthy people; lane 8, APE antisera. The molecular mass markers were as follows: phosphorylase *b*, 97 kDa; bovine serum, 68 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; beta-lactoglobulin, 18.4 kDa; lysozyme, 14.3 kDa.

well as those of lactic acid bacteria have previously been purified and characterized by several workers (8, 36). There appear to be no reports on the purification of *Brucella* APEs.

The present study describes the purification and characterization of an APE from *B. melitensis* not previously reported. 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 APE₉₇ 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.

APE₉₇ 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). APE₉₇ 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 APE₉₇ was almost completely inhibited in the presence of chelators, 1,10-phenanthroline, and EDTA. 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 APE₉₇. Zn²⁺ and Hg²⁺ caused complete inhibition of APE₉₇. Inhibition by the metal

cation Zn²⁺ has been reported for *S. salivarius* (22) and *L. lactis* (8). Altogether, these results strongly suggest that APE₉₇ is a metallo-APE.

APE₉₇ showed optimal activity at 40°C, a temperature similar to that found for other bacterial APE N's (PepN) (8, 22, 36). APE₉₇ 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. APE₉₇ released a single amino acid and was able to cleave a variety of N-terminal amino acids from *p*NA 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 *p*NA 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, APE₉₇ showed a strong preference for bonds involving the hydrophobic amino acid Ala and hydrophilic Lys N-terminal aminocyl 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 APE₉₇ as an APE N.

A high similarity between the APE₉₇ 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 metalloproteases 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). Fur-

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 down-regulation of ClpP expression in *B. melitensis*, it is possible that alternative proteases may compensate for its absence. In this context, the role of APE₉₇ 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 APE₉₇ of *B. melitensis* is immunoreactive, we determined whether patient serum reacted with the protein. Sera from patients with acute and chronic brucellosis recognized the protein while sera from healthy people did not. These results suggest that the described *Brucella* APE could be used diagnostically to identify *Brucella*-infected humans. Further studies should be undertaken with sera from humans with brucellosis and from people with other infections produced by gram-negative bacteria to discriminate 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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