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Purification and antigenicity of two recombinant forms of *Boophilus microplus* yolk pro-cathepsin expressed in inclusion bodies

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Abstract

The tick *Boophilus microplus* is a bovine ectoparasite present in tropical and subtropical areas of the world and the use of vaccines is a promising method for tick control. BYC is an aspartic proteinase found in eggs that is involved in the embryogenesis of *B. microplus* and was proposed as an important antigen in the development of an anti-tick vaccine. The cDNA of BYC was amplified by PCR and cloned for expression in two forms with and without thioredoxin fusion protein (Trx), coding recombinant proteins named rBYC-Trx and rBYC, respectively. Expression, solubility, and yields of the two forms were analyzed. The recombinant proteins were expressed in inclusion bodies (IBs) and three denaturant agents (*N*-lauroyl sarcosine, guanidine hydrochloride, and urea) were tested for IBs solubilization. The *N*-lauroyl sarcosine solubilized 90.4 and 92.4% of rBYC-Trx and rBYC IBs, respectively, and was the most efficient denaturant. Two recombinant forms were affinity-purified by Ni^{2+}-Sepharose under denaturing conditions. After dialysis, the yield of soluble protein was 84.1% for r-BYC-Trx and 5.9% for rBYC. These proteins were immune-reactive against sera from rabbit, mouse, and bovine previously immunized with native BYC, which confirms the antigenicity of the recombinant BYCs expressed in the *Escherichia coli* system.
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Keywords: *Boophilus microplus*; BYC; Thioredoxin; Inclusion bodies; *N*-Lauroyl sarcosine; Vaccine

The tick *Boophilus microplus* is an important cattle ectoparasite in South and Central Americas, Asia, Africa, and Oceania [1]. This ixodidae tick causes considerable economical losses to cattle breeding, either directly, due to the blood-sucking deleterious effects which cause losses in animal production, or indirectly by increasing pest control costs. Besides, *B. microplus* is a vector of diseases such as anaplasmosis and babesiosis. Conventional tick control methods are based mainly on the use of acaricides, in spite of the high costs and the rapid appearance of resistance in the tick population [2]. The presence of chemical residues in meat and milk emphasizes the need for novel control methods [3]. Therefore, efforts are being made to develop new methodologies to monitor the appearance of acaricide resistance [4] as well as to promote alternative approaches to tick control such as vaccines [5–9] and biological control [10–12]. An anti-tick vaccine is considered one of the most promising methods; however, its development still

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depends on the identification and characterization of one or more protective tick antigens [13,14]. The success of this strategy depends on the identification and cloning of key tick molecules and the characterization of their roles in arthropod physiology [14,15].

The *Boophilus* yolk pro-cathepsin (BYC)[1] is an aspartic proteinase present in *B. microplus* eggs [16]. The 54 kDa BYC is activated in vitro by auto-proteolysis when incubated in acidic pH, and converted to a 47-kDa polypeptide that can be detected in vivo, during embryogenesis. Recently, the role of BYC was demonstrated in the degradation of vitellin (VT), the major yolk protein, confirming its importance in embryogenesis [17]. Cattle immunized with purified BYC showed an immune response partially protective against tick infestation that was mostly due to an increase in the number of sterile eggs [7]. These data support BYC as an antigen candidate to design an anti-tick vaccine. Aspartic proteinases produced in *Escherichia coli* are usually expressed in inclusion bodies (IBs), and fusion proteins as thioredoxin (Trx) [18,19] or maltose-binding protein (MBP) have been used in order to improve solubility of recombinant proteins [20]. In the present study, the BYC cDNA was cloned in a vector with thioredoxin and His-tag as fusion protein and in other vector only with His-tag at the carboxy-terminus of the protein. Therefore, we report the expression of the two recombinant forms of BYC (rBYC) in *E. coli* as inclusion bodies, and the analysis of various denaturants to solubilize the IBs in order to purify rBYC by affinity Ni^{2+}-Sepharose chromatography. We also report the efficiency of solubility and the recognition of rBYC not fused and fused with Trx by hyperimmune sera raised in animals immunized with the native BYC protein.

Materials and methods

Cloning of BYC

The cDNA encoding BYC was previously cloned by RT-PCR from fat body of engorged tick females (Maria Nascimento-Silva, personal communication). According to the amino acid sequencing of BYC [16] the cDNA was incomplete, without the coding sequence for the six N-terminal amino acids (GenBank Accession No. AY966003). The full coding sequence was restored by PCR and the complete cDNA was cloned into two expression vectors, pET-32b (Novagen), which produces a recombinant protein fused with histidine tagged thioredoxin protein (Trx), and pET-19b (Novagen), which produces a recombinant protein fused with a histidine tag. For cloning into pET-32b vector via *Nco*I and *Xho*I sites, the following primers were used: sense, tatcaatt*ccatgg*ca**aaaatt cgcattccgctt**cgcaaggatcgtattattatgtc; antisense, atcagggc *tcgag*ttagtacacgattgggcgg (*Nco*I and *Xho*I restriction sites are in italics, the bases coding the missing amino acids are in bold). PCR amplification with Elongase polymerase (Invitrogen) was performed using 10 ng of template plasmid (pT7-Blue/BYC). Reaction conditions were: an initial 5-min denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 54 °C, 60 s at 68 °C, and a 5-min final extension at 68 °C. The amplified DNA (1106 bp) sequence encoding the exon of BYC was digested with *Nco*I and *Xho*I, and cloned into plasmid pET-32b at *Nco*I/*Xho*I restriction sites downstream to the gene of thioredoxin (Trx) and histidine tag to give the pET-32b/BYC plasmid. For ligation, digested insert and vector were purified in a MicroSpin S-400 HR column (Amersham Biosciences) and ligated with T4 DNA ligase (Invitrogen). After the cloning into pET-32b, the BYC cDNA was subcloned into pET-19b using the following primers: sense, tatcaatt*ccatgg*caaaaattcgcattc; antisense,atcagggc*tcgag*tta**gtggtggtggtggtggtg**gtacacgatt gggcgggtg (*Nco*I and *Xho*I restriction sites are in italics, the encoding histidine tag included into antisense primer are in bold). Reaction conditions were: an initial 5-min denaturation at 94 °C, 30 cycles of 30 s at 94 °C, 30 s at 55.9 °C, 60 s at 68 °C, and a 5-min final extension at 68 °C. The 1123 bp PCR product and plasmid were digested with *Nco*I and *Xho*I, purified in MicroSpin S-400 column (Amersham Biosciences), and ligated with T4 DNA ligase (Invitrogen) to give pET-19b/BYC plasmid. The resulting plasmids pET-32b/BYC and pET-19b/BYC, coding the rBYC-Trx and rBYC proteins, respectively, were transformed by electroporation into XL1 Blue *E. coli* cells (Novagen) and selected in Luria–Bertani (LB) medium containing ampicillin (100 μg ml^{-1}). The BYC cDNA integrity was confirmed by sequencing the plasmid DNA.

Expression and preparation of inclusion bodies

The recombinant plasmids pET-32b/BYC and pET-19b/BYC were transformed into *E. coli* strain AD494 DES LysS (Novagen) and plated onto LB agar plates containing ampicillin. Single colonies were inoculated into 2.5 ml SOB medium [21] containing ampicillin (100 μg ml^{-1}) and grown overnight at 37 °C with shaking at 180 rpm. The overnight cultures were harvested by centrifugation, resuspended in fresh medium, and used to inoculate 500 ml of SOB medium in 2-L flasks. These flasks were incubated at 37 °C with shaking at 180 rpm until an OD_{600} of 0.5 was reached. For induction, isopropylthio-β-D-galactoside (IPTG, Invitrogen) was added to a final concentration of 0.4 mM and the culture was

[1] *Abbreviations used:* BYC, *Boophilus* yolk pro-cathepsin; VT, vitellin; IBs, inclusion bodies; Trx, thioredoxin; MBP, maltose-binding protein; LB, Luria–Bertani; IPTG, isopropylthio-β-D-galactoside; GuHCl, guanidine hydrochloride; ORFs, open reading frames; CMC, critical micelle concentration.

grown at 30 °C for 17 h. The cell culture was centrifuged at 10,000*g* for 10 min at 4 °C and the cell pellet was resuspended in phosphate buffer (10 mM sodium phosphate, 150 mM NaCl, pH 7.4). For cell lysis, the suspension was frozen, thawed out and sonicated on ice. The protease inhibitor PMSF (1 mM) was added prior to sonication. The soluble and insoluble fractions were separated by centrifugation at 10,000*g* for 10 min at 4 °C. The pellet containing the IBs was resuspended with washing buffer (50 mM Tris–HCl, 100 mM NaCl, 1 M urea, and 1% Triton X-100, pH 8.0) and centrifuged at 8000*g* for 10 min at 4 °C. This step was repeated three times.

Solubilization of recombinant proteins

The IBs were treated with three denaturant agents (0.3% *N*-lauroyl sarcosine, guanidine hydrochloride, and urea), as described below. After each denaturant treatment, solubilization yields were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis 12% (SDS–PAGE) [22] and protein quantification was determined by the Bradford method [23] with bovine serum albumin as standard.

Solubilization with 0.3% N-lauroyl sarcosine

The washed IBs were denatured and solubilized in lysis buffer A (0.3% *N*-lauroyl sarcosine, 50 mM CAPS buffer, and 0.3 M NaCl, pH 11.0) for 1 h and centrifuged at 10,000*g* for 15 min at 4 °C. The rBYC-Trx and rBYC proteins, which contain His-tag, were affinity-purified by Ni^{2+}-Sepharose. In brief, the supernatant was applied to a His Trap HP column (Amersham Biosciences) and the column was then washed with washing buffer A (30 mM imidazole, 0.3% *N*-lauroyl sarcosine, 50 mM CAPS buffer, and 150 mM NaCl, pH 11.0). The proteins were eluted with a serial concentration increase of imidazole (50, 100, 200, and 500 mM) in elution buffer A (0.3% *N*-lauroyl sarcosine, 50 mM CAPS buffer, and 150 mM NaCl, pH 11.0). Eluted fractions were analyzed by 12% SDS–PAGE. The denatured purified proteins in the eluted fractions were dialyzed in 20 mM Tris–HCl, 150 mM NaCl buffer with gradual pH reduction (pH 10, pH 9, and pH 8). Each dialysis step was performed at 4 °C during 12 h against at least 20× sample volume. Samples at pH 9.0 were collected for SDS–PAGE analysis.

Solubilization with 6 M guanidine hydrochloride and 8 M urea

The washed IBs were denatured and solubilized with lysis buffer B (20 mM sodium phosphate, 500 mM NaCl, 6 M guanidine hydrochloride (GuHCl), and 1 mM β-mercaptoethanol, pH 7.4) for 1 h and centrifuged at 10,000*g* for 15 min at 4 °C. The solubilized proteins (rBYC-Trx and rBYC) were purified in Ni^{2+}-Sepharose column following the manufacturer's instructions (Amersham Biosciences). Purifications were performed under denaturing conditions. Samples were loaded and the columns washed with the same lysis buffer B containing 30 mM imidazole. Proteins of interest were eluted with 100 mM imidazole in elution buffer B (20 mM sodium phosphate, 500 mM NaCl, and 6 M GuHCl, pH 7.4). The purified proteins were dialyzed against 20 mM Tris–HCl, 300 mM NaCl (pH 7.4). Eluted fractions were analyzed by 12% SDS–PAGE. Urea (8 M) was also tested for solubilization of recombinant proteins. Washed IBs were solubilized with lysis buffer C (20 mM sodium phosphate, 500 mM NaCl, and 8 M urea, pH 7.4) for 1 h at room temperature. The samples were centrifuged at 10,000*g* for 15 min at 4 °C and the soluble fractions were analyzed by 12% SDS–PAGE.

Western blotting with anti-BYC antibody

To verify antigenicity, Western blot assays using serum against native BYC of different species were carried out. The recombinant proteins (rBYC-Trx and rBYC) were separated by 12% SDS–PAGE and transferred onto a nitrocellulose membrane and probed with bovine, rabbit or mouse sera (1:500) previously immunized with native BYC. Anti-IgG species-specific alkaline phosphatase conjugates were used as secondary antibodies and NBT/BCIP (Sigma) were used as substrate.

Results

Cloning of BYC cDNA

The DNA sequence analysis confirmed the deduced sequences of the cloned cDNAs, which possess open reading frames (ORFs) of 1560 bp in pET-32b/BYC, and of 1101 bp in pET-19b/BYC. These ORFs encode to polypeptides with predicted molecular masses of 57,563 Da (rBYC-Trx) and 41,257 Da (rBYC), respectively.

Expression of recombinant proteins

rBYC-Trx and rBYC were detected by 12% SDS–PAGE and immunoblotting against rabbit anti-BYC serum. The apparent molecular masses of rBYC-Trx and rBYC were of approximately 56 and 40 kDa, respectively (Fig. 1). Both supernatant and pellet of cell lysates were tested for the presence of recombinant proteins. As shown in Fig. 1, the majority of the expressed recombinant proteins was present in inclusion bodies. The predicted molecular mass of the BYC amino acid sequence is 40.4 kDa, but the native BYC is

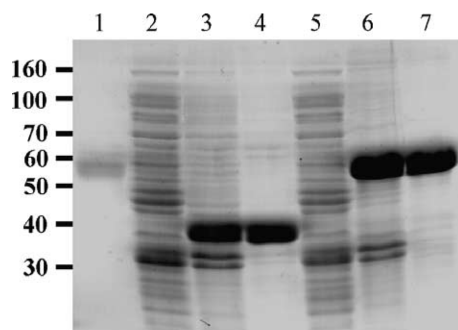

Fig. 1. SDS–PAGE (12% gel performed under reducing conditions) of recombinant BYC proteins in soluble and insoluble fractions of crude extracts stained with Coomassie blue G-250. Samples were lysed with SDS loading buffer and boiled for 5 min. Lane 1, native BYC; lane 2, supernatant of pET-19b/BYC bacterial extract; lane 3, pellet of pET-19b/BYC bacterial extract; lane 4, pET-19b/BYC inclusion bodies after washing; lane 5, supernatant of pET-32b/BYC bacterial extract; lane 6, pellet of pET-32b/BYC bacterial extract; and lane 7, pET-32b/BYC inclusion bodies after washing step. MW marker × 10^3.

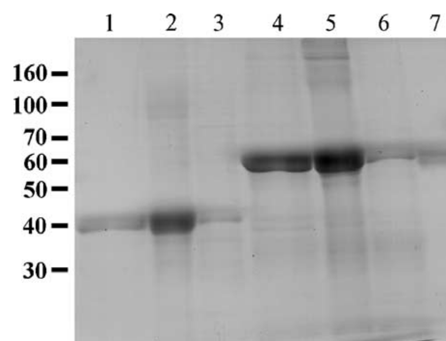

Fig. 3. Solubilization of IBs with three denaturant agents. Samples were analyzed by SDS–PAGE and stained with Coomassie blue G-250. Samples were resuspended directly in SDS loading buffer and boiled for 5 min. Lane 1, rBYC 6 M GuHCl treated; lane 2, rBYC *N*-lauroyl sarcosine treated; lane 3, rBYC 8 M urea treated; lane 4, rBYC-Trx 6 M GuHCl treated; lane 5, rBYC-Trx *N*-lauroyl sarcosine treated; and lane 6, rBYC-Trx 8 M urea treated. MW marker × 10^3.

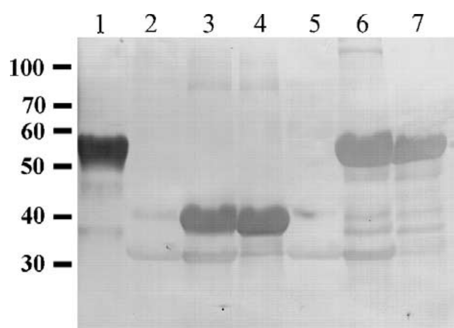

Fig. 2. Western blot of recombinant BYC proteins in soluble and insoluble fractions of crude extracts probed with rabbit BYC antiserum (1:500). Lane 1, native BYC; lane 2, supernatant of pET-19b/BYC bacterial extract; lane 3, pellet of pET-19b/BYC bacterial extract; lane 4, pET-19b/BYC inclusion bodies after washing; lane 5, supernatant of pET-32b/BYC bacterial extract; lane 6, pellet of pET-32b/BYC bacterial extract; and lane 7, pET-32b/BYC inclusion bodies after washing. Alkaline phosphatase-conjugated anti-rabbit IgG (1:5000) and NBT/BCIP were used. MW marker × 10^3.

a glycoprotein with apparent molecular mass of 54 kDa [16]. The major bands visualized in SDS–PAGE were confirmed as rBYC and rBYC-Trx proteins by Western blotting with rabbit anti-BYC serum and showed apparent molecular masses of 40 and 56 kDa, respectively (Fig. 2).

Solubilization of inclusion bodies

The IBs were partially purified after washing with buffer containing 1 M urea and 1% Triton X-100 (Fig. 1). Three denaturant agents were tested for solubilization of recombinant proteins. The alkalinized lysis buffer containing 0.3% *N*-lauroyl sarcosine was the most efficient for rBYC and rBYC-Trx IBs solubilization, as shown in Fig. 3 and Table 1. The highest yield of solubilization was achieved with *N*-lauroyl sarcosine, which was over 90% for both rBYC-Trx and rBYC (Table 1). Guanidine hydrochloride was also efficient and solubilized approximately 78% for rBYC-Trx and 65% for rBYC (Table 1). The IBs were only partially solubilized in 8 M urea with more than 80% of rBYC and rBYC-Trx proteins still residing in the precipitate after lysate centrifugation. As the recombinant protein-containing IBs were barely solubilized in 8 M urea, further purification was not carried out.

Purification of recombinant proteins

rBYC and rBYC-Trx solubilized with the *N*-lauroyl sarcosine treatment were purified under denaturing conditions using Ni^{2+}-Sepharose. This one-step purification yielded highly purified rBYC and rBYC-Trx proteins (Fig. 4). The recombinant proteins were eluted with both 50 and 100 mM imidazole, although they showed a higher degree of purity with 100 mM (Figs. 4A and B). The flowthrough was loaded a second time onto Ni^{2+}-Sepharose to increase the yield of recombinant protein recovery. Purification yields with *N*-lauroyl sarcosine are summarized in Table 2. The IBs solubilized with 6 M GuHCl were also affinity-purified by Ni^{2+}-Sepharose. However, rBYC and rBYC-Trx re-aggregated and precipitated during dialysis for GuHCl removal (data not shown).

Solubility of purified recombinant proteins

The purified protein was dialyzed for removal of the *N*-lauroyl sarcosine and gradual pH reduction. After each dialysis step (pH 10, pH 9, and pH 8), an aliquot was taken and centrifuged to separate soluble and insoluble fractions. Analysis by SDS–PAGE indicated that

Table 1
Efficiency of solubilization of inclusion bodies containing the recombinant BYC proteins with three denaturant agents

Recombinant protein	Denaturant	Total protein in washed IBs (mg)[a]	Total protein in solubilized IBs[a]	
			(mg)	(%)
rBYC	GuHCl	98	64.3	65.7
	N-Lauroyl sarcosine	98	90.5	92.4
	Urea	98	16.9	17.3
rBYC-Trx	GuHCl	133	104.4	78.5
	N-Lauroyl sarcosine	133	120.2	90.4
	Urea	133	21.0	15.8

[a] From 1 L of induced culture.

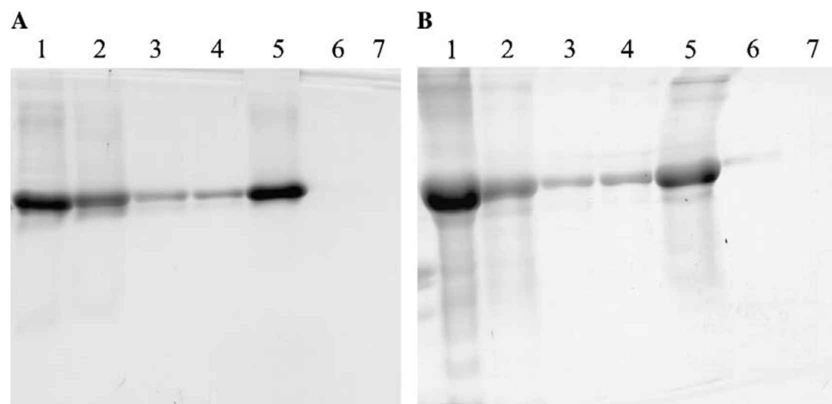

Fig. 4. SDS–PAGE of *N*-lauroyl sarcosine solubilized protein fractions after affinity purification in Ni^{2+}-Sepharose. Samples were resuspended directly in SDS loading buffer and boiled for 5 min. (A) rBYC; (B) rBYC-Trx. Lanes 1, solubilized IBs before purification; lanes 2, proteins in the flowthrough fraction; lanes 3, washing fractions with 30 mM imidazole; lanes 4, elution fraction with 50 mM imidazole; lanes 5, elution fraction with 100 mM imidazole; lanes 6, elution fraction with 200 mM imidazole; and lanes 7, elution fraction with 500 mM imidazole. Gel stained with Coomassie blue G-250.

Table 2
Purification and solubility rate of recombinant BYC proteins treated with *N*-lauroyl sarcosine

Recombinant protein	Total protein in solubilized IBs (mg)[a]	Purified protein with His Trap column (mg)	Soluble protein (mg)		Solubility rate pH 8.0 (%)	
			pH 9.0	pH 8.0	From IBs	From purification
rBYC	90.5	78.6	68.7	4.6	5.1	5.9
rBYC-Trx	120.2	64.9	63.0	54.5	45.4	84.1

[a] From 1 L of induced culture.

pH reduction was critical for solubility of recombinant proteins, especially rBYC. As shown in Fig. 5, rBYC was almost totally precipitated at pH 8.0 and partially precipitated at pH 9.0. On the other hand, recombinant thioredoxin-fusion protein (rBYC-Trx) remained in solution after *N*-lauroyl sarcosine removal at the pH values tested (Fig. 5). The solubility rates of both recombinant proteins are shown in Table 2.

Western blotting with anti-BYC antibodies

A Western blot with rabbit anti-BYC serum confirmed the expression of the recombinant proteins and indicated its immune-reactivity (Fig. 2). Another indication of antigenicity was the recognition by bovine and mouse anti-native BYC sera. Sera of bovines immunized with four doses of native BYC [7] also recognized the rBYC and rBYC-Trx, with a somewhat weaker intensity when compared to native BYC, as summarized in Fig. 6. Anti-native BYC mouse serum also recognized the recombinant proteins (Fig. 7). These results demonstrate that the immunogenicity potential was maintained in the rBYC and rBYC-Trx.

Discussion

Our group has characterized three proteinases from *B. microplus* eggs. Two aspartic proteinases named BYC [16] and THAP [24], and one cysteine proteinase [25].

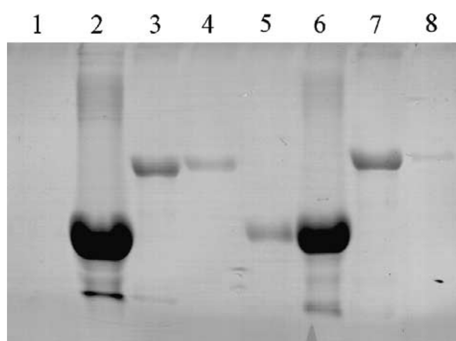

Fig. 5. Solubility of the purified proteins after dialysis. Samples were submitted to SDS–PAGE 12% and were stained with Coomassie blue G-250. Samples were resuspended directly in SDS loading buffer and boiled for 5 min. Lane 1, rBYC supernatant pH 8; lane 2, rBYC pellet pH 8; lane 3, rBYC-Trx supernatant pH 8;.lane 4, rBYC-Trx pellet pH 8; lane 5, rBYC supernatant pH 9; lane 6, rBYC pellet pH 9; lane 7, rBYC-Trx supernatant pH 9; and lane 8, rBYC-Trx pellet pH 9.

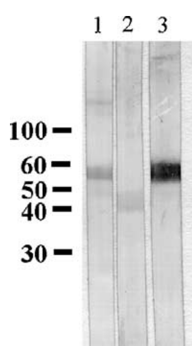

Fig. 6. Western blot of purified BYC and rBYC probed with bovine serum anti-native BYC. Lane 1, rBYC-Trx; lane 2, rBYC; and lane 3, native BYC. Alkaline phosphatase-conjugated anti-bovine IgG (1:5000). MW marker × 10^3.

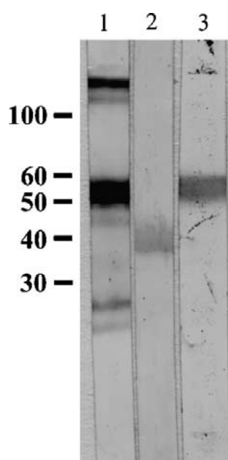

Fig. 7. Western blot of purified BYC and rBYC probed with mouse serum anti-native BYC. Lane 1, rBYC-Trx; lane 2, rBYC; and lane 3, native BYC. Alkaline phosphatase-conjugated anti-mouse IgG (1:5000). MW marker × 10^3.

The most abundant protein (BYC) is the precursor of an aspartic proteinase that is synthesized in the fat body and gut of the tick, secreted to the hemolymph, and stored in the oocyte [16]. This protease is activated by autoproteolysis under acidic conditions, a process that has been described in other invertebrates where the participation of different classes of acid proteases is also observed in digestion of egg yolk [26–28]. Recently, it was demonstrated (in vitro) that BYC is highly specific for vitellin [17], reinforcing the hypothesis that it plays a key role in the degradation of yolk proteins, an essential process for embryogenesis [16,17]. In previous reports, we demonstrated that cattle vaccination with native BYC induces an humoral response and functional anti-BYC antibodies were observed in the hemolymph of ticks feeding on vaccinated cattle [29], resulting in an overall protection between 14 and 36% of bovines challenged with *B. microplus* larvae. Consistent with the in vitro data, egg fertility and egg laying capacities were the principal parameters affected by immunization [7]. Other authors have provided evidence that anti-tick immunity induced by a cocktail vaccine is more effective as compared to a single antigen vaccine [5,30]. Based on these data we can suggest that BYC is an antigen with potential to become part of a cocktail vaccine against *B. microplus*.

The production of recombinant proteins in heterologous systems is a key process to vaccine development. High expression of foreign proteins in bacteria often results in aggregation and accumulation [31]. This may be due to a protein translation rate that may exceed the cell capacity to fold the newly synthesized proteins [32]. Aspartic proteinases produced in *E. coli* are generally expressed in IBs [20]. Thioredoxin is a small monomer which facilitates the soluble expression and refolding of recombinant proteins [18,19]. To compare the expression of rBYC and the role of a fusion protein in its solubility, BYC cDNA was cloned into two vectors, pET-32b and pET-19b, with and without Trx, respectively. Both expression vectors induced high levels of recombinant BYC expression in the *E. coli* system. However, rBYC accumulated in inclusion bodies independently of vectors and expression conditions tested. One way to overcome the formation of inclusion bodies consists in reducing the protein translation rate by inducing expression at a lower temperature [33]. We tested inductions at 23 and 18 °C, but the lower temperatures had no effect on the expression of soluble rBYC and rBYC-Trx (data not shown).

Guanidine hydrochloride and urea are denaturing agents commonly used for solubilization of proteins expressed in IBs. However, rBYC and rBYC-Trx obtained in IBs were not efficiently solubilized with 8 M urea. GuHCl, a strong denaturant, was efficient in rBYC and rBYC-Trx solubilization with yields of 65 and 78%, respectively. GuHCl-solubilized proteins were purified under denaturant conditions in Ni^{2+}-Sepharose chromatography (not shown). Both purified proteins were unable to refold efficiently due to aggregation and

precipitation of the proteins once the denaturant was gradually removed by dialysis. Gradual concentration reduction of denaturant agent and co-solvents as glycerol, reduced glutathione, oxidized glutathione, and L-arginine have been used to improve the refolding of recombinant proteins solubilized with GuHCl, but the results are different for each protein [31,34,35,37]. We have not tested these co-solvents in the refolding of rBYC, since *N*-lauroyl sarcosine proved to be a better denaturing agent for rBYC and rBYC-Trx.

N-Lauroyl sarcosine, an anionic denaturant, reduces hydrophobicity due to its capacity to interact with hydrophobic residues, and can be removed by dialysis when its concentration lies below critical micelle concentration (CMC) [35,36]. *N*-Lauroyl sarcosine was highly efficient for rBYC and rBYC-Trx solubilization with yields higher than 90%, the best result among the denaturants tested. The purification with His Trap column (Amersham Biosciences) was performed under denaturant conditions, indicating that the *N*-lauroyl sarcosine did not abolish adsorption of proteins to Ni^{2+} charged resin. The elution profile was not affected by Trx and most of the contents of both recombinant proteins were eluted with 100 mM imidazole and SDS–PAGE analyses showed few contaminating bands (Fig. 4) after a single purification step. The purified proteins were dialyzed for denaturant removal with gradual pH reduction. The Trx fusion partner increased the yield of soluble proteins (rBYC-Trx) after dialysis, which was also influenced by pH. In pH 9.0 solutions, the majority of the recombinant proteins was soluble. However, in pH 8.0 solutions rBYC was almost totally precipitated and rBYC-Trx remained soluble. Therefore, Trx facilitated the recovery of soluble protein after dialysis. The role of Trx and MBP in the increase of solubility during refolding of other recombinant aspartic proteases was previously described [20]. The predicted p*I*s of rBYC (5.77) and rBYC-Trx (5.36) are similar. Thus, the higher solubility of the rBYC-Trx compared to rBYC is remotely due to differences in the p*I*s of the two proteins. Sachdev and Chirgwin [20] suggested that during refolding fusion proteins block side-reactions, hence avoiding the aggregation of the recombinant proteins.

The BYC amino acid sequence indicates a molecular mass of 40.4 kDa, whereas the native glycoprotein has a molecular mass of 54 kDa—a difference due to glycosylation and phosphorylation [16]. Therefore, the possible involvement of glycosylation in the antigenicity of rBYC needs to be evaluated. In Western blot, the rBYC and rBYC-Trx were recognized by rabbit anti-BYC serum (Fig. 2). This, associated with Western blot assays with bovine and mouse anti-BYC sera, confirmed the presence of antigenicity for both recombinant forms (rBYC and rBYC-Trx). The recognition of rBYC by hyperimmune sera confirms that the recombinant protein displays epitopes that were immunogenic in the vaccination with native BYC. This characteristic is of paramount importance for a potential vaccine based on rBYC. However, investigation into the capacity of rBYC to induce protective humoral response in vaccinated bovines is required to authenticate its use as vaccine antigen. In this report, we described a method for expression, solubilization, and dialysis of the aspartic proteinase, a potential candidate to design a *B. microplus* vaccine. The presence of Trx as fusion protein in rBYC increased the solubility after dialysis of the recombinant protein. In addition, the aggregation dependence on pH values of dialysis solutions was demonstrated. This method might be applicable to other recombinant proteins. In this work, we verified that our recombinant proteins showed similar immunological properties as compared to native protein. Therefore, heterologous expression of recombinant protein could provide a valuable resource of BYC, which is a potential candidate as an antigen for multi-component vaccines against *B. microplus.*

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.pep.2005.07.009.

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