Available online at www.sciencedirect.com
ScienceDirect
Veterinary Immunology and Immunopathology 114 (2006) 341–345
Veterinary immunology and immunopathology
www.elsevier.com/locate/vetimm

Short communication

Vaccination of bovines with recombinant *Boophilus* Yolk pro-Cathepsin

Alexandre T. Leal [a,1], Adriana Seixas [a,1], Paula C. Pohl [a], Carlos A.S. Ferreira [b], Carlos Logullo [c], Pedro L. Oliveira [d], Sandra E. Farias [a,e], Carlos Termignoni [a,f], Itabajara da Silva Vaz Jr. [a,g], Aoi Masuda [a,h,*]

[a] *Centro de Biotecnologia, Universidade Federal do Rio Grande do Sul, Avenida Bento Gonçalves, 9500, Prédio 43421, Porto Alegre RS 91501-970, Brazil*
[b] *Laboratório de Imunologia e Microbiologia, Faculdade de Biociências, Pontifícia Universidade Católica do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[c] *Laboratório de Química e Função de Proteínas e Peptídeos, Universidade Estadual do Norte Fluminense, Campos de Goytacazes, RJ, Brazil*
[d] *Departamento de Bioquímica Médica, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brazil*
[e] *Departamento de Fisiologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[f] *Departamento de Bioquímica, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[g] *Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*
[h] *Departamento de Biologia Molecular e Biotecnologia, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil*

Received 21 July 2006; received in revised form 14 August 2006; accepted 23 August 2006

Abstract

Boophilus Yolk pro-Cathepsin (BYC) is an aspartic proteinase found in *Boophilus microplus* eggs that is involved in the embryogenesis and has been tested as antigen to compose an anti-tick vaccine. The vaccine potential of a recombinant BYC expressed in *Escherichia coli* (rBYC) was investigated. rBYC was purified and used to immunize Hereford cattle. The sera of bovines immunized with rBYC recognized the native BYC with a titer ranging from 125 to 4000. Furthermore, immunized bovines challenged with 20,000 larvae presented an overall protection of 25.24%. The partial protection obtained against *B. microplus* infestation with the recombinant protein immunization was similar to the already described for native BYC immunization.

Keywords: *Boophilus microplus*; Tick vaccine; BYC; Aspartic proteinase; Cytokine; Bovine

1. Introduction

The *Boophius microplus* ixodidae is the most economically damaging tick worldwide (Johnston et al., 1986). Conventional tick control methods have been based mainly on the use of acaricides; however, the rapid appearance of resistance in tick populations, and the presence of chemical residues in meat and milk are aspects that emphasize the need for novel control methods, such as vaccination (Willadsen et al., 1996) and biological control (Gindin et al., 2002). Although an anti-tick vaccine may be the most promising control method, its development still depends on the identification and characterization of one or more protective tick antigens (Imamura et al., 2005).

* Corresponding author. Tel.: +55 51 3316 6076; fax: +55 51 3316 7309.
E-mail address: aoi@cbiot.ufrgs.br (A. Masuda).
[1] Both authors contributed equally to this work.

0165-2427/$ – see front matter
doi:10.1016/j.vetimm.2006.08.011

The *Boophilus* Yolk pro-Cathepsin (BYC) is an aspartic proteinase present in *B. microplus* eggs (Logullo et al., 1998). The native 54-kDa BYC is activated in vitro by auto-proteolysis in acidic pH and its role has been demonstrated in the degradation of vitellin (VT), confirming the involvement of BYC in embryogenesis (Abreu et al., 2004). Cattle immunized with native BYC showed a partially protective immune response against tick infestation (da Silva Vaz et al., 1998). These data support BYC as a candidate antigen to compose an anti-tick vaccine.

Our preliminary studies with a recombinant BYC expressed in *Escherichia coli* (rBYC) demonstrated its antigenicity and immunogenicity in bovines (Leal et al., 2006). In the present study, we described the protection against *B. microplus* infestation induced by immunization with rBYC.

2. Materials and methods

2.1. Production of recombinant BYC

The rBYC was expressed and purified as previously described (Leal et al., 2006). Briefly, BYC cDNA was subcloned into vector pET-19b (Novagen) and expressed in *E. coli* strain AD494 (DE3)pLysS (Novagen). Expressed insoluble rBYC was solubilized with 6 M guanidine hydrochloride (GuHCl) and purified using a nickel-chelating Sepharose column. The eluted samples were then dialyzed against 20 mM Tris–HCl, 300 mM NaCl (pH 7.4), resulting in the precipitation of purified protein. rBYC concentration was determined by SDS-PAGE using a BSA curve as control.

2.2. Bovine immunization and challenge infestation

Eight 14-month-old Hereford breed females, from a tick-free area, were individually housed in tick-proof pens and divided into two groups: the control group (bovines 1, 2, 3, 4) and the treatment group (bovines 5, 6, 7, 8). The control group received emulsion composed of 1 ml PBS plus 1 ml of oil adjuvant (Montanide 888 and Marcol 52) per dose. The treatment group animals received emulsion of 1 ml of rBYC solution in PBS (1st/2nd dose = 50 μg; 3rd dose = 100 μg; 4th/5th dose = 200 μg) plus 1 ml adjuvant, at 10-day intervals. Ten days after the last inoculation, the bovines were challenged with 20,000 10-day-old larvae of *B. microplus* (Porto Alegre strain), *Babesia* spp. and *Anaplasma* spp. free.

2.3. Serological analysis

The humoral response was verified by ELISA and Western blot using native BYC as antigen, purified as previously described (Logullo et al., 1998). The ELISA was realized as previously described (da Silva Vaz et al., 1998), using 0.5 μg of native BYC per well. For IgG titration, sera samples collected on the challenge day were diluted from 1:125 to 1:4000 and detected with rabbit anti-IgG peroxidase conjugate. In Western blot, the native protein was separated by 12% SDS-PAGE (Laemmli, 1970) and transferred onto a nitrocellulose membrane of 0.45 μm. The nitrocellulose sheets were probed with sera of vaccinated or control bovines and anti-IgG alkaline phosphatase conjugates as previously described (Rosa de Lima et al., 2002).

2.4. Analysis of the ticks

From the 17th post-challenge day on until the end of the infestation period, ticks that detached spontaneously were collected once a day. Then the ticks and eggs were classified and incubated as previously described (da Silva Vaz et al., 1998). The protection was calculated by variation in number of fully engorged tick, egg laying capacity and egg fertility. The individual and overall protections were calculated by the interaction of these parameters with the formula (Willadsen et al., 1996; da Silva Vaz et al., 1998):

$$\text{Overall protection ratio} = 100 \times [1(\text{NFE} \times \text{WE} \times \text{WL})];$$

where NFE is the number of fully engorged ticks from vaccinated bovines/control bovines, WE is the egg laying capacity of ticks from vaccinated bovines/control bovines and WL is the egg fertility of ticks from vaccinated bovines/control bovines.

The statistical significance of the results shown in Table 1 was tested using *t*-test and ANOVA, as previously described (da Silva Vaz et al., 1998).

3. Results and discussion

3.1. Production of recombinant protein

The rBYC expressed in *E. coli* has a predicted molecular mass of 41.2 kDa and was visualized by SDS-PAGE and immunoblotting with rabbit anti-BYC serum (Fig. 1). The majority of rBYC was detected in the insoluble fraction (Fig. 1, lane 3), indicating its presence in inclusion bodies. High expression of foreign

Table 1
Biological parameters of detached *B. microplus* from cattle immunized with rBYC and control group

Group	Bovine	Fully engorged[a]		Index	
		Number	Weight (g)	Egg laying capacity[b]	Egg fertility[c]
Control	1	2419	701	0.416	0.325
	2	1915	494	0.399	0.320
	3	1198	309	0.433	0.286
	4	3245	916	0.402	0.309
	Total	8777	2420	1.651	1.240
	Mean	2194	605	0.413	0.310
	S.D.	861	262	0.015	0.017
rBYC	5	2099	583	0.432	0.339
	6	1129	267	0.398	0.310
	7	1891	498	0.396	0.257
	8	2076	583	0.395	0.260
	Total	7195	1931	1.621	1.166
	Mean	1799	483	0.405	0.292
	S.D.	456	149	0.018	0.040
	Difference (%)[d]	18.02*	20.21*	1.80*	5.96*

[a] Collected during 14 days in the course of the infestation period.
[b] The weight of eggs laid by samples of fully engorged ticks, collected daily during 14 days in the course of the infestation period, was used to calculate the proportion of the weight of ticks that was converted into eggs.
[c] Proportion between weight of fertile eggs and weight of eggs laid.
[d] Difference (%) = 100 × (1 − mean value of vaccinated group/control group).
* $p > 0.05$.

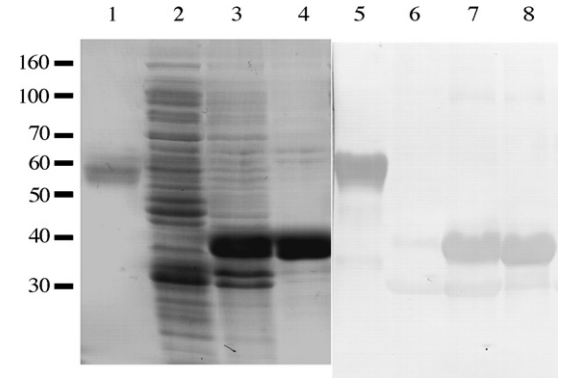

Fig. 1. SDS-PAGE (lanes 1–4) and Western blot (lanes 5–8) of recombinant BYC proteins in bacterial extract and purified form. SDS-PAGE stained with Coomassie blue G-250. Samples were lysed with SDS loading buffer and boiled for 5 min. Lane 1, native BYC; lane 2, supernatant of pET-19b/BYC bacterial extract; lane 3, pellet of pET-19b/BYC bacterial extract; lane 4, purified rBYC. Western blot probed with rabbit BYC antiserum (1:500). Lane 5, native BYC; lane 6, supernatant of pET-19b/BYC bacterial extract; lane 7, pellet of pET-19b/BYC bacterial extract; lane 8, purified rBYC. MW marker × 10^3.

proteins in bacteria often results in accumulation into insoluble aggregates (Hwang and Chung, 2002), and aspartic proteases produced in *E. coli* are generally expressed in inclusion bodies (Sachdev and Chirgwin, 1998). The guanidine solubilized rBYC was affinity purified with nickel–Sepharose resin under denaturant conditions (Fig. 1, lane 4), but rBYC aggregated and precipitated after dialysis (Leal et al., 2006). Other authors have suggested that recombinant proteins in a particulate form can result in an increase in immunogenicity (Rodriguez et al., 1994), a form which is often less immunogenic when compared to native antigens (Aucouturier et al., 2001).

As shown in Fig. 1 (lanes 5–8), Western blot with BYC antiserum confirmed the antigenicity of rBYC. Since the native BYC is a phospholipoglycoprotein (Logullo et al., 1998), the immunogenicity of the recombinant protein could have been affected. Previous studies demonstrated the antigenicity of rBYC (Leal et al., 2006). Here, the development of a specific humoral response by the vaccinated cattle also showed that rBYC is immunogenic and shares epitopes with native BYC. This characteristic is of paramount importance for a potential vaccine based on recombinant proteins.

3.2. Serological analysis

The ELISA and Western blot analysis, using native BYC as antigen, reveals that rBYC elicited specific anti-BYC IgG in vaccinated bovines. As observed in Fig. 2, the sera from immunized bovines recognized the native BYC in Western blot, whereas pre-immune and non-immunized bovine sera were negative. Analysis by ELISA demonstrated a gradual increase in antibodies titers (Fig. 3) as well as an individual variation in the humoral responses. Three bovines responded to immunization and presented titers between 1000 (bovines 6) and 4000 (bovines 5 and 7), while bovine 8 showed a lower IgG titer (125) in ELISA (data not shown). Individual variation in antibody titers is frequently observed in vaccination of non-isogenic animals; Rodriguez et al. (1995) described the vaccination of 98 bovines with Bm86 and observed 6% of animals with very low antibody titers. The titers obtained in this work are similar to those of bovines vaccinated with native BYC, which presented titers between 1600 and 6400 (da Silva Vaz et al., 1998).

3.3. Challenge and analysis of the ticks

The mean results for each biological parameter and the differences between groups are presented in Table 1.

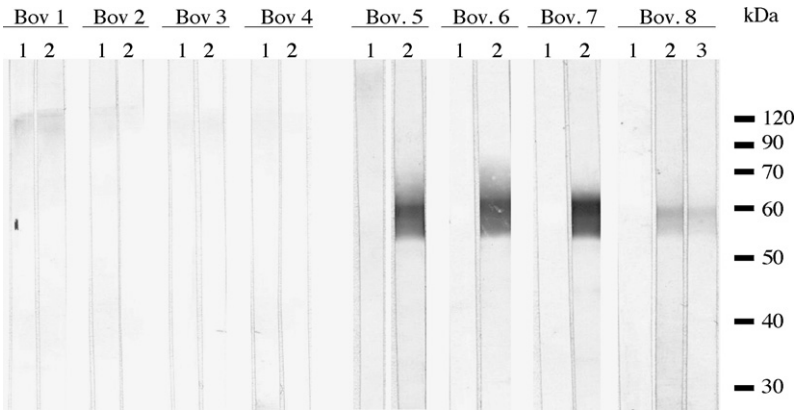

Fig. 2. Western blot with native BYC (SDS-PAGE, 12% gel performed under reducing conditions). Sera from control group (bovines 1–4) and rBYC vaccinated group (bovines 5–8). Bovines 1–7: lanes 1, pre-immune sera (1:250); lanes 2, immune sera (1:250); Bovine 8: lane 1, pre-immune serum (1:250); lanes 2 and 3, immune sera (1:125 and 1:250). MW marker $\times 10^3$.

The bovines vaccinated with rBYC showed a reduction of 18% and 20% in number and weight of engorged ticks, respectively, which were not statistically significant. The egg laying capacity and egg fertility presented, respectively, 1.8 and 5.9% reductions in vaccinated animals, which were also not significant. In this experiment, the overall efficacy ratio against *B. microplus* was 25.24% as compared with control group. This protection index was similar to that observed with native BYC vaccination, which demonstrated an overall protection between 14 and 36% (da Silva Vaz et al., 1998).

Previous studies with native BYC showed that ticks feeding on immunized bovines have active bovine antibodies in tick hemolymph and that fully engorged ticks inoculated with a monoclonal anti-BYC presented decreased survival rates and egg weights (da Silva Vaz et al., 1996, 1998). Willadsen et al. (1995) described an inverse correlation between the weight of eggs laid and anti-Bm86 antibody titers, while Kashino et al. (2005) observed higher levels of IgG anti-saliva in tick resistant bovines. Therefore, there is considerable experimental evidence that immunity against tick is antibody-dependent (Willadsen et al., 1995; Kashino et al., 2005), and our results suggest that BYC protection is mediated by IgG, and that the protection level could be improved by modifications in the immunization protocol to increase serum titers.

In this work, we verified that rBYC possesses similar immunological and protective properties as compared to the native protein. Based on these data, we can suggest that rBYC is a potential antigen to become part of a cocktail vaccine against *B. microplus*.

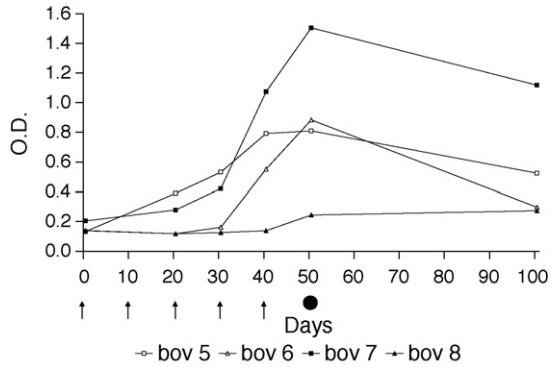

Fig. 3. Kinetics of the humoral response of bovines immunized with rBYC determined by indirect ELISA (sera diluted 1:250). Arrows indicate the days of immunization, and the dot the day of infestation.

Acknowledgment

This work was supported by grants from CNPq, FINEP, PRONEX and FAPERGS.

References

Abreu, L.A., Valle, D., Manso, P.P.A., Façanha, A.R., Pelajo-Machado, M., Masuda, H., Masuda, A., Vaz Jr., I., Lenzi, H., Oliveira, P.L., Logullo, C., 2004. Proteolytic activity of *Boophilus microplus* yolk pro-cathepsin D (BYC) is coincident with cortical acidification during embryogenesis. Insect Biochem. Mol. Biol. 34, 443–449.

Aucouturier, J., Dupuis, L., Ganne, V., 2001. Adjuvants designed for veterinary and human vaccines. Vaccine 19, 2666–2672.

da Silva Vaz, I., Martinez, R.H.M., Oliveira, A.T.D., Heck, A., Logullo, C., Gonzáles, J.C., Dewes, H., Masuda, A., 1996. Functional bovine immunoglobulins in *Boophilus microplus* hemolymph. Vet. Parasitol. 62, 155–160.

da Silva Vaz, I., Logullo, C., Sorgine, M., Velloso, F.F., Rosa de Lima, M.F., Gonzales, J.C., Masuda, H., Oliveira, P.L., Masuda, A., 1998. Immunization of bovines with an aspartic proteinase

precursor isolated from *Boophilus microplus* eggs. Vet. Immunol. Immunopathol. 66, 331–341.

Gindin, G., Samish, M., Zangi, G., Mishoutchenko, A., Glazer, I., 2002. The susceptibility of different species and stages of ticks to entomopathogenic fungi. Exp. Appl. Acarol. 28, 283–288.

Hwang, H., Chung, H., 2002. Preparation of active recombinant cathepsin K expressed in bacteria as inclusion body. Protein Expr. Purif. 25, 541–546.

Imamura, S., da Silva Vaz, I., Sugino, M., Ohashi, K., Onuma, M., 2005. A serine protease inhibitor (serpin) from *Haemaphysalis longicornis* as na anti-tick vaccine. Vaccine 23, 1301–1311.

Johnston, L.A.Y., Kemp, D.H., Pearson, R.D., 1986. Immunization of cattle against *Boophilus microplus* using extracts derived from adult female ticks: Effects of induced immunity on tick populations. Int. J. Parasitol. 16, 27–34.

Kashino, S.S., Resende, J., Sacco, A.M.S., Rocha, C., Proença, L., Carvalho, W.A., Firmino, A.A., Queiroz, R., Benavides, M., Gershwin, L.J., De Miranda Santos, I.K.F., 2005. *Boophilus microplus*: The pattern of bovine immunoglobulin isotype responses to high and low tick infestations. Exp. Parasitol. 110, 12–21.

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227, 680–685.

Leal, A.T., Pohl, P.C., Ferreira, C.A.S., Nascimento-Silva, M.C.L., Sorgine, M., Logullo, C., Oliveira, P.L., Farias, S.E., da Silva Vaz, I., Masuda, A., 2006. Purification and antigenicity of two recombinant forms of *Boophilus microplus* yolk pro-cathepsin expressed in inclusion bodies. Protein Expr. Purif. 45, 107–114.

Logullo, C., da Silva Vaz, I., Sorgine, M., Paiva-Silva, G.O., Faria, F.S., Zingali, R., De Lima, M.F.R., Abreu, L., Fialho Oliveira, E., Alves, E.W., Masuda, H., Gonzáles, J.C., Masuda, A., Oliveira, P.L., 1998. Isolation of an aspartic proteinase precursor from the egg of a hard tick, *Boophilus microplus*. Parasitology 116, 525–532.

Rodriguez, M., Rubiera, R., Penichet, M., Montesinos, R., Cremata, J., Fálcon, V., Sánchez, G., Bringas, R., Cordovés, C., Valdés, M., Lleonart, R., Herrera, L., De la Fuente, J., 1994. High level expression of the *B. microplus* Bm86 antigen in the yeast *Pichia pastoris* forming highly immunogenic particles for cattle. J. Biotechnol. 33, 135–146.

Rodriguez, M., Penichet, M.L., Mouris, A.E., Labarta, V., Lorenzo Luaces, L., Rubiera, R., Cordovés, C., Sánchez, P.A., Ramos, E., Soto, A., Canales, M., Palenzuela, D., Triguero, A., Lleonart, R., Herrera, L., De la Fuente, J., 1995. Control of *Boophilus microplus* population in grazing cattle vaccinated with a recombinant Bm86 antigen preparation. Vet. Parasitol. 57, 339–349.

Rosa de Lima, M.F., Ferreira, C.A.S., Freitas, D.R.J., Valenzuela, J.G., Masuda, A., 2002. Cloning and partial characterization of a *Boophilus microplus* (Acari: Ixodidae) glutathione S-transferase. Insect Biochem. Mol. Biol. 32, 747–754.

Sachdev, D., Chirgwin, J.M., 1998. Solubility of proteins isolated from inclusion bodies is enhanced by fusion to Maltose-binding protein or Thioredoxin. Protein Expr. Purif. 12, 122–132.

Willadsen, P., Bird, P., Cobon, G.S., Hungerford, J., 1995. Commercialisation of a recombinant vaccine against *Boophilus microplus*. Parasitology 110, S43–S50.

Willadsen, P., Smith, D., Cobon, G., Mckenna, R.V., 1996. Comparative vaccination of cattle against *Boophilus microplus* with recombinant antigen Bm86 alone or in combination with recombinant Bm91. Parasite Immunol. 18, 241–246.