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PURIFICATION AND CHARACTERIZATION OF THE ALKALINE PHOSPHATASE FROM *ECHINOCOCCUS GRANULOSUS* CYST MEMBRANES

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ABSTRACT: The purification to homogeneity and the characterization of *Echinococcus granulosus* alkaline phosphatase (AP; EC 3.1.3.1) from hydatid cyst membranes are described. After *n*-butanol extraction, the parasite enzyme was sequentially purified by affinity chromatography on concanavalin A-sepharose followed by gel filtration. The purified protein (210 kDa) had a tetrameric structure composed of 4 56-kDa subunits. Its isoelectric point (4.8) and its kinetic parameters were determined ($K_m = 0.24 \pm 0.05$ mmol/L; $V_m = 173 \pm 21$ nmol/min/mg protein for *p*-nitrophenylphosphate). The parasite enzyme differed from the host liver enzyme in its thermal stability, optimum reaction temperature, optimum pH, and catalytic parameters, but not in its apparent molecular weight. Furthermore, sera from patients infected with *E. granulosus* recognized the parasite AP on immunoblots, whereas uninfected controls were negative. These results as well as the role of this enzyme in the host-parasite relationship emphasize its potential importance as a diagnostic and prognostic antigen in the monitoring of hydatid infection.

Hydatidosis, a disease caused by the larval stage of *Echinococcus granulosus* (Cestoda), is an economic and public health problem in many countries. The hydatid cyst membranes (laminated and germinal layers) are key structures in the host-parasite interface and should provide useful antigens as efficient diagnostic markers; they should also reflect the parasite viability and thus help in monitoring the course of infection and therapy of the disease. In this respect, the membrane-bound metabolic enzyme, alkaline phosphatase (AP; EC 3.1.3.1), possibly involved in transport processes (Fujino et al., 1983), is a good candidate. Among helminths, AP has been mostly studied in adults of *Hymenolepis diminuta* (Pappas, 1991) and *Schistosoma mansoni*, where its antigenic importance has been emphasized (Balloul et al., 1987; Pujol and Cesari, 1990). McManus and Barrett (1985) used the enzyme as a membrane marker during the isolation of the tegumental surface of *E. granulosus* proto-scolecocytes. The APs from larval stages, e.g., metacystodes, have been far less studied; recently however, AP from *Echinococcus multilocularis* has been purified and characterized (Sarciron et al., 1991). In the present paper, the isolation and characterization of the *E. granulosus* (sheep strain) hydatid membranes' AP is described.

MATERIALS AND METHODS

Extract preparation

Livers of sheep infected with *E. granulosus* were collected at a slaughterhouse (Sisteron, France). The

hydatid fluid was aseptically aspirated and the cyst membranes were carefully removed and thoroughly washed in cold (4 °C) saline. The washed membranes were stored in liquid nitrogen until further use. The normal sheep liver was cut into small pieces, washed several times in cold saline to remove blood, and stored in liquid nitrogen.

Hydatid cyst membranes or sheep liver were cut in small pieces while frozen, suspended (1/1, w/v) in 10 mmol/L Tris/HCl, pH 7.6, containing 0.1 mmol/L *N*- α -*p*-tosyl-L-lysine chloromethyl ketone (TLCK), 2 mmol/L phenylmethylsulfonyl fluoride (PMSF), 0.5 mmol/L antipain and pepstatin (buffer A) and ground with a Polytron homogenizer. The procedure of Hua et al. (1986) was used. Briefly, the homogenate was mixed for 3 hr at 4 °C; cold (-20 °C) *n*-butanol was slowly added, and the mixture was stirred overnight in the cold room. After centrifugation for 30 min at 9,000 g, the pellet was re-extracted in buffer A for 3 hr. After centrifugation, the 2 supernatants were pooled, adjusted to pH 5 with acetic acid, and stirred for 30 min. The mixture was again centrifuged, and the supernatant containing the enzyme activity was adjusted to pH 7 with solid Na_2CO_3 . Cold acetone (-20 °C) was added dropwise to a final concentration of 30% (v/v), and after centrifugation, cold acetone was again added to the supernatant to a final concentration of 50% (v/v). The last acetone pellet (extract) was kept, dissolved in buffer A and frozen in aliquots at -196 °C.

Purification of the APs

Extracts (0.5-2 ml, protein content: 1-2 mg/ml) were thawed and applied to a 5-ml concanavalin A-sepharose column (Pharmacia Biotech, St-Quentin/Yvelines, France) previously equilibrated in buffer B (100 mmol/L Tris/HCl, pH 7.6, 100 mmol/L NaCl, 1 mmol/L CaCl_2 , 1 mmol/L MgCl_2) at a flow rate of 1 ml/min at 4 °C. After washing with at least 6 column volumes of the same buffer, retained glycoproteins were eluted with 5 column volumes of 0.5 mol/L α -methylmannoside in buffer B. After washing with at least 10 volumes of the same buffer, the solid phase was removed and AP recovered by 2 batchwise treatments

at 37 °C for 30 min with 4 volumes of 0.1 mol/L α -methylmannoside in buffer B containing 1% (w/v) Triton X-100. The supernatants were immediately cooled on ice and concentrated in an Amicon pressure cell (Amicon-Grace, Epernon, France) with a YM 10 membrane to a final volume of 0.5–1 ml. This fraction was subsequently applied to a Sephacryl S300 HR column (1.6 \times 70 cm; Pharmacia Biotech) equilibrated with buffer C (100 mM ammonium bicarbonate, pH 7.8, 20 mmol/L NaCl, 0.5 mmol/L $MgCl_2$), at a flow rate of 1 ml/min. The 2-ml collected fractions were assayed for AP activity, and the most active fractions were either freeze-dried or concentrated as above, reconstituted or diluted with buffer D (10 mmol/L Tris/HCl, pH 7.6, 0.1% Triton X-100, 0.1 mmol/L $MgCl_2$), and stored in aliquots at –20 °C.

The host liver extract was applied to the concanavalin A–sepharose column in the same way, except that the active fractions were eluted columnwise with 0.1 mol/L α -methylmannoside in buffer B. The most active fractions were pooled, concentrated, and processed as above.

Enzyme assays and protein determination

One hundred microliters of a 20-mmol/L *p*-nitrophenylphosphate (PNPP, Sigma 104; Sigma Chimie, L'Isle d'Abeau, France) solution was diluted in 1 mol/L diethanolamine, pH 9.0, containing 0.5 mmol/L $MgCl_2$, and the samples (10–200 μ l) were added to the prewarmed solution (2 ml final volume). The reaction was allowed to proceed at 37 °C for 30 min, and the amount of released *p*-nitrophenol was monitored at 420 nm, using the calculated absorption coefficient of $16.7 \times 10^3 \text{ M}^{-1} \text{ cm}^{-1}$.

The determination of the optimum reaction pH was performed using 100 mol/L sodium acetate buffer (pH 4.5–5.5), 100 mmol/L Tris/HCl (pH 6.5–8.5), and 1 mol/L diethanolamine (pH 9–11.5).

The kinetic parameters were determined in a temperature-controlled Beckman DU 40 spectrophotometer with 25 μ l (0.25–1 μ g protein) of purified APs, and the reaction rates were followed at 37 °C for 90 min. The reaction was carried out at the relevant optimum pH for each AP. The catalytic constants were calculated from the linear part of the curves using the double-reciprocal plot method (Lineweaver and Burk, 1934).

For inhibition experiments, the following inhibitors were purchased from Sigma (Sigma Chimie): ethylenediamine tetraacetic acid (EDTA), levamisole, L-leucine, L-phenylalanine, L-homoarginine. They were preincubated for 10 min with the purified APs in the reaction buffer before addition of the substrate.

The protein content of the samples was assayed by the procedure of Bensadoun and Weinstein (1976), using bovine serum albumin as a standard.

SDS-PAGE and immunoblotting

Proteins were resolved by SDS-PAGE in 5–20% gradient gels with the discontinuous system of Laemmli (1970). The samples were loaded after heating in the sample buffer with or without 2-mercaptoethanol as a reducing agent. Electrophoresis was carried out at a constant voltage (1 hr at 30 V, 15 hr at 100 V) at 4 °C. The bands were either stained with Coomassie blue R-250 or with silver by a modification of the methods

of Merrill et al. (1981) and Cupo et al. (1990), and the molecular weights were determined with Sigma standards. The proteins were transferred onto nitrocellulose membranes according to the method of Towbin et al. (1979). Nitrocellulose strips were incubated overnight at 4 °C in sera collected from hydatidosis patients, diluted 1:50 and the immunoreactive bands were visualized after incubation in horseradish peroxidase-conjugated anti-human IgG (1:500; Sigma) followed by thorough washing and subsequent incubation in H_2O_2 -activated 4-chloro-1-naphthol.

Isoelectric focusing

Isoelectric focusing was carried out in an LKB-Pharmacia Multiphor II apparatus with 2-mm-thick 5% polyacrylamide gels containing 2% (v/v) ampholine 4–6.5, according to the specifications of the manufacturer. The samples (20 μ l) were loaded after a 30-min pre-focusing step, and the focusing was carried out at 10 °C for 15 hr at 25 mA and 400 V. The gels were stained with silver as above, or for AP activity at 37 °C with β -naphthyl acid phosphate (1 mg/ml) and fast blue BB (1 mg/ml) in 100 mmol/L Tris, pH 9.0/1 mmol/L $MgCl_2$. The bath was changed when the reagents became brown, and the incubation was allowed to proceed in buffer alone until the bands were distinguishable. The isoelectric points of the proteins were determined with markers (Sigma Chimie) and by measuring the pH of gel strips according to Trépanier et al. (1976).

RESULTS

Table I shows the results of a typical extraction and purification procedure. It was almost impossible to elute the parasite AP bound to the column by conventional means; even with 1 mol/L α -methylmannoside, less than 10% of the total could be released. The batch elution at 37 °C with 0.1 mol/L α -methylmannoside and 1% Triton X-100 allowed the release of 25–50% of the concanavalin A-bound activity. On the contrary, the host liver enzyme bound classically to the concanavalin A–sepharose and was subsequently eluted with 0.1 mol/L α -methylmannoside.

The SDS-PAGE analysis (Fig. 1) of the partially purified and purified AP showed an apparent molecular weight of 210 kDa under non-reducing conditions. These results are consistent with the molecular weight observed in gel filtration (180–250 kDa, data not shown). Under reducing conditions, the AP displayed an apparent molecular weight of 56 kDa. It was thus extracted and purified as a homotetramer, the 110-kDa dimeric form appearing when heating of the sample was delayed or omitted. The sheep liver AP was undistinguishable in this system.

Isoelectric focusing experiments indicated pIs of 4.8 and 4.4 for the *E. granulosus* and host

TABLE I. Extraction (A) and purification (B) of *Echinococcus granulosus* cyst membrane alkaline phosphatase.

	Sample	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min/mg)	Purification (fold)	Yield (%)
A	Lysate	300	1,972.6	6.58	1	100
	Butanol extract	110.4	1,608.8	14.57	2.2	81.6
	50% acetone pellet	8.55	187.9	21.98	3.3	9.5
B	Extract	1.71	37.3	21.98	3.3	100
	Concanavalin A eluate	0.114	14.4	126.3	19.2	38.6
	Gel filtration	0.0096	10.4	1,083.3	164.6	27.9

enzyme, respectively (Fig. 2). No activity comparable to the parasite AP was detected in the liver extracts.

The parasite AP exhibited an optimum pH of 9.0 and a high optimum reaction temperature.

Conversely, the host enzyme had a higher optimum pH, with a small activity peak appearing near physiological pH (Fig. 3).

The kinetic parameters and temperature sensitivity experiments are shown in Table II. Using

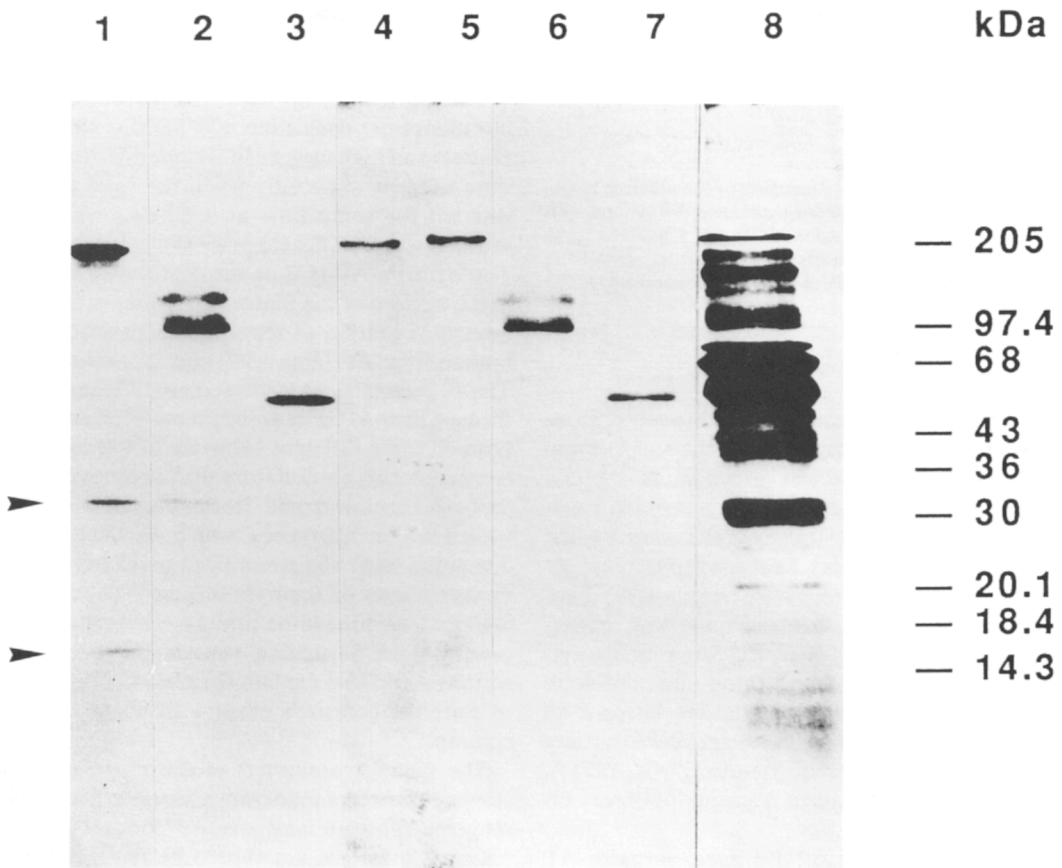


FIGURE 1. SDS-PAGE analysis of the purification steps and the different molecular species of the alkaline phosphatases. The gels were run and stained with silver as described in the Materials and Methods. The concentrated samples after affinity chromatography (lanes 1–3) and gel filtration (lanes 4–7) are shown. Partially purified AP, unreduced (lane 1), not heated (lane 2); note the presence of the dimer and of an intermediate species. The same, reduced (lane 3). Arrows point to concanavalin A released from the column. Pure host liver AP, unreduced (lane 4). Pure parasite AP, unreduced (lane 5), not heated (lane 6); the tetramer has disappeared. Pure parasite AP, reduced (lane 7). Starting parasite acetone extract (lane 8).

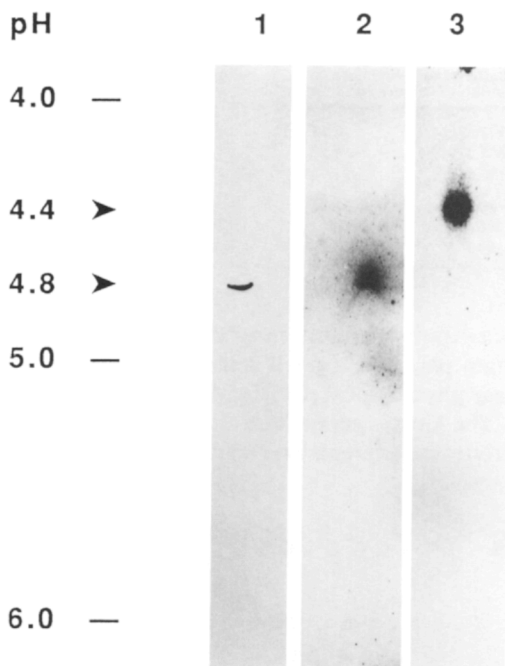


FIGURE 2. Isoelectric focusing of the alkaline phosphatases. Pure *Echinococcus granulosus* AP stained with silver (lane 1) and for AP activity for 6 hr (lane 2) as described in the Materials and Methods. Host liver acetone extract (lane 3); note the absence of parasite AP.

PNPP, the *E. granulosus* AP exhibited a moderate maximal velocity, whereas the host enzyme hydrolyzed this substrate much more rapidly. The parasite enzyme displayed a striking resistance to heat denaturation. On the contrary, the liver AP appeared very heat sensitive.

Known AP inhibitors were tested on the 2 enzymes. The *E. granulosus* enzyme was moderately inhibited by $ZnCl_2$, EDTA, L-homoarginine, and levamisole, and totally insensitive to L-leucine and L-phenylalanine. The sheep liver enzyme was inhibited by L-leucine and to a lesser extent by L-phenylalanine, whereas $ZnCl_2$, EDTA, and levamisole exhibited a strong inhibitory effect (Table III).

Immunoblotting of the pure parasite AP showed that it was recognized by sera from hydatidosis patients, whereas normal sera were unreactive (Fig. 4). Despite varying staining intensities, some of the antibodies were able to detect the various molecular species present in the unreduced lanes of the gels.

DISCUSSION

The extraction of the enzyme was performed according to the classical *n*-butanol and acetone precipitation method (Hua et al., 1986), and the activity was mainly concentrated in the last acetone precipitate. Concanavalin A-sepharose affinity chromatography had been successfully applied to human liver AP (Trépanier et al., 1976) and with modifications to AP from the related species *E. multilocularis* (Sarciron et al., 1991). The combination of temperature and detergent allowed the elution of most of the bound remaining activity. This procedure provided a partially purified AP fraction, which contained essentially various amounts of concanavalin A originating from the affinity column. The subsequent gel filtration permitted a 90–180-fold purification, a result comparable to those that Payares et al. (1984) obtained with the *Schistosoma mansoni* adult tegumental AP.

The pure enzyme appeared as a 210-kDa homotetramer consisting of 4 56-kDa subunits. Dimers and high molecular weight intermediates were evident, especially when the pure protein was left for some time at 4 C, e.g., when the samples were not heated prior to electrophoresis. The parasite AP is thus most probably located at the surface of the plasma membranes and extracted at pH 7.6 as tetramers as described for human liver AP (Hawrylak and Stinson, 1988). The *E. granulosus* AP showed no difference with the host liver AP in its apparent molecular weight. However, the different behavior of the parasitic enzyme during elution from the concanavalin A-sepharose column and isoelectric focusing revealed major differences, which are likely to be due to the sugar chain composition of the 2 APs; further studies on their amino acid sequence will help to determine if the protein backbone is also involved. In particular, protein-protein interactions could also explain the need for detergent to elute the parasite's enzyme from the affinity column.

The kinetic parameters of the *E. granulosus* enzyme showed a moderate affinity for the chromogenic substrate and a rather low maximum velocity, a feature not shared by the host liver AP. The apparently low AP-specific activity in the hydatid membranes thus reflects its catalytic properties rather than its importance as a major component of the starting extract.

The optimum reaction pH, the optimum reaction temperatures, and, above all, the thermal

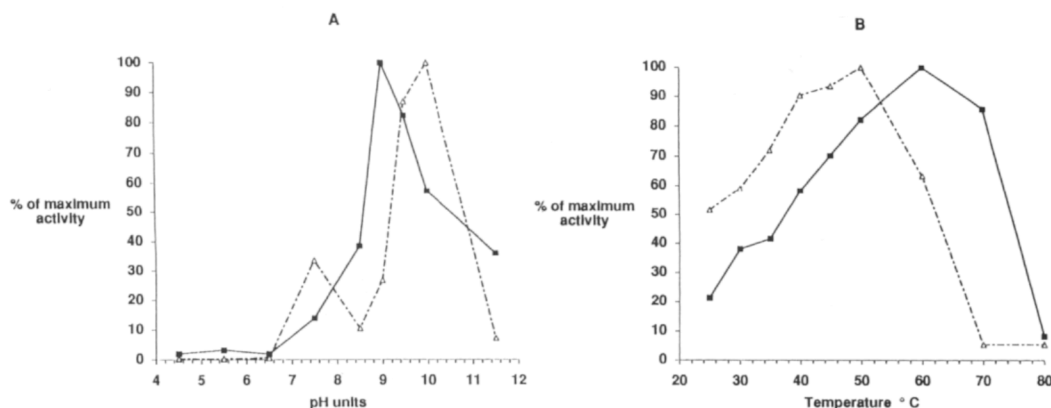


FIGURE 3. Optimum pH (A) and reaction temperatures (B) of the *Echinococcus granulosus* (—■—) and sheep liver (---△---) alkaline phosphatases. The maximum activities at the relevant optimum pH and temperature were taken as reference. Each point represents the mean of triplicate pure AP samples incubated separately.

stability of the *E. granulosus* AP are remarkable. It is believed that differential thermostability of the AP is due to organ/tissue-specific post-translational modifications such as glycosylation (Stinson and Seargeant, 1981). These results can be compared with those of the thermostable *E. multilocularis* AP (Sarciron et al., 1991) and are similar to those of the mammalian placental APs, which are reported as thermostable (Harris, 1989). A shrimp AP also displays the same optimum reaction pH and the same subunit molecular weight (Chuang, 1990); a clam AP comparable to our *E. granulosus* enzyme in its thermal stability has also been described (Chuang and Yang, 1990). This could be important from an evolutionary perspective.

The effect of inhibitors that differentiate be-

tween the placental, intestinal, and bone/liver/kidney APs (Harris, 1989) showed that the *E. granulosus* AP cannot be grouped with any of these 3 types. Conversely, the sheep liver AP behaved like its mammalian counterparts, with IC_{50} values comparable to those already reported (Harris, 1989).

The parasite origin of the purified AP is supported by the following: (1) no thermostable activity was found in sheep liver extracts, and (2) isoelectrofocusing showed no activity band in the *E. granulosus* AP region. Preliminary experiments on crude parasite extracts had shown that the material was largely free of host tissue. The fraction eluted with 0.1 M α -methylmannoside (less than 10% of the total) could represent contamination by the host soluble AP. The presence of host serum components in the hydatid cyst fluid has been known for several years (Varela-

TABLE II. Catalytic parameters and resistance to heat denaturation of the *Echinococcus granulosus* and the host liver alkaline phosphatases. The experiments were performed at the relevant optimum reaction pH: 9.0 for the parasite AP; 10.0 for the host liver AP. Data represent mean \pm SD for 3 determinations.

	Cyst membrane enzyme	Host liver enzyme
K_m (mmol/L)	0.24 ± 0.05	0.85 ± 0.14
V_m (nmol/min/mg)*	173 ± 21	876 ± 174
Remaining activity 56 C, 30 min (%)†	82.2 ± 6.7	38.8 ± 0.6
Remaining activity 65 C, 30 min (%)	73.3 ± 4.5	0
Remaining activity 65 C, 60 min (%)	6.7 ± 3.3	0

* Results are expressed in mg of pure AP.

† The remaining activities were assayed for 30 min at 37 C after the relevant incubations and compared to the unaffected activity.

TABLE III. Inhibition of the parasite and the host alkaline phosphatases. The triplicate samples were separately preincubated for 10 min with the inhibitors before the addition of the substrate.

Inhibitor	IC_{50} (mM)*	
	Parasite enzyme	Host liver enzyme
L-Leucine	N.I.†	18
L-Phenylalanine	N.I.	24
L-Homoarginine	0.80	3
Levamisole	0.55	0.06
EDTA	0.25	0.02
ZnCl ₂	0.60	0.03

* Concentration needed to achieve a 50% inhibition of the purified enzymes ($\sim 0.1 \mu\text{g}$ pure protein) at 37 C at their optimum reaction pH.

† Not inhibitory.

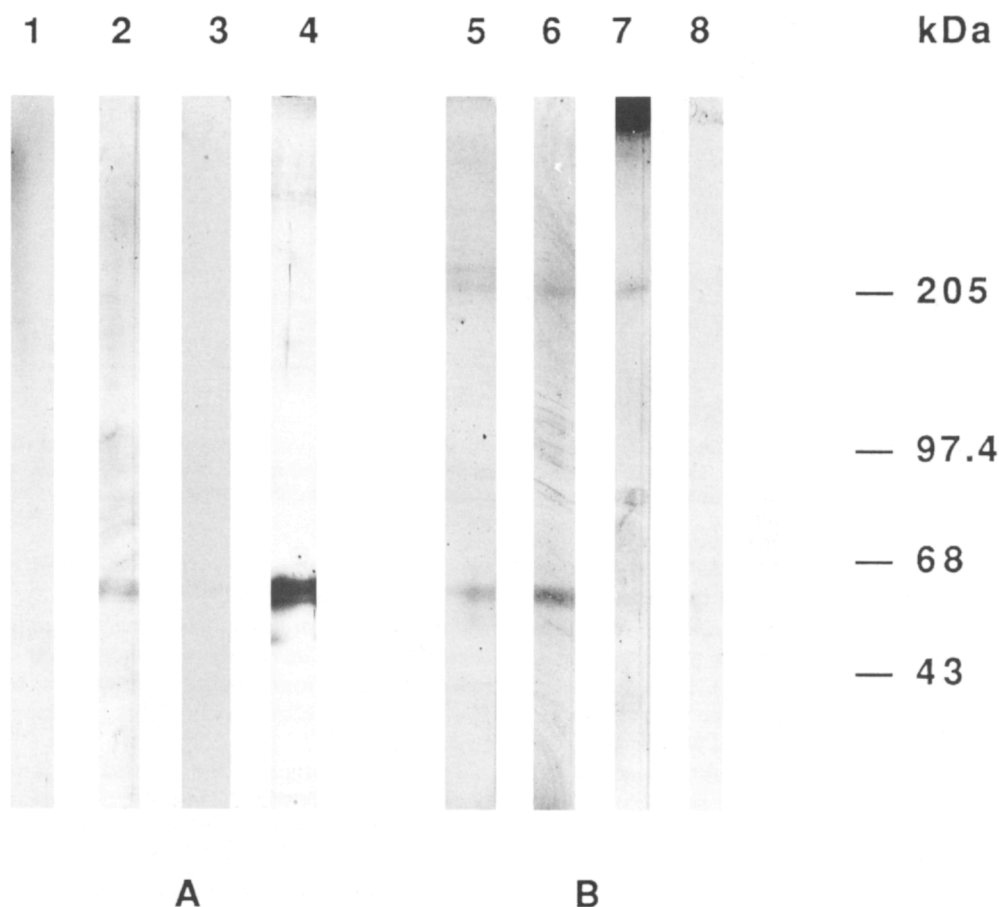


FIGURE 4. Immunological recognition of the *Echinococcus granulosus* alkaline phosphatase by hydatidosis sera. The pure reduced (A) and unreduced AP (B) were incubated with non-immune serum (lanes 1, 8) and sera from 3 hydatidosis patients (lanes 2, 7; 3, 6; and 4, 5). Under nonreducing conditions, some of the sera recognize the tetramer, the intermediate species, and trace amounts of the monomer.

Diaz and Coltorti, 1972), the cyst membranes being an exchange zone between the host and the larva. The hydatid membranes we used consisted mainly of the laminated and germinal layers, with a few brood capsules containing budding protoscoleces. Preliminary experiments with free protoscoleces indicated the presence of a thermostable activity, suggesting that there is only 1 species of AP in the *E. granulosus* hydatid cyst.

The *E. granulosus* cyst membrane AP thus appears as an original enzyme, differing from the known types of APs and from the host liver AP. Its presence in the cyst membranes is evidence for an important role in the host-parasite relationship, possibly in the regulation of membrane transport. Like its counterpart from *S. mansoni* adults (Payares et al., 1984; Balloul et al., 1987)

and *Plasmodium falciparum* aldolase (Srivastava et al., 1990), the *E. granulosus* AP, which is recognized immunologically by patients, could be a good diagnostic antigen candidate, but it may also be a marker of parasite viability. In this respect, studies are under way to use the anti-AP immune response to monitor the course of infection and the therapeutic status of hydatidosis patients.

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