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Echinococcus granulosus, E. multilocularis and mammalian liver-type alkaline phosphatases: a comparative study

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The alkaline phosphatases (EC 3.1.3.1) from *Echinococcus granulosus* and *E. multilocularis* (Cestoda) were compared to each other and to a liver-type enzyme. The purified proteins (210 and 220 kDa, respectively) had a tetrameric structure composed of 4, 56/53 kDa subunits. Enzymatic removal of their *N*-linked sugar moieties abolished the differences in their apparent molecular weight under reducing conditions. After phase separation in Triton X-114, the *E. multilocularis* enzyme was the most amphiphilic, and treatment with PI-PlC reduced the amount of the parasite alkaline phosphatases that were in a hydrophobic form by about 50%. Both parasite enzymes were highly resistant to heat denaturation and insensitive to the inhibitors L-phenylalanine and L-leucine. In addition, L-homoarginine, levamisole and $ZnCl_2$ can be used to differentiate the parasite and mammalian liver-type enzymes from each other. The *Echinococcus* alkaline phosphatases have original biochemical properties when compared to the mammalian liver-type enzyme.

Key words: *Echinococcus*; *E. granulosus*; *E. multilocularis*; Alkaline phosphatases; Characterization; Biochemical properties; GPI-anchor.

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Introduction

The hepatic larval stages—metacestodes—of *Echinococcus granulosus* and *E. multilocularis* (Cestoda) are the causative agents of hydatidosis and alveolar hydatid disease, which are economic and public health problems in many countries as well as a real therapeutic challenge. In the search for parasite viability markers and chemotherapeutic targets, alkaline phosphatase (AP; EC.3.1.3.1) as a membrane-bound metabolic enzyme, is a good candidate. Despite decades of research, its physiological role is still controversial. Although possibly involved in transport processes (Arme and Pappas, 1983; Fujino *et al.*, 1983), its importance in protein phosphorylation/dephosphorylation has recently been hypothesized (Sarrouilhe *et al.*, 1992) and denied (Fedde *et al.*, 1993). Used as a membrane marker during the isolation of the tegumental surface of *E. granulosus* protoscoleces (McManus and Barrett, 1985), AP has been mostly studied in other parasites such as *Hymenolepis diminuta* (Pappas, 1991) and *Schistosoma mansoni*, where its antigenic importance has been underlined (Payares *et al.*, 1984; Balloul *et al.*, 1987; Pujol and Cesari, 1990). The alkaline phosphatases from larval

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Abbreviations—AP, alkaline phosphatase; EDTA, Ethylenediamine tetraacetic acid; PMSF, phenylmethylsulfonyl fluoride; pNPP, *p*-nitrophenyl phosphate; TLCK, Nα-p-tosyl-L-lysine chloromethyl ketone.

stages have been recently purified and characterized in *E. multilocularis* metacestodes (Sarciron *et al.*, 1991) and in the *E. granulosus* (sheep strain) hydatid membranes (Lawton *et al.*, 1994). We provide here evidence that these two enzymes are different from each other and from the mammalian liver-type APs.

Materials and Methods

Materials

All biochemicals were of the purest quality available and were purchased from Sigma Chimie (L'Isle d'Abeau, France). The chromatography media were from Pharmacia Biotech (St-Quentin/Yvelines, France) and the electrophoresis reagents were obtained from Serva (Tebu, Le Perray/Yvelines, France).

Enzyme preparations

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. An uninfected sheep liver, processed as above, served as a source for the reference mammalian liver-type AP. *E. multilocularis* metacestodes (Savoie strain) were maintained in jirds (*Meriones unguiculatus*) by intraperitoneal inoculation (Sarciron *et al.*, 1993) and were processed similarly.

The parasite material was cut in small pieces while frozen, suspended (w/v) in 10 mM Tris/HCl, pH 7.6 containing 0.1 mM TLCK, 2 mM PMSF, 0.5 mM antipain and pepstatin (Buffer A) and processed as previously described (Lawton *et al.*, 1994) except that the pellet was re-extracted either in buffer A for 3 hr (*E. granulosus*) or with *n*-butanol (*E. multilocularis*) and that after centrifugation, the 2 supernatants were directly used as starting extracts after being pooled, freeze-dried, dissolved in buffer A and frozen in aliquots at −196°C.

The liver-type AP was extracted by the classical acetone precipitation method (Hua *et al.*, 1986).

Purification of the APs

The parasite APs were purified as previously described (Lawton *et al.*, 1994). Briefly, extracts (0.5 to 2 ml, protein content: 1–2 mg/ml) were thawed and applied to a Concanavalin A-Sepharose column and the APs were recovered by two batchwise elutions with 100 mM α-methyl mannoside and 1% Triton X-100 at 37°C followed by a gel filtration step on a Sephacryl S300 HR column (1.6 × 70 cm). The 2 ml collected fractions were assayed for AP activity and the most active fractions were concentrated and stored in aliquots at −20°C.

The liver AP was similarly purified, but was conventionally eluted from the affinity column with 100 mM α-methyl mannoside.

Enzyme assays and protein determination

One hundred μl of a 20 mM pNPP solution (Sigma 104; final concentration 1 mM) was diluted in 1 M diethanolamine, pH 9.0 containing 0.5 mM $MgCl_2$ and the samples (10–200 μl) were added to the pre-warmed 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 $16.7 \times 10^3\ M^{-1} \cdot cm^{-1}$.

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

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 30V, 15 hr at 100V) at 4°C. The bands were either stained with Coomassie blue R-250 or with silver by a modification of the method of Kirkeby *et al.* (1993) and the apparent molecular weights were determined with Sigma standards.

Deglycosylation

The purified APs were diluted v/v with buffer B (200 mM phosphate buffer, pH 6.5, EDTA 20 mM, 1 U/ml Endoglycosydase F (EC 3.2.1.96) according to Karcz *et al.* (1988) and incubated at 37°C overnight. Controls were incubated in the same buffer without endoglycosidase F.

Phase separation in Triton X-114

The purified APs were diluted v/v with 10 mM Tris-HCl, pH 7.6, 300 mM NaCl, 2% Triton X-114 (buffer C) and processed as described by Bordier (1981), except that the sucrose cushion was omitted and the incubation was performed at 37°C. The aqueous phase was washed twice with the detergent, whereas the detergent-enriched phase was rinsed with buffer C without Triton X-114. The samples

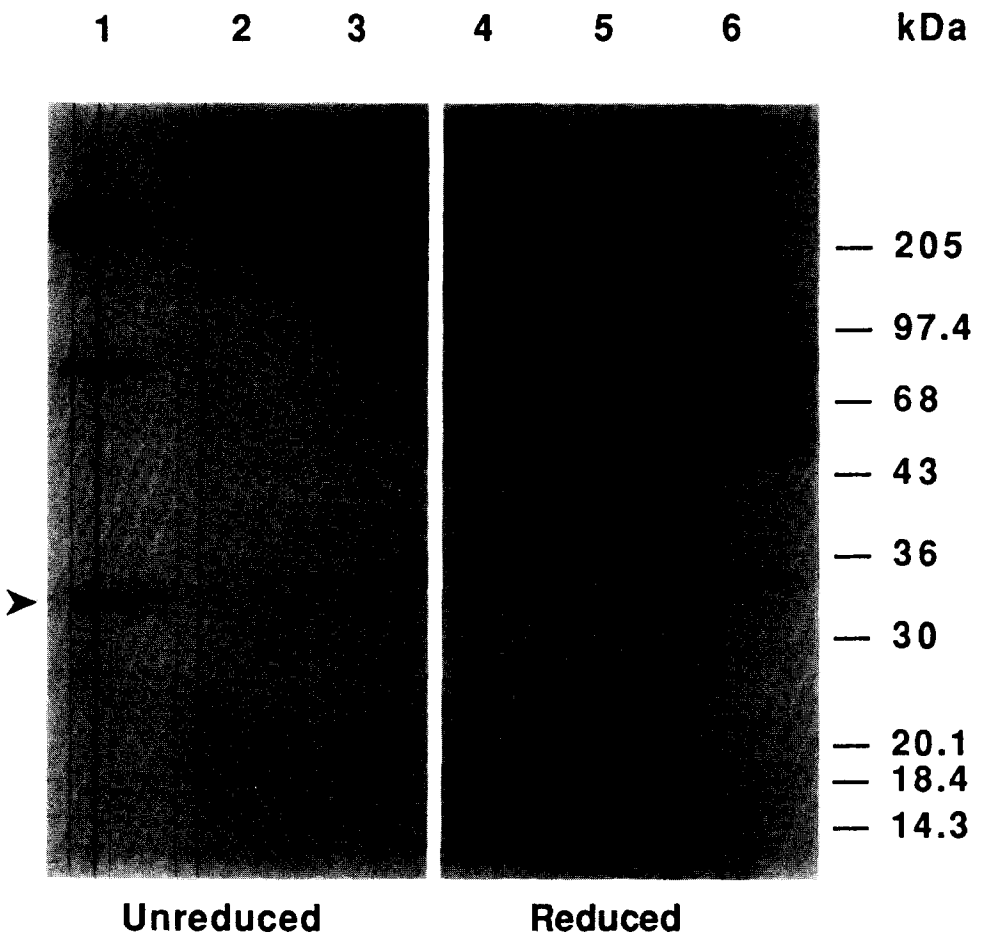

Fig. 1. SDS-PAGE analysis of the purified *Echinococcus* alkaline phosphatases. The gels were run and stained with silver as described in Materials and Methods. Average apparent molecular weights are indicated. Unreduced samples: (1) *E. multilocularis* AP after the first purification step; (2) purified *E. multilocularis* AP; (3) purified *E. granulosus* AP. Reduced samples: (4) purified *E. granulosus* AP; (5) purified and (6) prepurified *E. multilocularis* AP; Arrow: concanavalin A from the column.

were adjusted to the same volume and the AP activity was assayed as above.

Effect of phosphatidylinositol-specific phospholipase C (PI-PLC)

The purified *Echinococcus* APs were incubated with 1 mM $CaCl_2$ and 80 mU/ml PI-PLC for 5 hr at 37°C prior to phase partitioning. Controls were processed similarly in the absence of PI-PLC.

Effect of inhibitors

The following inhibitors: EDTA, $ZnCl_2$, levamisole, L-leucine, L-phenylalanine, L-homoarginine were preincubated for 10 min with triplicates of the purified APs (approximatively 0.1 μg of pure protein) in the reaction buffer before addition of the substrate. The IC_{50} were graphically determined from the relevant curves with 5 different inhibitor concentrations.

Results

The SDS-PAGE analysis of the purified polypeptides showed tetrameric structures of 210 and 220 kDa apparent molecular weights for the *E. granulosus* and *E. multilocularis* APs, respectively, under non-reducing conditions; gel filtration gave similar results (not shown). Under reducing conditions, the *E. multilocularis* enzyme subunit appeared as a lower molecular weight band. After the first purification step, a 80 kDa glycoprotein was also present with the *E. multilocularis* AP (Fig. 1).

N-deglycosylation experiments evidenced that the differential behavior of the two parasite APs in SDS-PAGE was at least partly due to the *N*-linked carbohydrate moieties. The subunit apparent molecular weights of all APs, including the liver enzyme, were identical after treatment with endoglycosidase F

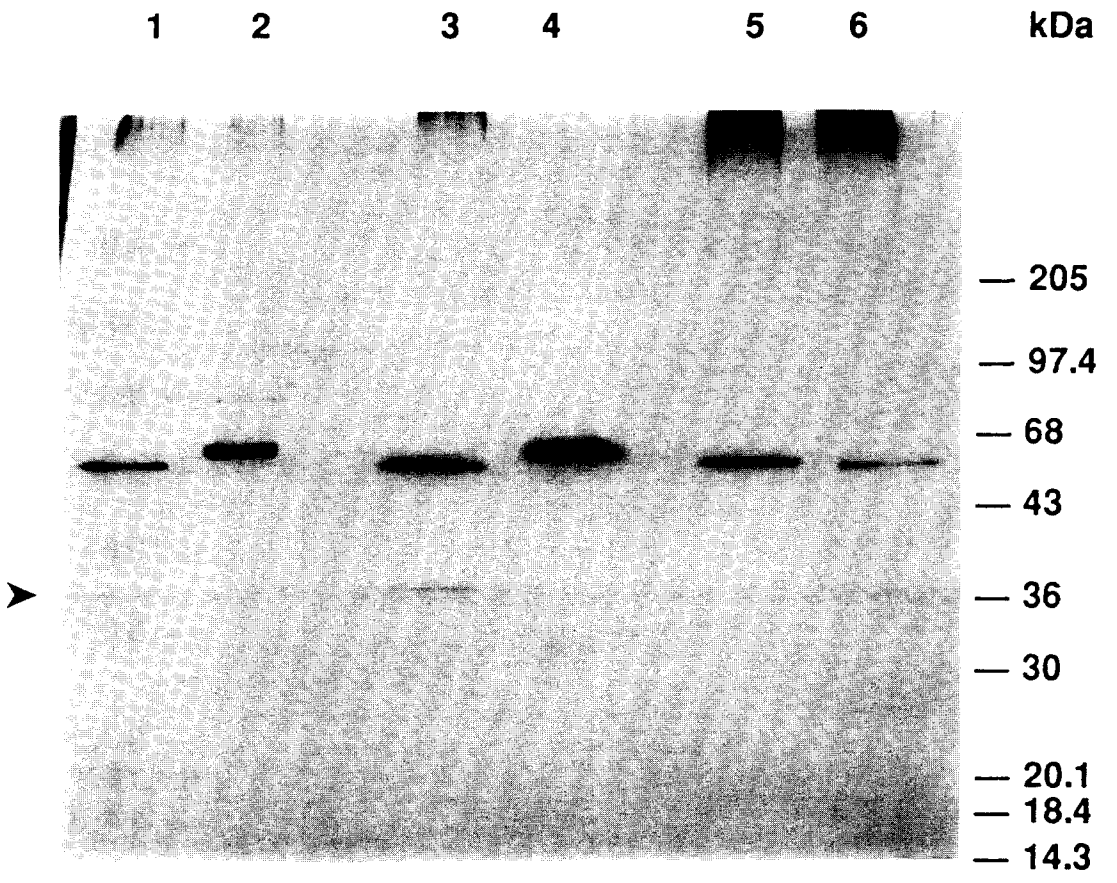

Fig. 2. Effect of *N*-deglycosylation on the apparent molecular weight of the purified APs. Lanes 1,3,6: pure APs after removal of the *N*-linked carbohydrates; Lanes 2,4,5: control enzymes. (1,2) Liver AP; (3,4) *E. granulosus* and (5,6) *E. multilocularis* enzymes. Arrow: Endoglycosydase F.

(Fig. 2). Periodate oxidation gave similar results, except that the electrophoretic migration of the treated enzymes was slightly reduced (not shown).

After Triton X-114 phase separation, the *E. granulosus* AP was mostly recovered in the detergent-depleted phase, but the *E. multilocularis* AP tended to be distributed in both phases, with most of the activity being concentrated in the detergent-enriched phase. After treatment with PI-PLC, the percentage of hydrophobic parasite APs was reduced by about 50% (Table 1).

The temperature sensitivity is shown in Figure 3: the parasite enzymes displayed a striking resistance to heat denaturation, whereas the liver-type AP appeared very heat-sensitive.

Known AP inhibitors were tested on both parasite enzymes (Table 2), which were insensitive to L-leucine and L-phenylalanine and reacted quite similarily to EDTA, whereas $ZnCl_2$, L-homoarginine and levamisole discriminated the parasites and the reference liver enzymes.

Discussion

Our previous studies on the *Echinococcus* APs had evidenced original features concerning their isoelectric points and their catalytic parameters when compared to their respective host liver counterparts (Sarciron *et al.*, 1991; Lawton *et al.*, 1994). Since their potential immunological importance is likely to be due to their structure, a comparative study was then undertaken to highlight their main differential characteristics. A liver AP was chosen as ref-

Table 1. Percentages of hydrophobic forms of the purified APs recovered after phase separation in Triton X-114 before and after removal of their GPI anchor with phosphatidylinositol-specific phospholipase C (PI-PLC)

	Without PI-PLC (%)	With PI-PLC (%)
E. granulosus AP	17.5 ± 2.9 (3)	8.6 ± 3.8 (2)
E. multilocularis AP	78.8 ± 3.5 (3)	45.3 ± 6.0 (3)
Sheep liver AP	1.2 ± 1.1 (3)	ND*

Triplicates were assayed for AP activity. Mean ± S.D. of (*N*) different experiments.
*Not determined.

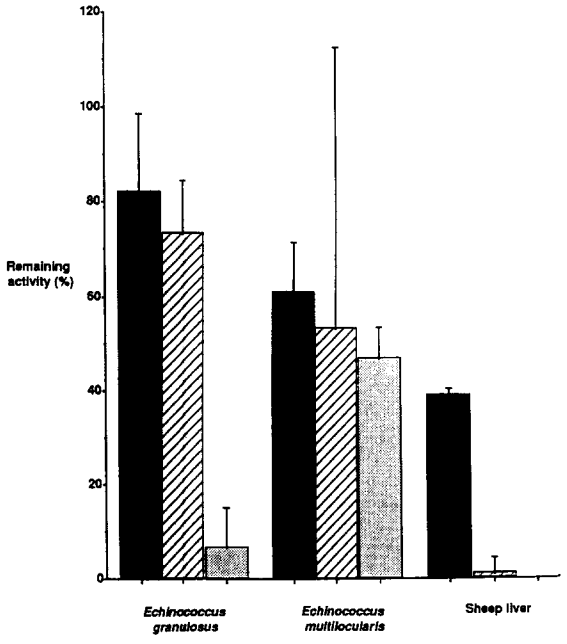

Fig. 3. Resistance to heat denaturation of the parasites AP compared to the liver-type AP. The pure enzymes were incubated for 30 min at 56°C (■), 30 min at 65°C (▨) and 60 min at 65°C (□). Triplicates were then assayed for AP activity and compared to the relevant controls. Results from 3 separate experiments.

erence since both parasites are preferentially located in this organ.

The parasite enzymes were extracted using two different protocols, the *E. multilocularis* AP being more resistant to *n*-butanol (Sarciron *et al.*, 1991). They remained tightly bound to the Concanavalin A-Sepharose column and we thus used the combination of temperature and detergent to elute most of the bound activity. This procedure provided partially purified AP fractions which contained various amounts of concanavalin A. In the *E. multilocularis* preparations, a 80 kDa glycoprotein could also be detected; we do not know if it represents part of an *in vivo* complex or an unrelated protein also tightly bound to the affinity column. This band was not recovered after the subsequent gel filtration step. The enzymes were purified as 210/220 kDa homotetramers, as described for the human liver AP (Hawrylak and Stinson, 1988), consisting of 4, 56/53 kDa subunits. When compared to the *E. granulosus* enzyme, the *E. multilocularis* AP gave a higher band under non-reducing conditions and displayed a lower apparent molecular weight under reducing conditions. However, when treated with endoglycosidase F, both molecules as well as the liver AP, exhibited the same apparent molecular weights indicating that the sugar chain composition of the parasite APs probably account for their electrophoretic properties. Differences in the carbohydrate composition of mammalian APs are common and specific for cell types and/or tissues (Komoda *et al.*, 1989). However, APs being multilocus enzymes (Seargent and Stinson, 1979; Henthorn *et al.*, 1988; Harris, 1989) their protein backbone is probably also involved.

The parasite APs behaved differently during phase separation in Triton X-114: the *E. granulosus* enzyme was mostly distributed like its mammalian host counterpart in the detergent-depleted phase, while the *E. multilocularis* AP displayed marked amphiphilic properties, a difference which could account for its relative resistance to *n*-butanol and acetone extraction (Sarciron *et al.*, 1991). APs are members of the family of glycan phosphatidylinositol (GPI)-anchored proteins (Ogata *et al.*, 1988) and a recent report evidenced the presence of a phosphatidylinositol phospholipase D (Stinson and Hamilton, 1994) present on liver membranes which converts the amphiphilic AP tetramers into hydrophilic dimers. In our case however, the APs remained tetrameric. Our results are closer to those of Bublitz *et al.* (1993) who evidenced 5 types of calf intestinal AP, of which the anchor-containing forms were hydrophobic tetramers and octamers. Treatment of the parasite enzymes with PI-PLC leaves about 50% of their hydrophobic moiety, which could then possess a third

Table 2. Effect of AP inhibitors on the *Echinococcus* and liver-type enzymes

	IC_{50} (mM)		
Inhibitor	*E. granulosus* AP	*E. multilocularis* AP	Sheep liver AP
L-Leucine	N.I.*	30	18
L-Phenylalanine	N.I.	>50	24
L-Homoarginine	0.80	8.80	3
Levamisole	0.55	0.08	0.06
EDTA	0.25	0.40	0.02
$ZnCl_2$	0.60	0.10	0.03

The activity was measured at the relevant optimum pH: 9.0 for the parasite APs; 10.0 for the liver-type enzyme.
*Not inhibitory.

inositol-linked fatty acid residue (Bublitz *et al.*, 1993).

The thermal stability of the *Echinococcus* APs is remarkable. Differential thermostability is thought to be due to organ/tissue-specific, post-translational modifications such as glycosylation (Stinson and Seargeant, 1981) and recently Miura *et al.* (1994) reported that selective removal of the O-linked sugar moieties and sialic acid linkage alters the sensitivity to heat exposure of liver- and bone-type APs. Thermoresistance is also a characteristic of the mammalian placental APs (Harris, 1989), and has also been described in a shrimp AP (Chuang, 1990) and a fruit fly AP (Bourtzis *et al.*, 1993).

Inhibitors which differentiate between the placental, intestinal and bone/liver/kidney alkaline phosphatases (Harris, 1989) showed that the *Echinococcus* APs cannot be grouped with any of these 3 types. In addition, L-homoarginine, levamisole and $ZnCl_2$ can be useful to discriminate between the 2 parasite enzymes and the liver-type AP.

The *Echinococcus* APs thus differ from the mammalian liver-type APs by their isoelectric point (Sarciron *et al.*, 1991; Lawton *et al.*, 1994) and their resistance to heat denaturation; the 3 enzymes can be further distinguished with selected inhibitors and finally, the apparent molecular weight and amphiphilic character discriminate between the *E. granulosus* and the *E. multilocularis* AP, which therefore appears as the most original molecule. Preliminary experiments showed that both enzymes are recognized immunologically by patients, like their counterpart from *S. mansoni* adults (Lien *et al.*, 1992) and that their biochemical interest as chemotherapeutic targets should be enhanced by their immunological importance as markers of parasite viability.

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