



## Isolation, characterization, and ELISA applications of alkaline phosphatase and acetylcholinesterase from *Moniezia expansa*

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### Abstract

*Moniezia expansa* worms are a significant source of alkaline phosphatase (ALP) and acetylcholinesterase (AChE) enzymes. The current study presents a simple and reproducible ALP and AChE purification method from *Moniezia expansa* helminthes by precipitating the proteins with ammonium sulfate and chromatography on the Sephacryl S-300 column. The *M. expansa* ALP purified at 1070.8 U/mg, displaying 6.0 purification folds and 53.6% yield, while *M. expansa* AChE is at 5250 U/mg, displaying 2.0 purification folds and 43% yield. The *M. expansa* ALP isoenzyme displayed its optimum activity at pH 9.6, while the *M. expansa* AChE isoenzyme displayed its optimum activity at pH 8.0. The affinity of *M. expansa* ALP for several substrates revealed that p-nitrophenyl phosphate preferentially cleaved with a Km value of 4.4 mM. *M. expansa* AChE preferentially cleaved acetylthiocholine iodide with a Km value of 0.9 mM. *M. expansa* ALP is strongly stimulated with Co<sup>2+</sup>, Mn<sup>2+</sup>, Ni<sup>2+</sup>, and Mg<sup>2+</sup> and reduced with Zn<sup>2+</sup>, Cu<sup>2+</sup>, Ca<sup>2+</sup>, EDTA and DTT. On the other hand, *M. expansa* AChE is significantly induced with Co<sup>2+</sup>, Zn<sup>2+</sup>, and Ni<sup>2+</sup> and inhibited with Mg<sup>2+</sup>, Ca<sup>2+</sup>, EDTA, 1,10-phenanthroline and eserine. The antisera of the purified *M. expansa* ALP and AChE found effective for determining the two enzymes in different unknown sera from different animal species, including humans, sheep and fish. These results may provide a possible future application of such enzymes in producing ALP and AChE-coated ELISA plates for research purposes.

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### Introduction

Tapeworms *Moniezia expansa* (*M. expansa*) is considered one of the substantial sheep parasites that causes monieziasis and is a fundamental problem in sheep breeding (1,2). The electron microscopy and cytochemical studies of *M. expansa* revealed that the tegumental tissues and the interproglottidal glands of the worms are responsible for secreting alkaline phosphatase and acetylcholinesterase enzymes, signifying the endocrine pathway of its secretion (3,4). The *M. expansa* worms have various cell organelles, secretory vesicles, and glands responsible for secretions of

varied enzymes (5). Alkaline phosphatase catalyzes the hydrolysis of various phosphate monoesters to give the phosphate group and the corresponding alcohol, phenol, or sugar (6). The ALPs are membrane-bound metalloenzymes consisting of different isoenzymes encoded by four gene loci: tissue-nonspecific, placental, intestinal, and germ-cell isoenzymes. The liver, kidney, and bone express comparatively large amounts of the same ALP isoform that designated tissue-nonspecific ALP. Intestinal (IntALP), placental (PALP), and germ cell (GCALP) are designated tissue-specific ALPs (7,8). The tissue-nonspecific ALP widely distributed in nature in virtually all species with high

extents of homology and implicated in different biochemical operations (9). Several ALPs have purified from tissues of many mammals like the liver, placenta, kidney and intestine and commercially used for various purposes (10). The alkaline phosphatase used extensively in molecular procedures as a biological reagent like (i) Removal of the phosphate group from ends of DNA or RNA fragments, (ii) Hybrid protein formation for studying gene expression, (iii) Enzyme attachment to antibodies for ELISA and Immunoblotting assays, (iv) Activation of phosphorescent substrates for DNA and RNA non-radioactive detections. Enzyme-linked immunosorbent Assay (ELISA) and different immunoassays are substantial in quality control and monitoring various biotechnological industries such as pharmaceuticals (11,12). Cholinesterases (ChEs) are specialized carboxylic ester hydrolases that break down the neurotransmitter acetylcholine into choline and acetate at a synaptic cleft. This terminates neurotransmission, allowing another signal to receive. Two ChEs types have specified in mammal tissues, acetylcholinesterase (AChE) and butyrylcholinesterase (BChE), based on their substrate specificities and sensitivity to inhibitors (13). AChE is a membrane-bound enzyme in muscles, the brain, cholinergic nerves, and erythrocytes in various molecular isoforms. A significant neuron enzyme exists in vertebral and invertebrate animals' neuromuscular junctions and cholinergic synapses. Acetylcholinesterase hydrolyzes the acetylcholine to restore the synapse excitability and transmission of the following nervous signals (14). Acetylcholinesterase targets different neurotoxic substances like organophosphates and their nerve agents. Acetylcholine is clinically significant in many neuronal diseases like Alzheimer's and myasthenia gravis (MG), characterized by a sharp decrease in acetylcholine content and diminished cholinergic functions (15). MG is an autoimmune disease characterized by skeletal muscle weakness due to the antibody-mediated processes in which antibodies manufactured having a tropism for acetylcholine receptors or their related proteins situated in postsynaptic membranes of the neuromuscular junctions (16). AChE activity is a suitable biomarker for exposure to many types of pollution in the surrounding environment. In fish, AChE activity is a suitable biomarker for exposure to many pollutants, such as organophosphates and polycyclic aromatic hydrocarbons. Firstly, the excess pollutant molecules trigger the gene expression of AChE till the pollutant concentration is high enough to inhibit AChE (17).

The current study isolates and characterizes two essential enzymes, ALP and AChE, from a new biological source, the helminthic parasite *M. expansa*. Preparation of ALP and AChE antibodies in rabbits and determination of ALP and AChE protein levels in different sheep, human and fish samples by ELISA test carried out.

## Materials and methods

### Ethical approval

The experiment carried out according to the institutional guidelines of the National Research Centre's Animal Research Committee under registration number 19-145: project number 1201034 NRC, Egypt.

### Samples collection

Adult worms of *M. expansa* acquired from the intestine of lambs immediately after slaughter, then washed in 30% salted water at 37 °C and used immediately (18).

### Chemicals

Acetylthiocholine iodide (AcSChI), molecular weight marker kits for gel filtration. benzoylthiocholine iodide (BzSChI), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB), disodium *p*-nitrophenyl phosphate (*p*-NPP). Sephacryl S-300, bovine serum albumin (BSA), glucose 6-phosphate, creatine phosphate, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, phosphoenolpyruvate, propionylthiocholine iodide (PrSChI), 1,4 dithiothritol, butyrylthiocholine iodide (BuSChI),  $\beta$ -mercaptoethanol and *o*-phenylenediamine dihydrochloride (OPD) were purchased from Sigma Chemical Co. Molecular weight marker kits for SDS polyacrylamide gel electrophoresis were products of Pharmacia Fine Chemicals, Uppsala, Sweden. The other chemicals were of analytical grade.

### Alkaline phosphatase activity assay

ALP assay mixture is 500 $\mu$ l of 1M diethanolamine-HCl pH 10.0, including 1 mM MgCl<sub>2</sub>, 4 mM *p*-NPP, and enzyme solution was incubated for 20 min at 37° C and terminated with 500 $\mu$ l 1 N NaOH. The released *p*-nitrophenol monitored at 405 nm, and one ALP unit represents 1 $\mu$  mol *p*-nitrophenol produced per minute (19).

### Acetylcholinesterase activity assay

In the AChE activity assay, 1.0 mL of 60 mM Tris-HCl buffer pH 8.0, comprising 1 mM AcSChI, 1 mM DTNB, and enzyme solution, incubated for 1 hr at 37 °C and the OD was read at 412 nm. One AChE unit was the enzyme amount hydrolyzing 1 $\mu$ mol substrate per hour (20).

### Protein measurement

Determination of protein contents achieved by the dye-binding assay utilizing BSA as a standard (21).

### Purification of *M. expansa* ALP and AChE enzymes

All purification proceedings carried out at 4° C unless reported otherwise. 20 gm of *Moniezia expansa* worms homogenized by Teflon-pestled homogenizer with one volume of 0.05 M Tris-HCl buffer pH 8.0 and centrifuged for 30 min at 12.000 xg and 4° C to acquire the supernatant that designated crude extract. The crude extract was

gradually saturated to 80%  $(\text{NH}_4)_2\text{SO}_4$  with stirring for 30 min at 4 °C and centrifuged for 30 min at 12,000  $\times g$  to acquire the pellet, which was dissolved and extensively dialyzed in 0.05 M Tris-HCl buffer pH 8.0. The concentrated  $(\text{NH}_4)_2\text{SO}_4$  fraction was fractionated utilizing a Sephacryl S-300 resin (142 cm  $\times$  2.4 cm) that previously equilibrated with the Tris-HCl buffer pH 8.0 and run with the same buffer at 30 ml / h flow rate. This chromatographic step repeated two times, the first for separating *M. expansa* ALP and the second for *M. expansa* AChE. In each chromatographic step, the column fractions tested for the activity of the desired enzyme (14).

#### Determination of the native molecular mass

For estimation of the native molecular weights of *M. expansa* ALP and *M. expansa* AChE, the Sephacryl column calibrated with the protein ferritin, catalase, alcohol dehydrogenase, bovine serum albumin, and myoglobin of the known molecular weights 440, 240, 150, 67 and 17 kDa respectively as standard markers (4).

#### Electrophoretic analysis

Electrophoresis analysis of *M. expansa* ALP and AChE was carried out on 12% SDS-PAGE, the subunit molecular weights of both enzymes were determined, and the protein staining was achieved utilizing 0.25% Coomassie BBR-250 (22).

#### Substrate specificity and enzyme Kinetics

The substrate specificity of *M. expansa* ALP screened toward the substrates, *p*-nitrophenyl phosphate (*p*-NPP), glucose 6-phosphate, creatine phosphate, adenosine monophosphate, adenosine diphosphate, adenosine triphosphate, thiamine pyrophosphate, and phosphoenol pyruvate. The *K<sub>m</sub>* value of *M. expansa* ALP derived from the plot constructed for the reaction velocity (V) versus the substrate (*p*-NPP) concentrations (0.1 - 16 mM). In addition, the substrate specificity of *M. expansa* AChE screened toward the substrates, AcSChI-, PrSChI-, BuSChI-, and BzSChI iodides. The *K<sub>m</sub>* derived from the plot constructed for the reaction velocity (V) versus the substrate (AcSChI) concentrations (0.1 - 3.0 mM) (23).

#### Preparation of *M. expansa* ALP and AChE antibodies in rabbits

Enzyme antibodies were prepared according to the technique adopted by Toaleb *et al.* (24). Briefly, four rabbits (two for each enzyme of an average weight of 1.25 Kg/each) were subcutaneous immunized using  $\approx 40$   $\mu\text{g}$  from each of *M. expansa* ALP and AChE proteins in Freund's complete adjuvant. A second  $\approx 40$ - $\mu\text{g}$  protein booster dose in Freund's incomplete adjuvant was provided on day 15. Two further doses of about  $\approx 40$ - $\mu\text{g}$  protein each without adjuvant also given on days 21 and 28. Serum samples were collected four

days after the last immunization. These sera were stored at -20 °C until used in ELISA to determine ALP and AChE protein levels in random serum samples.

#### Collection of different serum samples

Different serum samples from different hosts (sheep, human, and fish) subjected to different circumstances were collected and stored at -20°C until analysis was used for detection of ALP and AChE (2).

#### Enzyme-linked immunosorbent assay (ELISA)

*M. expansa* ALP and AChE protein levels determined by a semi-quantitative ELISA test (25). The optimum antigen, serum, and conjugate concentrations previously determined by check board titration (26). Enzyme antisera diluted to  $\approx 30$   $\mu\text{g/ml}$  concentration in 0.05 M Na-bicarbonate buffer pH 9.5 and 100  $\mu\text{l}$ , which adsorbed to a polystyrene 96-well microtiter plate overnight at 4°C. The plates subjected to three washes using Tween-20 phosphate-buffered saline (TPBS) pH 7.4, leaving the last wash in the wells for about 5 min as a soaking step. The wells then blocked for unspecific binding using 200  $\mu\text{l}$  of 5% non-fat dry milk in PBS for 1h at room temperature. After washing, 100  $\mu\text{l}$  of fish, sheep, and human sera at dilution 1:50 in PBS were added to the wells and incubated at 37°C for 2h. After washing, 100- $\mu\text{l}$  secondary antibody (Horseradish peroxidase-conjugated Anti-sheep IgG for sheep samples, Anti-human for human samples, and protein A for fish samples at 1:1000 dilution in PBS) added to the wells and incubated at 37 °C for 1h. The last washing step was repeated 5 times and followed by adding 100  $\mu\text{l}$  OPD 60 mg tablets in 0.15 M Na-phosphate + 0.05 M Na-citrate buffer, pH 5.7 + 0.012%  $\text{H}_2\text{O}_2$ . The reaction was developed for about 30 min, terminated by adding 100  $\mu\text{l}$  1N NaOH, and absorbance was monitored at 450 nm utilizing an ELISA plate reader (27).

#### Results

##### Purification of *M. expansa* ALP and AChE

The starting specific activity of the ALP in the crude extract of the *M. expansa* worms found to be 178.1 units/mg protein (Table 1). This crude extract brought to 80%  $(\text{NH}_4)_2\text{SO}_4$  saturation, stirred, and centrifuged for 30 min at 12,000- $\times g$ , and the obtained pellet dissolved and dialyzed in 0.05 M Tris-HCl buffer pH 8.0. Most of the ALP activity acquired in this fraction, and 80.1% enzyme activity retrieved, yielding a specific activity of 248.3 units/mg protein. The 80%  $(\text{NH}_4)_2\text{SO}_4$  fraction was fractionated through a Sephacryl S-300 column, resulting in one peak exhibiting ALP activity resolved and designated *M. expansa* ALP (Figure 1a). The *M. expansa* ALP-specific activity was increased to 1070.8 units/mg protein, representing 6 purification folds with recovery of 53.6% (Table 1).

Table 1: A typical purification scheme of *M. expansa* worms ALP and AChE

Purification steps		Total protein (mg)	Total activity (unit)	Specific activity	Yield (%)	Fold purification
ALP	Worm crude extract	83	14785	178.1	100	1.0
	80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> Fraction	47.7	11843	248.3	80.1	1.4
	Sephacryl S-300 fraction	7.4	7924	1070.8	53.6	6.0
AChE	Worm crude extract	42	110000	2619	100	1.0
	80% (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> fraction	29	93234	3213	84.8	1.23
	Sephacryl S-300 fraction	9	47254	5250	43	2

Concerning AChE, the starting specific activity in the crude extract of the *M. expansa* worms found to be 2619 units/mg protein. This crude extract brought to 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> saturation, stirred, and centrifuged for 30 min at 12,000-x g, and the obtained pellet dissolved and dialyzed in 0.05 M Tris-HCl buffer pH 8.0. Most of the AChE activity acquired in this fraction, and 84.8% enzyme activity retrieved, yielding a specific activity of 3213 units/mg protein. The 80% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> fraction fractionated through a Sephacryl S-300 column, which showed one peak exhibited AChE activity was resolved and designated *M. expansa* AChE (Figure 1b). The *M. expansa* AChE-specific activity increased to 5250 units/mg protein of 2 purification folds and recovery of 43% (Table 1).

### Molecular weight determination

The native molecular weight of both enzymes deduced from their elution volumes from the gel filtration column to be 312 kDa for *M. expansa* ALP (Figure 1a) and 270 kDa for *M. expansa* AChE (Figure 1b)

### Electrophoretic analyses of *M. expansa* ALP and AChE enzymes

To determine subunit molecular weights of the separated *M. expansa* ALP and AChE, the denatured ALP and AChE electrophoretically analyzed on 12% SDS-PAGE compared to molecular weight marker proteins. On SDS-PAGE, *M. expansa* ALP was found as one band of 312 kDa (Figure 2a), while *M. expansa* AChE was one band of 90 kDa subunit (Figure 2b).

### Estimation of optimum pH

The impact of pH on *M. expansa* ALP activity examined in diethanolamine buffer, while AChE in Tris-HCl buffer of various pH values. The *M. expansa* ALP pH profile displayed its maximum activity at pH 9.6 (Figure 3a), while *M. expansa* AChE displayed its maximum activity at pH 8.0 (Figure 3b).

### Substrate specificity and enzyme Kinetics

The substrate specificity of *M. expansa* ALP and AChE screened toward various substrates. *M. expansa* ALP cleaved preferentially *p*-NPP (100% relative activity) followed by ATP 17.3% and displayed the lowest activity toward phospho-enol-pyruvate 3.8%. Furthermore, *M. expansa*

AChE cleaved preferentially AcSChI (100% relative activity) followed by PrSChI 55.7% and displayed the lowest activity toward BzSChI 13.4% (Table 2).

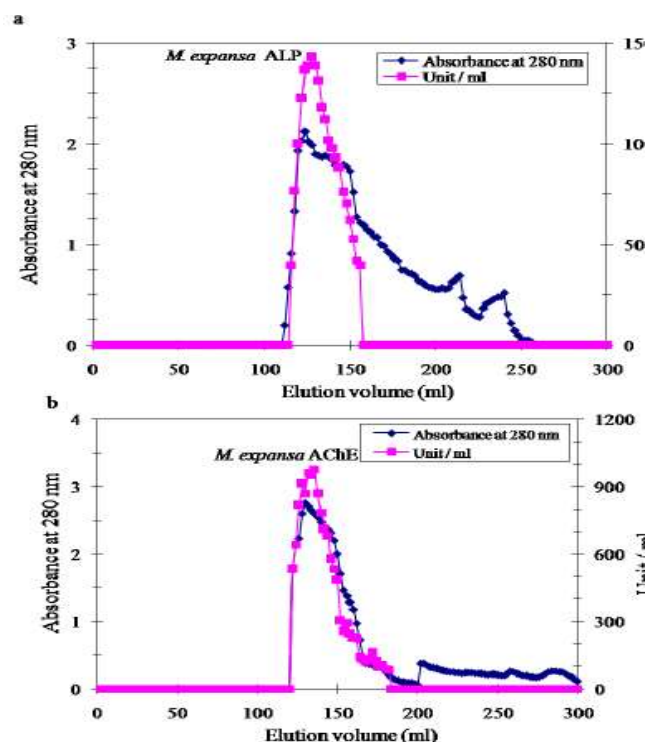


Figure 1: A typical elution profile for the chromatography of the *M. expansa* ammonium sulfate fraction on Sephacryl S-300 column (142 cm x 2.4 cm) previously equilibrated with 0.05 M Tris-HCl buffer, pH 8.0 (a): *M. expansa* ALP and (b): *M. expansa* AChE.

The *M. expansa* ALP incubated with increasing concentrations of *p*-NPP, and a definite amount of the enzyme was utilized to construct the plot for the reaction velocity (V) versus the substrate concentration [S] (Figure 3c). The *M. expansa* ALP obeyed the Michaelis-Menten kinetics, and a linear relationship between (V) and [S] obtained and ended with a steady state at the maximum velocity. *M. expansa* AChE incubated with increasing concentrations of AcSChI, and a definite amount of the enzyme utilized to construct the plot for

the reaction velocity (V) versus the substrate concentration [S] (Figure 3d). The *M. expansa* AChE obeyed the Michaelis-Menten kinetics, and a linear relationship between (V) and [S] obtained and ended with a steady state at the maximum velocity. The Km value found to be 0.9 mM AcSChI.

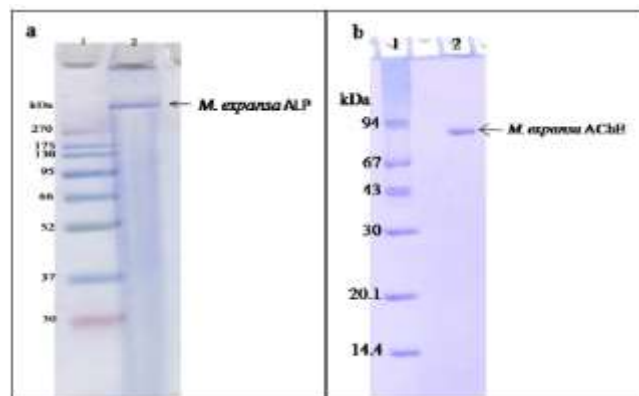


Figure 2: Subunit molecular weight determination by electrophoretic analysis of *M. expansa* ALP and AChE on 12% SDS-PAGE (a): *M. expansa* ALP and (b): *M. expansa* AChE.

#### Effect of divalent cations and inhibitors

The purified *M. expansa* ALP and AChE pre-incubated with 2 and 4 mM of each cation at 37°C, and the activity then assayed. The activity of *M. expansa* ALP increased in the presence of 4 mM CoCl<sub>2</sub>, MnCl<sub>2</sub>, NiCl<sub>2</sub>, and MgCl<sub>2</sub>. Contrariwise, ZnCl<sub>2</sub>, CuCl<sub>2</sub>, and CaCl<sub>2</sub> were potent inhibitors of *M. expansa* ALP. Further, the activity of *M. expansa* AChE increased in the presence of CoCl<sub>2</sub>, ZnCl<sub>2</sub>, and NiCl<sub>2</sub> and decreased with MgCl<sub>2</sub> and CaCl<sub>2</sub> ions (Table 3).

In addition, the purified *M. expansa* ALP and AChE pre-incubated with each inhibitor at 37°C for 5 min, and the residual activity then assayed. EDTA and DL-dithiothreitol (DTT) inhibited *M. expansa* ALP activity at 76.6 and 93.8%,

respectively, while a considerable resistance of the enzyme was detected toward the inhibitors β-mercaptoethanol and 1, 10-phenanthroline. On the other hand, EDTA, 1, 10-phenanthroline, and eserine potently inhibited *M. expansa* AChE activity at 92.7, 50.8 and 70.1%, respectively, while a moderate, inhibition of the enzyme detected toward β-mercaptoethanol and DTT (Table 4).

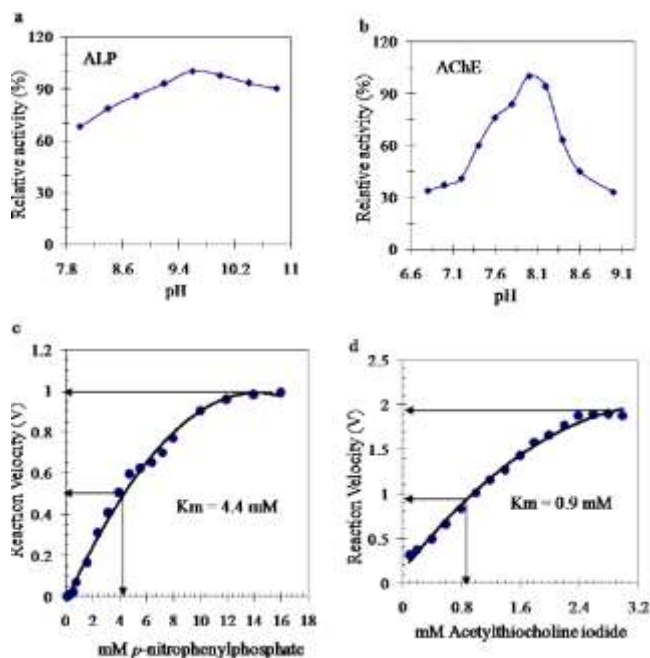


Figure 3: (a): Effect of pH on the purified *M. expansa* ALP using diethanolamine buffer, pH (8.0-10.8). (b): Effect of pH on the purified *M. expansa* AChE using Tris-HCl buffer pH (6.8 - 9). (c): Effect of the substrate *p*-nitrophenyl phosphate concentration in mM on the reaction velocity of the purified *M. expansa* ALP. (d): Effect of the substrate acetylthiocholine iodide (AcSChI) concentration in mM on the reaction velocity of the purified *M. expansa* AChE.

Table 2: Substrate specificity of *M. expansa* ALP and AChE enzymes

Substrate	Concentration	Rate of hydrolysis	Relative Activity (%)	
ALP	<i>p</i> -Nitrophenyl phosphate (p-NPP)	4 mM	0.684	100.0
	Glucose-6-phosphate sodium salt	4 mM	0.104	15.2
	Adenosine monophosphate (AMP)	4 mM	0.08	11.7
	Adenosine diphosphate (ADP)	4 mM	0.038	5.6
	Adenosine triphosphate (ATP)	4 mM	0.118	17.3
	Naphthyl phosphate	4 mM	0.092	13.6
	Creatine phosphate	4 mM	0.035	5.1
	Phospho-enol-pyruvate	4 mM	0.026	3.8
AChE	Acetylthiocholine iodide (AcSChI)	1 mM	0.524	100.0
	Propionylthiocholine iodide (PrSChI)	1 mM	0.292	55.7
	Butyrylthiocholine iodide (BuSChI)	1 mM	0.104	19.8
	Benzoylthiocholine iodide (BzSChI)	1 mM	0.07	13.4

Table 3: Effect of divalent cations on the *M. expansa* ALP and AChE enzymes

Reagent	Concentration (mM)	Residual activity (%)	
		ALP	AChE
Control	-----	100.0	100.0
CoCl <sub>2</sub>	2.0	102.2	121.9
	4.0	110.1	186.8
MnCl <sub>2</sub>	2.0	140.7	98.0
	4.0	189.5	95.6
FeCl <sub>2</sub>	2.0	93.5	101.3
	4.0	105.9	98.4
ZnCl <sub>2</sub>	2.0	52.6	119.3
	4.0	42.1	167.1
CuCl <sub>2</sub>	2.0	93.9	94.9
	4.0	77.6	90.8
NiCl <sub>2</sub>	2.0	104.3	151.7
	4.0	123.6	184.2
MgCl <sub>2</sub>	2.0	100.8	69.2
	4.0	114.4	56.4
CaCl <sub>2</sub>	2.0	78.9	98.2
	4.0	57.8	97.3

\* These values represent % of the control and the means of triplicate experiments.

Table 4: Effect of inhibitors on the *M. expansa* worms ALP and AChE

Reagent	Concentration (mM)	Inhibition (%)	
		ALP	AChE
Control	-----	0.0	0.0
EDTA	5 mM	76.6	92.7
DTT	5 mM	93.8	14.2
β-Mercaptoethanol	5 mM	1.2	30.4
1,10 Phenanthroline	5 mM	0.8	50.8
Eserine	5 μM	-	70.1

\* These values represent % of the control and the means of triplicate experiments.

**Determination of *M. expansa* ALP and AChE protein levels in sheep, human, and fish samples**

Concerning the *M. expansa* ALP level in random sheep samples by ELISA, the OD values ranged from 0.201 to 0.427. In humans, the OD values of *M. expansa* ALP recorded higher values than sheep ones. They ranged from 0.444 to 1.218. In fish samples, OD values of *M. expansa* ALP recorded different values in the different treated samples; values in B-a-P treated samples ranged from 0.453 to 0.858, values in Zn treated samples ranged from 0.612 to 0.793, and the pesticides group recorded OD values ranged from 0.503 to 0.672 (Figure 4).

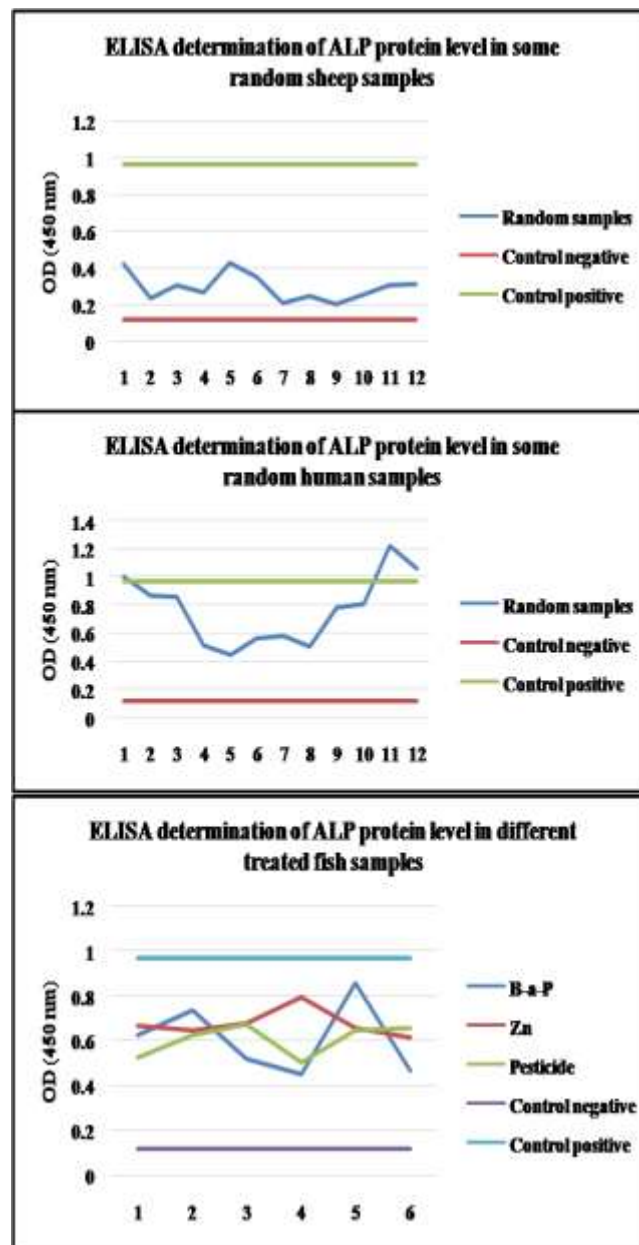


Figure 4: ELISA determination of *M. expansa* ALP protein level in different sheep, human, and fish samples.

Concerning the *M. expansa* AChE level in some random sheep samples by ELISA test, the OD values ranged from 0.149 to 0.499. Similarly, human samples recorded more *M. expansa* AChE values than sheep samples, ranging from 0.522 to 1.208. While in fish samples, the highest *M. expansa* AChE values recorded in B-a-P treated samples, followed by pesticides-treated samples, and finally, the Zn treated ones, at ranges of 0.708 to 0.922, 0.565 to 0.785, and 0.432 to 0.596, respectively (Figure 5).

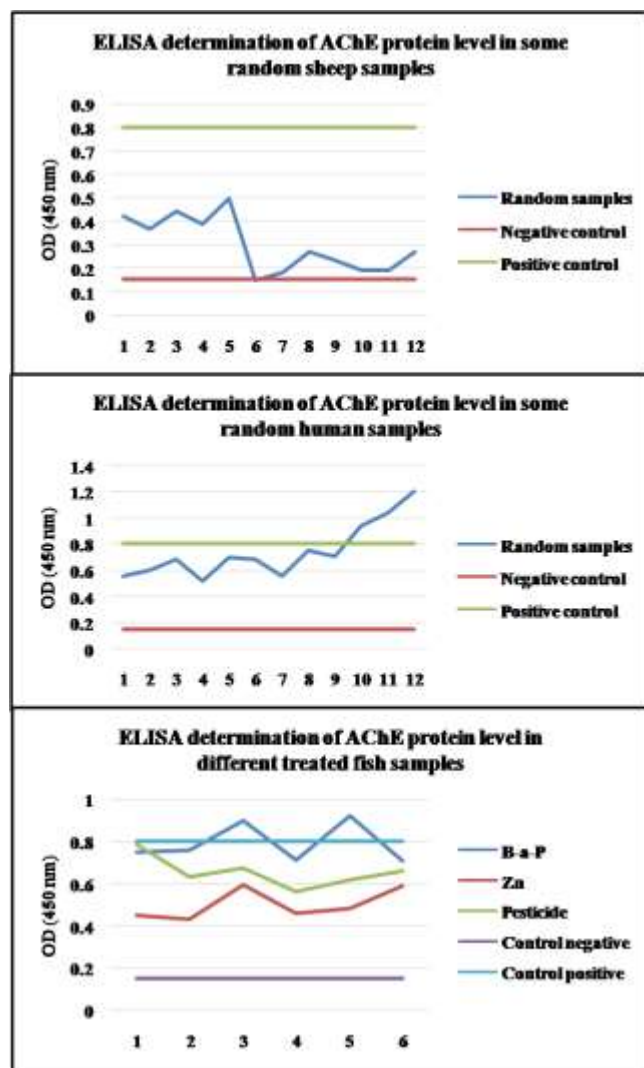


Figure 5: ELISA determination of *M. expansa* AChE protein level in different sheep, human, and fish samples.

## Discussion

The tegumental tissues and the interproglottidal glands of *M. expansa* considered a significant source of ALP and AChE enzymes (3). ALP utilized exhaustively as a reagent in various molecular biological tests such as studying gene expression, ELISA, and the non-radioactive detection of DNA and RNA (11,12). It previously purified from different sources, such as the brain of *Fenneropenaeus merguensis* crustaceans (28), from hen's egg yolk (29), and bovine milk (30). The activity and inhibition of AChE have known as specific human biomarkers for pesticide toxicity. Measuring AChE inhibition or activity has been utilized as a biomarker for pesticide exposure effect on the nervous system in environmental and occupational medicine. The success of using AChE as a biomarker was due to the ease of measuring

the response, the dose-dependent behavior to toxin exposure, the sensitivity, and exhibiting a link to health harm effects (31).

The chromatography of *M. expansa*  $(\text{NH}_4)_2\text{SO}_4$  fraction on the Sephacryl S-300 column showed one activity peak for both ALP and AChE enzymes. The specific activity of *M. expansa* ALP is 1070.8 U/mg, displaying 6.0 purification folds and 53.6% yield. Furthermore, the specific activity of *M. expansa* AChE is 5250 U/mg, displaying 2 purification folds and 43% yield. A broad range of purification folds and recovery percentages for ALP and AChE reported. ALP was purified from non-pasteurized milk with 17.67 folds and 56% yield (32), water buffalo liver with 10.56 and 17.92 folds with 17.1 and 24.5% yields, and from lactic acid bacteria with 36.07 folds with 23.74% yield (33). The enzyme AChE was purified from human erythrocytes 658 folds with a 23.5% yield (34). Based on the protein molecular weights, *M. expansa* ALP molecular mass deduced from the gel filtration chromatography as 312 kDa, confirmed as a monomer protein by the visualization on the SDS PAGE. These results are different from that of 190 and 180 kDa beef brain ALPs, 172 kDa bovine kidney (35), 146 kDa human liver (36), and 188 and 194 kDa water buffalo liver (33). On the other hand, *M. expansa* AChE molecular mass deduced from the gel filtration chromatography as 270 kDa, while by the visualization on the SDS PAGE, it seen as 90 kDa representing a homotrimer protein. This *M. expansa* AChE molecular mass is more significant than 245.5 kDa quail brain AChE (37), 201.5 kDa sheep liver AChE (38), and lower than 340 kDa cattle AChE (39).

The  $K_m$  value of the purified *M. expansa* ALP was 4.4 mM *p*-NPP, more significant than 0.927 mM for non-pasteurized milk ALP (32) and 2.14 mM for lactic acid bacteria ALP (40). The divalent ions  $\text{Co}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Mg}^{2+}$  increased the activity of *M. expansa* ALP, while  $\text{Zn}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Ca}^{2+}$  inhibited it. On the other hand,  $\text{Co}^{2+}$ ,  $\text{Zn}^{2+}$ , and  $\text{Ni}^{2+}$  increased the activity of *M. expansa* AChE, while  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  hindered it. In agreement with this report, the metal ions  $\text{Mg}^{2+}$ ,  $\text{Ca}^{2+}$ ,  $\text{Ba}^{2+}$ ,  $\text{Mn}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Ni}^{2+}$ , and  $\text{Cd}^{2+}$  activated the green crab ALP, while  $\text{Hg}^{2+}$ ,  $\text{Ag}^+$ ,  $\text{Bi}^{2+}$ ,  $\text{Cu}^{2+}$ , and  $\text{Zn}^{2+}$  inhibit the enzyme (6) and the lactic acid bacteria ALP was activated by  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  and inhibited with  $\text{Cu}^{2+}$  and  $\text{Zn}^{2+}$  (40). Furthermore, red palm weevil AChE was greatly enhanced with  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$  (32), and the entomopathogenic nematode AChE was strongly inhibited with  $\text{Hg}^{+2}$  and  $\text{Ni}^{+2}$  and stimulated by  $\text{Mn}^{+2}$  (20).

ALP plays a substantial role in growth through cell signal transduction and regulation of the cell cycle and used as a clinical diagnosis biomarker in various diseases (9). It represents a high research interest enzyme since it has many industrial applications. The ALP labeling routinely used in enzyme immunoassays, such as the ELISA technique. In addition, it can used in pharmaceutical and clinical analysis by using different ALPs conjugated to Zn and Mg (40). On the other hand, AChE activity is a suitable biomarker for

exposure to many types of pollution in the surrounding environment. In fish, AChE activity is a suitable biomarker for exposure to many pollutants such as organophosphates and polycyclic aromatic hydrocarbons. Excess pollutant molecules activate gene expression of AChE until the pollutant concentration is high enough to inhibit AChE (17).

## Conclusion

In conclusion, this study presents an established, easy and reproducible purification procedure of alkaline phosphatase and acetylcholine esterase enzymes from tapeworms, *M. expansa*, as a new biological available source. Production of both *M. expansa* ALP and AChE enzymes on a large scale will allow their use in various applications. The antisera of the purified *M. expansa* ALP and AChE are effective for determining both enzymes in different unknown sera from different animal species. This provides a possible future application of purified enzyme antibodies in producing ALP and AChE-coated ELISA plates, which will less charged than those will imported for research purposes.

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## Conflict of interest

The authors have declared no conflict of interest.

## References

1. Yan H, Bo X, Liu Y, Lou Z, Ni X, Shi W, Zhan F, Ooi H, Jia W. Differential diagnosis of *Moniezia benedeni* and *M. expansa* (Anoplocephalidae) by PCR using markers in small ribosomal DNA (18S rDNA). *Acta Vet Hung.* 2013;61(4):463-472. DOI: [10.1556/AVet.2013.035](https://doi.org/10.1556/AVet.2013.035)
2. Mahmoud MA, Ghazy AA, Shaapan RM. Review of diagnostic procedures and control of some viral diseases causing abortion and infertility in small ruminants in Egypt. *Iraqi J Vet Sci.* 2021;35:513-521. DOI: [10.33899/ijvs.2020.127114.1461](https://doi.org/10.33899/ijvs.2020.127114.1461)
3. Hassanain MA, Shaapan RM, Khalil FA. Sero-epidemiological value of some hydatid cyst antigen in diagnosis of human cystic echinococcosis. *J Parasit Dis.* 2016;40(1):52-56. DOI: [10.1007/s12639-014-0443-5](https://doi.org/10.1007/s12639-014-0443-5)
4. Bashtar A, Hassanein M, Abdel-Ghaffar F, Al-Rasheid K, Hassan S, Mehlhorn H, AL-Mahdi M, Morsy K, Al-Ghamdi A. Studies on monieziasis of sheep I. Prevalence and antihelminthic effects of some plant extracts, a light and electron microscopic study. *Parasitol Res.* 2011;108:177-186. DOI: [10.1007/s00436-010-2060-2](https://doi.org/10.1007/s00436-010-2060-2)
5. Abdel-Shafy S, Ghazy AA, Shaapan RM. Applications of electron microscopy in ticks: Description, detection of pathogens, and control. *Comp Clin Pathol.* 2019;28(3):585-592. DOI: [10.1007/s00580-018-2786-2](https://doi.org/10.1007/s00580-018-2786-2)
6. Azpiazu D, Gonzalo S, Villa-Bellosta R. Tissue non-specific alkaline phosphatase and vascular calcification: A potential therapeutic target.

*Curr Cardiol Rev.* 2019;15(2):91-95. DOI: [10.2174/1573403X14666181031141226](https://doi.org/10.2174/1573403X14666181031141226)

7. Matsushita M, Irino T, Oh-Le K, Komoda T. Specific gel electrophoresis method detects two isoforms of human intestinal alkaline phosphatase. *Electrophoresis.* 2000;21:281-284. DOI: [10.1002/\(SICI\)1522-2683\(2000101\)21](https://doi.org/10.1002/(SICI)1522-2683(2000101)21)
8. Liedtke D, Hofmann C, Jakob F, Klopocki E, Graser S. Tissue-nonspecific alkaline phosphatase—a gatekeeper of physiological conditions in health and a modulator of biological environments in disease. *Biomol.* 2020;10(12):1648. DOI: [10.3390/biom10121648](https://doi.org/10.3390/biom10121648)
9. Bartlett CL, Cave EM, Crowther NJ, Ferris WF. A new perspective on the function of tissue non-specific alkaline phosphatase: from bone mineralization to intra-cellular lipid accumulation. *Mol Cell Biochem.* 2022;477:2093-2106. DOI: [10.1007/s11010-022-04429-w](https://doi.org/10.1007/s11010-022-04429-w)
10. Lallès J. Intestinal alkaline phosphatase: novel functions and protective effects. *Nutr Rev.* 2014;72:82-94. DOI: [10.1111/nure.12082](https://doi.org/10.1111/nure.12082)
11. Roy I, Gupta MN. Purification of alkaline phosphatase from chicken intestine by expanded-bed affinity chromatography on dye-linked cellulose. *Biotechnol Appl Biochem.* 2000;32:81-87. DOI: [10.1042/ba20000021](https://doi.org/10.1042/ba20000021)
12. Li Y, Ma X, Xu Z, Liu M, Lin Z, Qiu B, Guo L, Chen G. Multicolor ELISA based on alkaline phosphatase-triggered growth of Au nanorods. *Analyst.* 2016;141(10):2970-2976. DOI: [10.1039/c6an00117c](https://doi.org/10.1039/c6an00117c)
13. Heo JH, Eom BH, Ryu HW, Kang M, Park JE, Kim D, Kim J, Park D, Oh S, Kim H. Acetylcholinesterase and butyrylcholinesterase inhibitory activities of khellactone coumarin derivatives isolated from *Peucedanum japonicum* Thunberg. *Sci Rep.* 2020;10:21695. DOI: [10.1038/s41598-020-78782-5](https://doi.org/10.1038/s41598-020-78782-5)
14. Sarkar B, Alam S, Rajib TK, Islam SS, Araf Y, Asad Ullah M. Identification of the most potent acetylcholinesterase inhibitors from plants for possible treatment of Alzheimer's disease: A computational approach. *Egypt J Med Hum Genet.* 2021;22:10 DOI: [10.1186/s43042-020-00127-8](https://doi.org/10.1186/s43042-020-00127-8)
15. Haga T. Molecular properties of muscarinic acetylcholine receptors. *Proc Jpn Acad Ser B Phys Biol Sci.* 2013;89(6):226-56. DOI: [10.2183/pjab.89.226](https://doi.org/10.2183/pjab.89.226)
16. Koneczny I, Herbst R. Myasthenia gravis: Pathogenic effects of autoantibodies on neuromuscular architecture. *Cells.* 2019;8(7):671. DOI: [10.3390/cells8070671](https://doi.org/10.3390/cells8070671)
17. Olivares-Rubio HF, Espinosa-Aguirre JJ. Acetylcholinesterase activity in fish species exposed to crude oil hydrocarbons: A review and new perspectives. *Chemosphere.* 2021;264 (1):128401. DOI: [10.1016/j.chemosphere.2020.128401](https://doi.org/10.1016/j.chemosphere.2020.128401)
18. Shaapan RM, Toaleb NI, Abdel-Rahman EH. Significance of a common 65 kDa antigen in the experimental fasciolosis and toxoplasmosis. *J Parasit Dis.* 2015;39:550-556. DOI: [10.1007/s1263901303942](https://doi.org/10.1007/s1263901303942)
19. Iyer SK, Daron HH, Aull JL. Purification and properties of alkaline phosphatase from boar seminal plasma. *J Reprod Fert.* 1988;82:657-664. DOI: [10.1530/jrf.0.0820657](https://doi.org/10.1530/jrf.0.0820657)
20. Ellman GL, Courtney D, Andros V, Featherston RM. A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochem Pharmacol.* 1961;7(2):88-95. DOI: [10.1016/0006-2952\(61\)90145-9](https://doi.org/10.1016/0006-2952(61)90145-9)
21. Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976;72:248-254. DOI: [10.1006/abio.1976.9999](https://doi.org/10.1006/abio.1976.9999)
22. Laemmli UK. Cleavage of structural proteins during the assembly of the head of Bacteriophage T4. *Nature.* 1970;227:680-685. DOI: [10.1038/227680a0](https://doi.org/10.1038/227680a0)
23. Le Du MH, Lamoure C, Muller BH, Bulgakov OV, Lajeunesse E, Menez A, Boulain JC. Artificial evolution of an enzyme active site: structural studies of three highly active mutants of *Escherichia coli* alkaline phosphatase. *J Mol Biol.* 2002;316:941-953. DOI: [10.1006/jmbi.2001.5384](https://doi.org/10.1006/jmbi.2001.5384)
24. Toaleb NI, Shaapan RM, Abdel-Rahman EH. Adoption of immuno-affinity isolated *Fasciola gigantica* fraction for diagnosis of ovine toxoplasmosis. *Global Vet.* 2014;12(1):140-145. [\[available at\]](#)



25. Shaapan RM, Toaleb NI, Abdel-Rahman EH. Detection of *Toxoplasma gondii*-specific immunoglobulin (IgG) antibodies in meat juice of beef. *Iraqi J Vet Sci*. 2021;35:319-324. DOI: [10.33899/ijvs.2020.126829.1390](https://doi.org/10.33899/ijvs.2020.126829.1390)
26. Alanazi AD, Abdullah S, Helps C, Wall R, Puschendorf R, ALHarbi SA, Abdel-Shafy S, Shaapan RM. Tick-borne pathogens in ticks and blood samples collected from camels in Riyadh province, Saudi Arabia. *Int J Zool Res*. 2018;14(1):30-36. DOI: [10.3923/ijzr.2018.30\\_36](https://doi.org/10.3923/ijzr.2018.30_36)
27. Elaadli H, Abo El-Makarem H, Abd Elrahma AH, Shaapan RM, Bessat M. Prevalence and associated risk factors of *Toxoplasma gondii* infection in sheep and aborted women in Egypt. *Iraqi J Vet Sci*. 2023;37(2):437-445. DOI: [10.33899/ijvs.2022.135777.2518](https://doi.org/10.33899/ijvs.2022.135777.2518)
28. Homaei A. Purification and biochemical properties of highly efficient alkaline phosphatase from *Fenneropenaeus merguensis* brain. *J Mol Catal B: Enzym*. 2015;118:16-22. DOI: [10.1016/j.molcatb.2015.04.013](https://doi.org/10.1016/j.molcatb.2015.04.013)
29. Farzi-Khajeh H, Safa KD, Dastmalchi S. Arsanilic acid modified superparamagnetic iron oxide nanoparticles for purification of alkaline phosphatase from hen's egg yolk. *J Chromatogr B Analyt Technol Biomed Life Sci*. 2017;1061-1062: 26-33. DOI: [10.1016/j.jchromb.2017.06.04](https://doi.org/10.1016/j.jchromb.2017.06.04)
30. Farzi-Khajeh H, Jafari B, Safa KD, Dastmalchi S. Magnetic iron oxide nanoparticles modified with vanadate and phosphate salts for purification of alkaline phosphatase from the bovine skim milk. *Colloids Surf B Biointerfaces*. 2019;175:644-653. DOI: [10.1016/j.colsurfb.2018.12.051](https://doi.org/10.1016/j.colsurfb.2018.12.051)
31. Lionetto MG, Caricato R, Calisi A, Giordano ME, Schettino T. Acetylcholinesterase as a biomarker in environmental and occupational medicine: new insights and future perspectives. *Biomed Res Int*. 2013;321213. DOI: [10.1155/2013/321213](https://doi.org/10.1155/2013/321213)
32. Upadhyay LB, Verma N. A three-step approach for the purification of alkaline phosphatase from non-pasteurized milk. *J Food Sci Technol*. 2015;52(5):3140-3146. DOI: [10.1007/s13197-014-1578-1](https://doi.org/10.1007/s13197-014-1578-1)
33. Darwish DA, Ibrahim MA, Ghazy AM. Purification and characterization of alkaline phosphatase from water buffalo liver (*Bubalus bubalis*). *Res J Pharm Biol Chem Sci*. 2016;7(1):1869-1880. [\[available at\]](#)
34. Kaya HB, Özcan B, Şişecioglu M, Ozdemir H. Purification of acetylcholinesterase by 9-amino-1,2,3,4-tetrahydroacridine from human erythrocytes. *Appl Biochem Biotechnol*. 2013;170:198-209. DOI: [0.1007/s12010-013-0177-3](https://doi.org/10.1007/s12010-013-0177-3)
35. Cathala G, Brunel C. Bovine kidney alkaline phosphatase. Catalytic properties, subunit interactions in the catalytic process, and mechanism of  $Mg^{2+}$  stimulation. *J Biol Chem*. 1975;250:6046-6053. DOI: [10.1016/S0021-9258\(19\)41156-3](https://doi.org/10.1016/S0021-9258(19)41156-3)
36. Trepanier JM, Seargeant LE, Stinson RA. Affinity purification and some molecular properties of human liver alkaline phosphatase. *Biochem J*. 1976;155:653-660. DOI: [10.1042/bj1550653](https://doi.org/10.1042/bj1550653)
37. Son JY, Shin S, Choi KH, Park IK. Purification of soluble acetylcholinesterase from Japanese quail brain by affinity chromatography. *Int J Biochem Cell Biol*. 2002;34(2):204-210. DOI: [10.1016/s1357-2725\(01\)00082-6](https://doi.org/10.1016/s1357-2725(01)00082-6)
38. Askar KA, Kudi C, Moody J. Purification of soluble acetylcholinesterase from sheep liver by affinity chromatography. *Appl Biochem Biotechnol*. 2011;165:336-346. DOI: [10.1007/s12010-011-9254-7](https://doi.org/10.1007/s12010-011-9254-7)
39. Ralston JS, Rush RS, Doctor BP, Wolfe AD. Acetylcholinesterase from fetal bovine serum. Purification and characterization of soluble G4 enzyme. *J Biol Chem*. 1985;260(7):4312-4318. DOI: [10.1016/S0021-9258\(18\)89265-1](https://doi.org/10.1016/S0021-9258(18)89265-1)
40. Chu Y, Yu X, Jin X, Wang Y, Zhao D, Zhang P, Sun G, Zhang Y. Purification and characterization of alkaline phosphatase from lactic acid bacteria. *RSC Adv*. 2019;9:354. DOI: [10.1039/c8ra08921c](https://doi.org/10.1039/c8ra08921c)

## عزل وتوصيف وتطبيقات اختبارات الإليزا لإنزيمات الفوسفاتيز القلوية والأستيل كولين إستيريز من ديدان المونيزيا اكسبانزا

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### الخلاصة

تعد ديدان المونيزيا اكسبانزا مصدرًا مهمًا لإنزيمات الفوسفاتيز القلوية وإنزيمات أستيل كولين إستيريز التي تستخدم بشكل عام كمواد كاشفة في الاختبارات البيولوجية المناعية المختلفة مثل اختبارات الإليزا. وتقدم هذه الدراسة الحالية طريقة تنقية بسيطة وقابلة للتكرار لإنزيمي الفوسفاتيز القلوي والأستيل كولين إستيريز من الديدان الطفيلية المونيزيا اكسبانزا عن طريق ترسيب البروتينات بكبريتات الأمونيوم متبوعًا بالفصل الكروماتوغرافيا بالترشيح الهلامي على عمود السفاكريل S300. قمت تنقية إنزيم الفوسفاتيز القلوي بدرجة نقاوة تصل إلى ٦ مرات وبدرجة نشاط نوعي ١٠٧٠،٨ وحدة نشاط / ملجم بروتين ونسبة استرداد ٥٣،٦٪، بينما تمت تنقية إنزيم الأستيل كولين إستيريز بدرجة نشاط نوعي ٥٢٥٠ وحدة / ملجم بروتين وبدرجة نقاوة تصل إلى ٢ مرة ونسبة استرداد ٤٣٪. تم استنتاج الأوزان الجزيئية لإنزيمي الفوسفاتيز القلوي والأستيل كولين إستيريز باستخدام الفصل الكهربائي ك ٣١٢ و ٢٧٠ كيلو دالتون على التوالي. أظهر إنزيم الفوسفاتيز القلوي نشاطه الأمثل عند الرقم الهيدروجيني ٩،٦، بينما أظهر إنزيم الأستيل كولين إستيريز نشاطه الأمثل عند الرقم الهيدروجيني ٨،٠. تم تحفيز إنزيم الفوسفاتيز القلوي بقوة باستخدام أيونات الكوبلت والمنغنيز والنيكل والمغنيسيوم وتم تثبيطه باستخدام الزنك والنحاس والكالسيوم والايديتا والديثيوتريوتول. من ناحية أخرى، تم تحفيز نشاط إنزيم الأستيل كولين إستيريز بشكل كبير باستخدام أيونات الكوبلت والزنك والنيكل وتم تثبيطه باستخدام المغنيسيوم والكالسيوم والايديتا والفينانثرولين والايذيرين. وقد وجدت الأمصال المضادة للإنزيمين المنقيين فعالة لتحديد الإنزيمين في أمصال الإنسان والأغنام والأسماك. وتوفر هذه النتائج تطبيقًا مستقبليًا محتملاً لهذه الإنزيمات في إنتاج اختبارات الإليزا المقترنة بأنزيمي الفوسفاتيز القلوي والأستيل كولين إستيريز لاستخدامها في الأغراض البحثية.