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Isolation and Biochemical Characterization of Acid Phosphatase from Venom of Egyptian Honeybee *Apis Mellifera Lamarchii* with Antibacterial and Anticancer Impacts

Mohamed M. Abdel-Monsef[1,2]*, Doaa A. Darwish[1,2], Mohamed S. Helmy[1,2], Ahmed A. Hamed[3], Hind A. Zidan[4], Hassan M.M. Masoud[1,2]

[1]*Molecular Biology Department, National Research Centre, El-Tahrir st., Dokki, Giza, P.O. 12622, Egypt*

[2]*Proteome Research Laboratory, National Research Centre, El-Tahrir st., Dokki, Giza, P.O. 12622, Egypt*

[3]*Microbial Chemistry Department, National Research Centre, Dokki, Giza, P.O. 12622, Egypt*

[4]*Plant Protection Research Institute, Agricultural Research Center, Giza, Egypt*

Abstract

ACID PHOSPHATASE is one of the most important enzymes in venom of honeybees that has many medical and pharmaceutical applications. In this study, an acid phosphatase was isolated and homogeneously purified from venom of *Apis mellifera lamarckii* that was named BVACP. The purification process was carried out using column chromatography on CM-cellulose and Sephacryl S-300 resins. BVACP specific activity of 305.4 Umg^{-1} was obtained with 33.2% recovery and 3.9 times purification was realized. BVACP was found monomer protein of 46 kDa by gel electrophoresis and gel filtration chromatography. BVACP displayed its maximum activity at pH 5.0, with K_m value of 3.3 mM *p*-NPP and *Vmax* was 0.45 Umg^{-1}. It was activated with $MnCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$ and $CaCl_2$, while $FeCl_2$, $CuCl_2$ and $ZnCl_2$ inhibited it. Ammonium molybdate and sodium fluoride inhibited BVACP non-competitively with *Ki* values of 1.2 µm and 0.37 mM, respectively. BVACP had diverse inhibition ratios against specific bacterial strains and had no effect on others, referring to a selective spectrum of activity. It selectively and potently affects A549, HOS and HCT116 tumour cells. These potent antitumor and antibacterial activities of BVACP could be useful for many clinical and medical applications.

Keywords: Acid phosphatase, Honeybee Venom, Characterization and Purification, Antitumor, Antimicrobial.

Introduction

Acid phosphatase (ACP) is an enzyme that catalyzes hydrolysis of monoesters and anhydrides of phosphoric acid to inorganic phosphate at acidic medium [1-3]. Phosphatases were categorized into acid phosphatases and alkaline phosphatases on the basis of the optimum pH necessary for enzyme catalytic activity [4]. ACP is one of ubiquitous enzymes which widely distributed in nature [5]; microorganisms [6, 7], plants [8, 9] and animals [10, 11]. The wide distribution of ACP in higher and lower organisms recommends its involvement in essential reactions, it acts as scavenger for (Pi) phosphate, mobilize phosphate under conditions of stress and during growth [12]. Microbial and plant ACPs play vital roles in the transportation, production, and recycling of phosphate, while ACP in animals is key step enzyme for autolysis and cell detection and is involved in metabolic regulation, energy metabolism, and pathways of cellular signal transduction [13-15]. ACP plays an important role in various applications of food processing; milk casein de-phosphorylation to decrease content of phosphate so decreasing phosphate toxicity [16]. It used as biomarker in cancer diseases diagnosis, plant salt stress tolerance, deficiency of water and marine pollution [17, 18]. It was studied from liver of human, carp, catfish, frog, bovine, rat, porcine, brain, bovine heart, human erythrocytes and placenta [14]. It has been found and studied in venoms of ants, wasps and bees [19-21].

Apis mellifera lamarckii (Egyptian honeybee) is a subspecies of the honeybees that was known since ancient Egyptians civilization and it is native to

Corresponding author: Mohamed M. Abdel-Monsef, E-mail: mosaadnrc@yahoo.com, Tel.: 01114452529
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southern Egypt. It is different from honey bees found in Europe and it is resistant to diseases of bees as Varroa disease [22-26]. Bee venom is secreted by bee sting apparatus for protecting from enemies and it is widely recognized for its active components with medicinal properties. It is a complicated secretion contained medical active components of peptides, proteins and enzymes including hyaluronidase, phospholipase, acid phosphatase, α-glucosidase, proteases, peptidases, esterases and protease inhibitors [27-29]. It is a clear, scent-filled fluid that has a strong odor, an unpleasant taste and a pH ranging from 4.5 to 5.5 [30-32]. When it is left at room temperature for about five to ten minutes, it turns into a yellowish-white powder similar to flour. A single drop of bee venom is mostly composed of water (88%) and contains only 0.1 µg of dry venom [33, 34]. Bee venom therapy has been used in traditional remedy for various conditions such as arthritis, back pain, muscular and skeletal pain, heart diseases, cancerous tumors, anticancer treatments, and multiple sclerosis [35, 36]. It was found to have anti-protozoan, anti-microbial, anti-inflammatory, anti-arthritic and anti-cancer properties, so it used in folk medicine for treatment of diseases [37-39]. ACP in venom of honeybees is denominated and is one of the most potent allergens in venom together with the hyaluronidase and phospholipase A2. The aim of the present study is purification of ACP from venom of *Apis mellifera lamarchii* bees and studying their biochemical characteristics comprehensively, with emphasis on their potential anti-cancer and anti-microbial activities to provide a basis for future therapies for different diseases.

Material and Methods

Venom collection

Egyptian subspecies *Apis mellifera lamarckii* bees were brought from governorate of Asuit and venom was extracted from 500 foraging bee workers. Bees were caught at entry of the colony and rapidly immobilized at -20 °C. Venom reservoirs and sting devices were acquired after dissection; disrupted with distilled H_2O in Eppendorf tubes, centrifuged for 5 min at 12000 *xg* and 4˚C and the supernatant was acquired as the crude venom.

Chemicals

p-NPP (p-Nitrophenyl phosphate), Creatine phosphate, Phospho-enolpyruvate, Glucose-6-phosphate, ADP (Adenosine diphosphate), AMP (Adenosine monophosphate), ATP (Adenosine triphosphate), CM-cellulose, DTT (dithiothreitol), trypan blue dye, 1,10 phenanthroline, BSA (bovine serum albumin), crystal violet, blue dextran, Naphthyl phosphate, Sephacryl S-300 and gel filtration marker molecular weight kits were from Sigma Co. Marker proteins of molecular weight for SDS were from Pharmacia Co., DMEM, Fetal Bovine serum, HEPES buffer solution, gentamycin, L-glutamine, RPMI-1640 were from Lonza, Belgium.

Acid phosphatase assay

Assay of acid phosphatase was carried out by adding 0.1 ml of 0.05 M p-NPP to 0.8 ml of 20 mmol L^{-1} Na-acetate buffer (pH 5.0) and 0.1 ml of enzyme extract, then incubate at 37 ºC for 15 minutes, and reaction was terminated by adding 1 ml of 0.1 M NaOH. The liberated pnitrophenol (p-NP) was determined at 405 nm by a spectrophotometer (JASCO V-730). One ACP unit was determined as amount of enzyme liberating 1 micromole of p-NP during one minute at 37ºC [40].

Staining of acid phosphatase activity on PAGE

Staining of acid phosphatase activity was determined according to [41]. After electrophoresis; gels were washed 3 times with 50 ml of buffer, 0.1 Na-acetate (pH 5.0). After washing, the gels were incubated in buffer, 0.1 Na-acetate (pH 5.0) containing 100 mg Fast Garnet GBC and 100 mg p-naphthyl phosphate until reddish brown bands appeared.

Purification of bee venom acid phosphatase

Chromatography on CM-cellulose column

All the steps of ACP purification were done at 4 °C unless otherwise stated. The acid phosphatase activity and protein contents were estimated throughout different purification steps. Crude venom was dissolved in Na-acetate buffer, pH 5.0 and chromatographed on column CM-cellulose (2.4 x 12 cm), previously equilibrated with 20 mmol L^{-1} Na-acetate, pH 5.0. The flow rate was maintained at 60 ml/h and adsorbed proteins were eluted with stepwise NaCl gradient contained in equilibration buffer. Fractions of 3 ml volumes were obtained, and then the acid phosphatase specific activity were estimated in all fractions. The fractions which contain ACP activity were pooled, then dialyzed, and concentrated by sucrose.

Chromatography on column Sephacryl S-300

Concentrated ACP fractions were applied onto column of Sephacryl S-300 (1.75 x 142 cm) pre-equilibrated and run using the buffer (20 mmol L^{-1} Na-acetate, pH 5.0) at 30 ml/h rate of flow and fractions of 2 ml were collected. For determination of the native molecular mass of the ACP, The equilibrated Sephacryl S-300 column was calibrated with protein markers in range from (17 - 440 kDa) as standards.

Electrophoretic analysis

Native 7% PAGE was carried out utilizing Smith method [42]. The purified acid phosphatase subunit molecular weight was done utilizing 12% SDS-PAGE [43]. The gels were submerged in Coomassie

brilliant blue solution (0.25%) for proteins staining on the PAGE.

Protein determination

Estimation of protein concentrations were determined spectrophotometrically according to Bradford method during all purification steps [44].

Optimum pH, substrate specificity and Michaelis-Menten constant (K_m)

Bee venom ACP activity changes were studied at different pHs utilizing 10 mmol L^{-1} (Na acetate, pH 3.4 -5.6) and (Na phosphate, pH 5.7 - 8.0) at 37˚C. From the above information, a plot of the ACP relative activity (%) against pH was done for determination of pH optima. Substrate specificity of bee venom ACP was examined toward different substrates (ATP, ADP, AMP glucose-6-phosphate, p-NPP, creatine phosphate and phospho-enol pyruvate) of 5 mM each in 50 mmol L^{-1} Na-acetate, pH 5.0. The Km and Vmax values were located by incubating the ACP enzyme with increasing concentrations (0.5 - 8 mM) of p- NPP substrate and constructing the Lineweaver–Burk plot.

Effect of ions and inhibitors on acid phosphatase activity

Impact of divalent ions on bee venom ACP activity was achieved by incubation of purified ACP enzyme with 5 mM Cu^{2+}, Fe^{2+}, Mg^{2+}, Ca^{2+}, Co^{2+}, Zn^{2+}, Ni^{2+} and Mn^{2+} at 37˚C for 5 min before activity test. Thereafter, activity of ACP was evaluated and a control test (with no ions) was done as 100% for comparison.

Action of different inhibitor compounds on the activity of bee venom ACP was achieved by incubating the purified enzyme with 5 µM ammonium molybdate, 2 mM sodium fluoride and 5 mM from each of sodium tartrate, dithiothreitol, EDTA, mercaptoethanol and phenanthroline for 5 min at 37. Thereafter, activity of ACP was evaluated and a control test without any inhibitor was done as 100% for comparison. The inhibition kinetic of bee venom ACP with ammonium molybdate and sodium fluoride was achieved for detecting the values of maximum inhibition, I_{50} (50% inhibition), inhibition type and Ki for ACP inhibition.

Antimicrobial activity determination

To assess the inhibition ratio for the purified bee venom ACP against various strains of microbes, we employed a Microtiter Plate assay (MTP) [45]. Bacterial strains including *Pseudomonas aeruginosa* ATCC 90902, *Listeria monocytogenes* ATCC 7644, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* 0157 ATCC 700728, *Shigella sonnei* ATCC 25931, *Escherichia coli* ATCC 8739 lot 03801105 and *Bacillus cereus* ATCC 10876 lot 0860505 were cultured individually in sterile growth media and adjusted to a standardized concentration using phosphate-buffered saline (PBS). Simultaneously, a range of dilutions of the purified ACP was prepared in a buffer, with a control well containing only the buffer for background correction. The microtiter plate was set up by dispensing the diluted protein and bacterial cultures into wells, ensuring consistent volumes. Following an incubation period at optimal temperatures, bacterial growth was measured by assessing O.D at 600 nm using a microtiter plate reader. Inhibition ratios were calculated for each strain by this formula; (O.D. $_{Control}$ − O.D. $_{Treatment}$ / O.D. $_{Control}$) × 100, where a higher ratio indicates greater inhibition. The resulting data provided quantitative insights into the antimicrobial activity of the purified protein against the specified bacterial strains, facilitating a thorough analysis of its inhibitory effects (Streptomycin was the positive control). The test microbes were acquired from the Culture Collection unit - Microbial Chemistry Dept., - NRC, Egypt.

Antitumor activity determination

Cytotoxic effects of purified ACP on human cell lines were determined. Normal Skin fibroblast (BJ1), lung carcinoma cell line (A549), hepatocellular carcinoma cell line (HePG2), Osteosarcoma cell line (HOS), colon cell line (HCT116), breast adenocarcinoma (MCF7), skin cancer (A431) and prostate cell line (PC3) were used in assessment the antitumor activity of purified ACP. Cells were suspended in DMEM for A549, 1% L-glutamine, 1 % antibiotic-antimycotic mixture (Amphotericin B (25 µg/ml), Streptomycin Sulfate (10,000 µg/ml), Potassium Penicillin (10,000 U/ml)) at 37 ºC in 5% CO_2. Cells were batch cultured for 10 days and seeded at conc. of $10x10^3$ cells/well in a fresh complete growth medium with a 96-well microtiter plates in 5% CO_2, 37 ºC for 24 hr. Media was aspirated, fresh medium was added, cells were incubated alone as a negative control and with different concentrations of purified ACP. After 48 hr incubation, medium was aspirated; MTT salt was added (40 µl of 2.5µg/ml) to all wells and incubated under 5% CO_2 at 37 ºC for 4 hr. 200 µL of 10% SDS was added to all wells to stop reaction and incubated at 37ºC for 24 hr. Doxorubicin was used as a positive control under the same conditions (natural cytotoxic agent gives 100% lethality). Absorbance was then determined by a microtiter reader at 595 nm. The viability change percentage was determined by (Reading of ACP / Reading of negative control) -1) x 100. IC_{50} was determination by SPSS 11 program. IC_{50}: ACP lethal conc. which causes 50 % cells death in 48 hrs [46-47]. Cell lines were obtained from Bioassay-Cell Culture Lab., NRC.

Results

Purification of bee venom acid phosphatase

Honey bee crude venom was found to have ACP specific activity of 78.4 Umg^{-1} and a summary of

acid phosphatase purification steps from bee venom is given in Table 1. Chromatography on CM-cellulose and Sephacryl S-300 columns was very effective in purifying bee venom ACP. The elution profile of bee venom ACP CM-cellulose column (Fig. 1a) showed one ACP activity peak that eluted by 20 mmol L^{-1}Na-acetate, pH 5.0, and designated BVACP. This step increased BVACP specific activity 1.5 fold more than crude venom with 58.1% recovery. The BVACP elution profile from the Sephacryl column displayed one peak of ACP activity (Fig. 1b), which led to an increase in the specific activity to 305.4 Umg^{-1} representing 3.9 purification times and 33.2% yield. Native molecular weight of BVACP enzyme obtained by gel filtration was found to be 46 kDa.

Electrophoretic analysis

The consecutive purification steps of BVACP (crude venom, CM-cellulose and Sephacryl S-300 fractions) were electrophoretically investigated on native 7% PAGE. One protein band (Fig. 2a) was agreed with BVACP activity band (Fig. 2b) denoting the tentative homogeneity of the preparation. The SDSPAGE of the final BVACP preparation showed a major band matching to 46 kDa (Fig. 2c) indicating a monomeric structure of the purified protein.

Effect of pH, substrate specificity and Michaelis-Menten constant (K_m)

The effect of pH on BVACP activity was achieved utilizing acetate and phosphate buffers of different pHs. The highest BVACP activity was attained at pH 5.0 (Fig.3a). BVACP substrate specificity was assessed toward different substrates (Table 2). BVACP cleaved specifically *p*-NPP with relative activity of (100%) followed by AMP (14.2%).

BVACP activity was estimated at different p-NPP concentrations with plotting of the reciprocals of reaction velocity (1/V) against substrate concentration (1/[S]) (Fig. 3b). According to Lineweaver-Burk plot, both Km and Vmax values were calculated as 3.3 mM and 0.45 Umg^{-1} respectively.

Effect of cations and inhibitors

The pure BVACP enzyme was initially incubated with 5 mM of different divalent metal ions and acid phosphatase activity was then measured. Each of $MnCl_2$, $MgCl_2$, $NiCl_2$ and $CoCl_2$ increased BVACP activity, while $CuCl_2$, $FeCl_2$ and $ZnCl_2$ inhibited it (Table 3). The effect of inhibitors on activity of purified BVACP (Table 4) showed that, ammonium molybdate, sodium fluoride and sodium tartrate were found to be potent inhibitors for BVACP activity.

Inhibition kinetics BVACP

The most potent inhibitors on the purified BVACP activity were sodium fluoride and ammonium molybdate. Effect of various concentrations of sodium fluoride on BVACP activity (Fig. 4a) displayed that I_{50} is 0.4 mM with the maximum inhibition of 98.4% was attained at 2 mM. In the Hill plot, when values of (log Vi / Vmax – Vi) were drawn against log [I] for sodium fluoride (Fig. 4b), the slope of the obtained straight line was 1.04 indicating one binding site for the inhibitor on BVACP. BVACP was non-competitively inhibited with sodium fluoride (Fig. 4c) with a *Ki* value of 0.37 mM (Fig. 4d). On the other hand, effect of varying concentrations of ammonium molybdate on BVACP activity (Fig. 5a) displayed that I_{50} is 1.2 µM with the maximum inhibition of 91.3% was attained at 5 µM. In the Hill plot, when values of (log Vi / Vmax – Vi) were drawn against log [I] for ammonium molybdate (Fig. 5b), the slope of the obtained straight line was 0.92 indicating one binding site for ammonium molybdate on BVACP. BVACP was also non-competitively inhibited with ammonium molybdate (Fig. 5c) with a *Ki* value of 1.2 µM (Fig. 5d).

Antimicrobial activity

The purified BVACP was screened for its antimicrobial effect against different bacterial strains, visualized in the form of a bar graph, and Streptomycin was used as reference standard (Fig. 6). The inhibition ratios of 42.1% against *Staphylococcus aureus*, 14.4% against *Listeria monocytogenes*, 33.4% against *Pseudomonas aeruginosa*, 55.5% against *Shigella sonnei* and 68.1% against *Bacillus cereus* underscore the protein's potent antimicrobial properties. Interestingly, no inhibitory effect was observed against the *Escherichia coli* strains.

Antitumor activity

Cancer cell lines were acquired from the Bioassay-Cell Culture Lab., National Research Centre, Egypt. The screening of purified BVACP antitumor activity was determined using different human tumour cell lines (BJ1, A549, HOS, HCT116, HepG2, MCF7 and PC3) using MTT cell viability assay in comparison with doxorubicin as a positive control. BVACP displayed potent cytotoxicity against the lung cell line (A549), osteosarcoma cell line (HOS) and colon cell line (HCT116) with 8.2, 13.7 and 18.7 µg/ml IC_{50} values in comparison with 28.3, 29.1 and 37.6 µg/ml for doxorubicin respectively (Fig. 7). BVACP have no inhibitory effect against normal skin fibroblast (BJ1), hepatic cell line (HePG2), breast adenocarcinoma (MCF7) and prostate cell line (PC3).

Discussion

Bee venom is a rich source of many active pharmacological components [48, 49]. Acid phosphatase is one of the major components of the venom of honeybees [21]. ACP has been studied in

various parasitoids venoms such as honeybee *Apis mellifera* and endoparasitic wasp *Pteromalus puparum* [50, 51, 20]. This study presents a reproducible and simple purification method for acid phosphatase from venom of *Apis mellifera lamarchii* by two steps of chromatography. The CM-cellulose column profile displayed a well-defined fraction designated as BVACP. The specific activity of collected fraction of BVACP from CM-cellulose was increased 1.5 fold more than the starting extract with 58.1% recovery. BVACP Fraction was then subjected to Sephacryl S-300 column by which the specific activity of BVACP was raised to 305.4 U/mg protein representing 3.9 fold purification and yield of 33.2%. Acid phosphatase was isolated from many organisms with different specific activities; ACP of honey bee *Apis mellifera* isolated with specific activity of 135 U/mg [52], from Garlic Seedling was 168 U/mg [12], from ripened banana fruit was 745 U/mg [53], and from *Drosophila melanogasteris* fly was 252.5 U/mg [54]. Steps of purification; crude venom, CM-cellulose and Sephacryl S-300 fractions were investigated by electrophoresis on 7% native PAGE. One protein band was agreed with band of BVACP activity denoting BVACP preparation purity. BVACP exhibited a monomeric structure of 46 kDa as deduced from both Sephacryl S-300 column and SDS-PAGE. Many molecular weights for ACP were reported; 64 kDa for honey bee *Apis cerana* [21], 40 kDa for ripened banana fruit [53], 44 kDa for cactus cladodes [55], 40 kDa for larvae of the camel tick [3], 55 kDa for *Macrotyloma uiflorum* seeds [54], 57.8 kDa for *Trichoderma harzianum* fungi [1], 58 kDa for Garlic Seedling [12], 66 kDa for the lady beetle egg [56], 72.5 kDa for *Schizophyllum commune* mushroom [18], 80 kDa from *seeds of Nelumbo nucifera* [15], and 145 kDa and consists of four identical 39 kDa subunits from *Agaricus bisporus* mushroom [57].

In this study, the effect of pH on BVACP activity revealed that, the highest BVACP activity was found at pH 5.0. Similar pH optimum ranges of ACP was reported to be 4.6 from *Schizophyllum commune* mushroom [18], 4.75 from commercial mushroom [57], 4.8 from honey bee *Apis cerana, Trichoderma harzianum* and larvae of the camel tick [21, 1, 3], 5.0 from *Macrotyloma uiflorum* seeds, and seeds of *Nelumbo nucifera* [54, 15], 5.5 from cactus cladodes [55], 5.7 from Garlic Seedling [12], 5.8 from banana fruit [53] and 6.0 from *Spirodela oligorrhiza* aquatic plant [58]. BVACP showed high substrate specificity towards p-NPP when it was checked with various substrates. These results are in accordance with ACP from *Macrotyloma uiflorum* seeds [54], *Schizophyllum commune* mushroom [18], *Trichoderma harzianum* [1], *Spirodela oligorrhiza* aquatic plant [58], *Agaricus bisporus* mushroom [57] and larvae of the camel tick [3]. The purified BVACP value of K_m was 3.3 mM *p*-NPP with a *Vmax value* of 0.45 U/mg indicating a high affinity of BVACP toward *p*-NPP. Different K_m values for ACP toward *p*-NPP were reported; for fish liver was 0.25 mM and Vmax was 1.10 U/mg [14], 0.164 mM for the lady beetle egg [56], 0.248 mM for *Agaricus bisporus* mushroom [57], 0.37 mM for *Schizophyllum commune* mushroom [18], 0.934 mM for *Macrotyloma uiflorum* seeds [54], 0.132 mM for seeds of *Nelumbo nucifera* [15], 1.25 mM for larvae of the camel tick [3].

In this study, $MnCl_2$, $MgCl_2$, $CoCl_2$, $NiCl_2$ and $CaCl_2$ elevated BVACP activity while $FeCl_2$, $CuCl_2$ and $ZnCl_2$ inhibited it. These results are in accordance with ACP of cactus cladodes activity which was stimulated by Ca^{+2} and inhibited with Cu^{+2} , Fe^{+2} and Zn^{+2} [55]. *Macrotyloma uiflorum* seeds ACP activity was enhanced with Mg^{+2} [54]. *Nelumbo nucifera* seeds ACP activity was increased by Mg^{+2} and inhibited by Zn^{+2} and Cu^{+2} [15]. *Schizophyllum commune* mushroom ACP activity was inhibited strongly by Fe^{+2} but enhanced by Co^{+2}, Mg^{+2}, and Ca^{+2} [18], Cu^{+2} showed strong inhibitory effects on garlic seedling ACP activity while Ca^{+2} was significant activator [12]. Banana ACP was activated by Mn^{+2} and Mg^{+2} and inhibited by Cu^{+2} and Zn^{+2} [53]. Camel tick larvae ACP was enhanced with Co^{2+}, Ca^{2+}, Ni^{2+}, Mg^{2+} and Zn^{2+} and inhibited with Cu^{2+}, $Mn^{2+}$and Fe^{2+} [3].

The effect of different inhibitors on BVACP activity showed that, ammonium molybdate and sodium fluoride were found the most potent inhibitors of the enzyme, since 5 µM ammonium molybdate inhibited BVACP activity 91.3%, while 2 mM sodium fluoride inhibited BVACP activity 98.4 %. Sodium tartrate was found a moderate inhibitor for BVACP activity, since 5.0 mM inhibited BVACP activity 49.6%. Dithiothreitol (5 mM) inhibited BVACP activity by 18.8%, which may refer to the importance of the SH- group for activity of the enzyme. 1, 10 phenanthroline, EDTA and β-mercaptoethanol had negligible effects on BVACP activity. Similarly, ammonium molybdate and sodium fluoride were found potent inhibitors for ACP from banana fruits [53], egg of the lady Beetle [56] and liver of *Labeo rohita* [14], while ACP of camel tick larvae was inhibited with fluoride and tartrate [3]. 2 mM sodium fluoride inhibited 98.4% of BVACP activity with an I_{50} = 0.4 mM, and a straight relation was achieved on plotting the Hill plot with a slope of 1.04 representing one binding site for the inhibitor on BVACP. Sodium fluoride non-competitively inhibited BVACP since presence of the inhibitor reduced *Vmax* value but did not alter the K_m value with a *Ki* value of 0.37 mM proving the potency of the inhibitor. On the other hand, 5 µM ammonium molybdate inhibited 91.3% of BVACP activity with an I_{50} = 1.2 µM, and a straight relation was achieved on plotting the Hill plot with a slope of 0.92 representing one binding site for the inhibitor on

BVACP. It also non-competitively inhibited BVACP since presence of the inhibitor reduced *Vmax* value but did not alter the K_m value with a *Ki* value of 1.2 µM proving the potency of the inhibitor. ACP from rohu fish liver was inhibited by molybdate and fluoride with *Ki* values of 0.02 mM and 0.29 mM respectively [14].

In this investigation, the antimicrobial activity results showed that BVACP have an antimicrobial interest. The inhibition ratio of 42.16 % against *Staphylococcus aureus* ATCC 25923 suggests a notable inhibitory effect, holding promise for potential applications in treating infections caused by this bacterium. In the case of *Listeria monocytogenes* ATCC 7644, the moderate inhibition ratio of 14.38 % indicates a degree of effectiveness, prompting further exploration of the purified BVACP potential role in food safety. The inhibition ratio of 33.41 % against *Pseudomonas aeruginosa* ATCC 90902 is particularly noteworthy, considering the bacterium's association with hospital-acquired infections, hinting at the BVACP potential use in healthcare settings. Interestingly, no inhibitory effect was observed against *Escherichia coli* strains, indicating a selective spectrum of activity. Conversely, the robust inhibition ratio of 55.53 % against *Shigella sonnei* and an impressive 68.08 % against *Bacillus cereus* underscore the BVACP potent antimicrobial properties, offering exciting prospects for therapeutic and food safety applications. These diverse inhibition ratios highlight the purified BVACP potential in targeting specific bacterial strains, warranting further investigation into its mechanisms and practical applications. Honey bee venom was reported to have antimicrobial impact against a wide range of fungi, bacteria and viruses. Studies have shown its potential as a natural alternative for combating antibiotic-resistant pathogens [59].

The BVACP was tested for its antitumor activity on different human cancer cell lines utilizing MTT cell viability assay compared with doxorubicin as a positive control. The purified BVACP selectively and potently affect A549, HOS and HCT116 tumour cells with IC_{50} values of 8.2, 13.7 and 18.7 µg/ml respectively, which was found much less than that of 28.3, 29.1 and 37.6 µg/ml for doxorubicin indicating the potency of the enzyme as anticancer agent. BVACP don't affect BJ1, HePG2, MCF7 and PC3 cell lines indicating its specificity and selectivity against certain tumour cells. Various studies had found that specific compounds in honey bee venom have the ability to inhibit the growth of cancer cells and cause them to die [37, 60, 61]. Also, some raw honey bee venom components had shown anti-tumor effects against prostate, lung, breast, liver, bladder, leukemia, ovarian, and melanoma cancers [60, 62].

Conclusion

In conclusion, this study illustrated a simple, reproducible and fast purification protocol for acid phosphatase from the Egyptian honeybee venom. The purified BVACP found to have antitumor and antimicrobial activities that can be useful for many clinical and medical applications and could have a part in treatment of various diseases as anti-cancer and antibacterial agent.

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Declaration of Conflict of Interest

The authors declare that there is no conflict of interest.

Ethical of approval

This article involves application of the ethics practices under approval number of 09410324.

TABLE 1. A typical purification scheme of bee venom acid phosphatase (BVACP)

Purification step	Total protein (mg)	Activity (unit)	Specific activity	Recovery (%)	Fold purification
Honey bee crude venom	28.2	2210	78.4	100.0	1.00
CM-cellulose fraction	10.7	1285	120.1	58.1	1.5
Sephacryl S-300 fraction	2.4	733	305.4	33.2	3.9

TABLE 2. Substrate specificity of the purified BVACP

Substrate (5 mM)	BVACP	
	Rate of hydrolysis	Relative activity
p-Nitrophenyl phosphate (*p*-NPP)	1.067	100.0
Adenosine monophosphate (AMP)	0.152	14.2
Adenosine diphosphate (ADP)	0.096	9.0
Adenosine triphosphate (ATP)	0.105	9.8
Creatine phosphate	0.049	4.6
Glucose-6-phosphate sodium salt	0.081	7.5
Phospho-enol-pyruvate	0.113	10.6

TABLE 3. Effect of divalent cations on the purified BVACP

Reagent (5 mM)	Residual activity (%)
Control	100.0
$CaCl_2$	108.3
$CoCl_2$	142.7
$CuCl_2$	27.2
$FeCl_2$	96.3
$MgCl_2$	154.3
$MnCl_2$	175.3
$NiCl_2$	133.0
$ZnCl_2$	77.8

TABLE 4. Effect of various inhibitors on the purified BVACP

Reagent	Conc.	Inhibition (%)
Control	-----	0.0
Ammonium molybdate	5.0 µM	91.3
Sodium fluoride (NaF)	2.0 mM	98.4
Sodium tartrate	5.0 mM	49.6
DL-Dithiothreitol (DTT)	5.0 mM	18.8
β-Mercaptoethanol	5.0 mM	4.6
EDTA	5.0 mM	5.4
1,10 Phenanthroline	5.0 mM	2.4

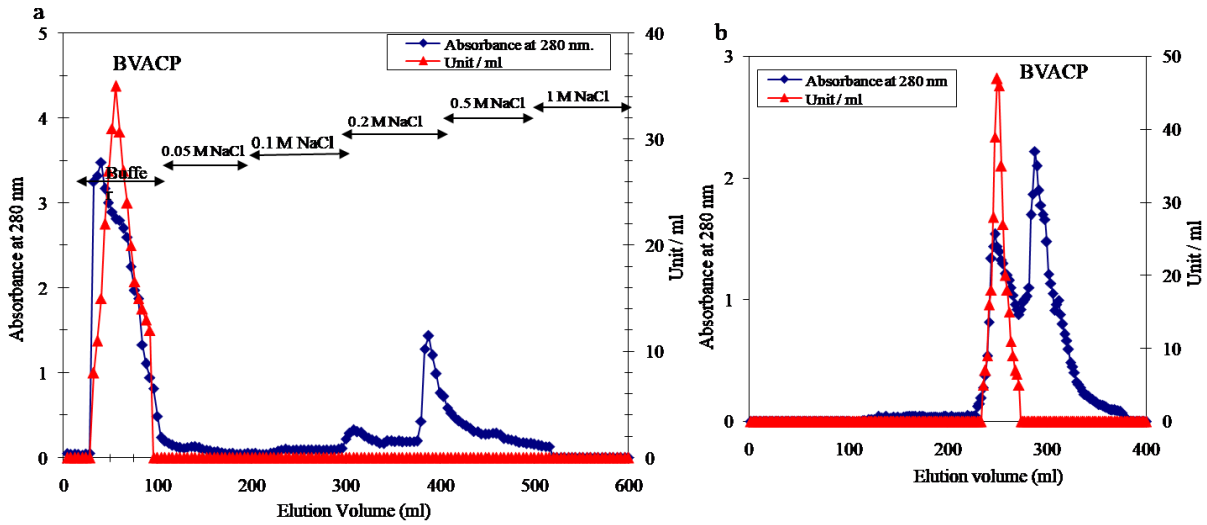

Fig. 1. (a) A chromatographic typical elution profile for honeybee venom crude extract on CM-cellulose column (12 cm x 2.4 cm) previously equilibrated with 20 mmol L^{-1} Na-acetate buffer (pH 5.0). (b) A chromatographic typical elution profile for the concentrated pooled CM-cellulose fractions containing ACP enzyme activity on Sephacryl S-300 column (142 cm x 1.75 cm) previously equilibrated with 20 mmol L^{-1} Na-acetate buffer (pH 5.0).

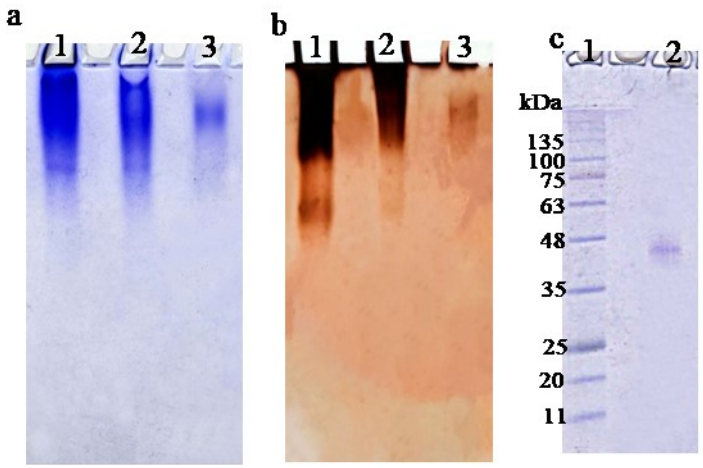

Fig. 2. (a) Electrophoretic analysis of BVACP protein pattern of the different purification steps on 7% native polyacrylamide gel: (1) crude venom, (2) CM-cellulose fraction and (3) Sephacryl S-300 purified fraction of BVACP. (b) Electrophoretic analysis of BVACP isoenzyme pattern of the different purification steps on 7% native polyacrylamide gel: (1) crude venom, (2) CM-cellulose fraction and (3) Sephacryl S-300 purified fraction of BVACP. (c): Subunit molecular weight determination by electrophoretic analysis of purified BVACP on 12% SDS-polyacrylamide gel: (1) molecular weight marker proteins and (2) purified BVACP.

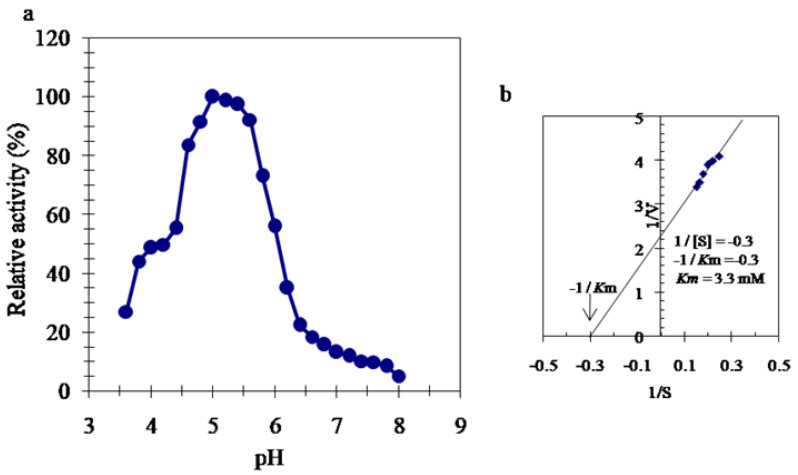

Fig. 3. (a) pH effect on the purified BVACP using 20 mmol L^{-1} Na-acetate buffer (pH 3.6 to 5.6) and Na-phosphate buffer (pH 5.7 to 8.0). (b) Lineweaver-Burk plot relating the reciprocal of the reaction velocity of the purified BVACP to *p*-NPP concentration in mM.

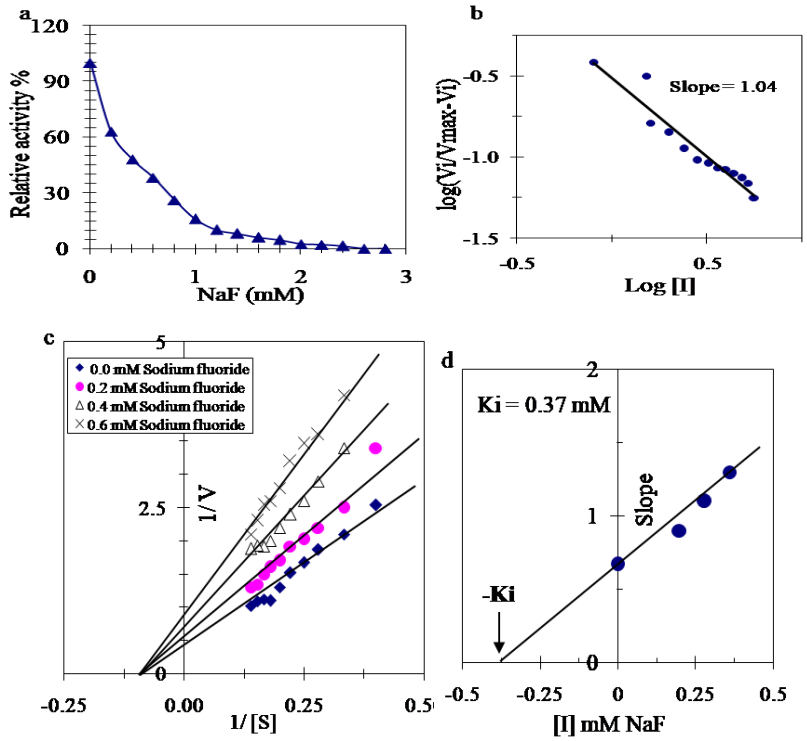

Fig. 4. (a) Inhibition of the purified BVACP by varying concentrations of sodium fluoride. (b) Hill plot for inhibition of BVACP by varying concentrations of sodium fluoride. (c) Lineweaver-Burk plots showing the type of inhibition of BVACP by sodium fluoride. (d) Determination of the inhibition constant (*Ki*) value for the inhibition of BVACP by sodium fluoride.

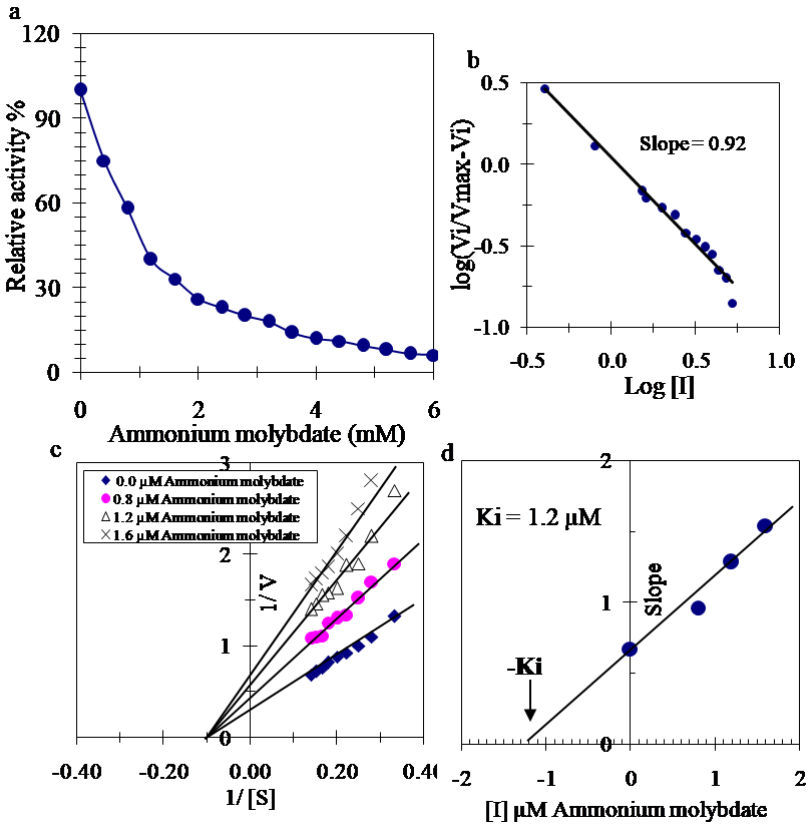

Fig. 5. (a) Inhibition of the purified BVACP by varying concentrations of ammonium molybdate. (b) Hill plot for inhibition of BVACP by varying concentrations of ammonium molybdate. (c) Lineweaver-Burk plots showing the type of inhibition of BVACP by ammonium molybdate. (d) Determination of the inhibition constant (*Ki*) value for the inhibition of BVACP by ammonium molybdate.

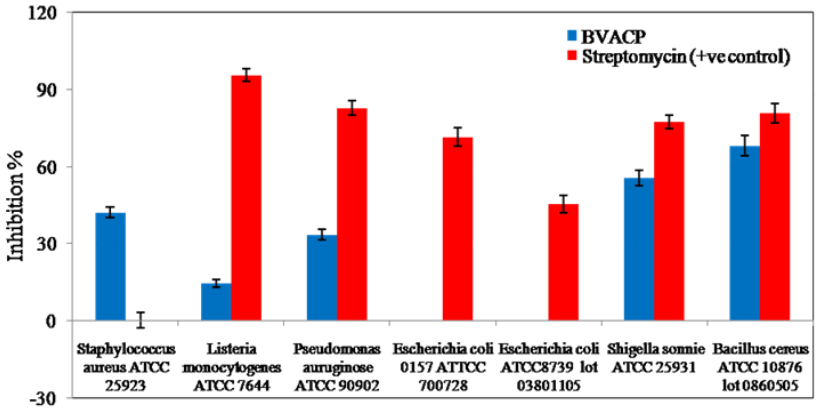

Fig. 6: Antimicrobial activity of the purified BVACP on various bacterial strains.

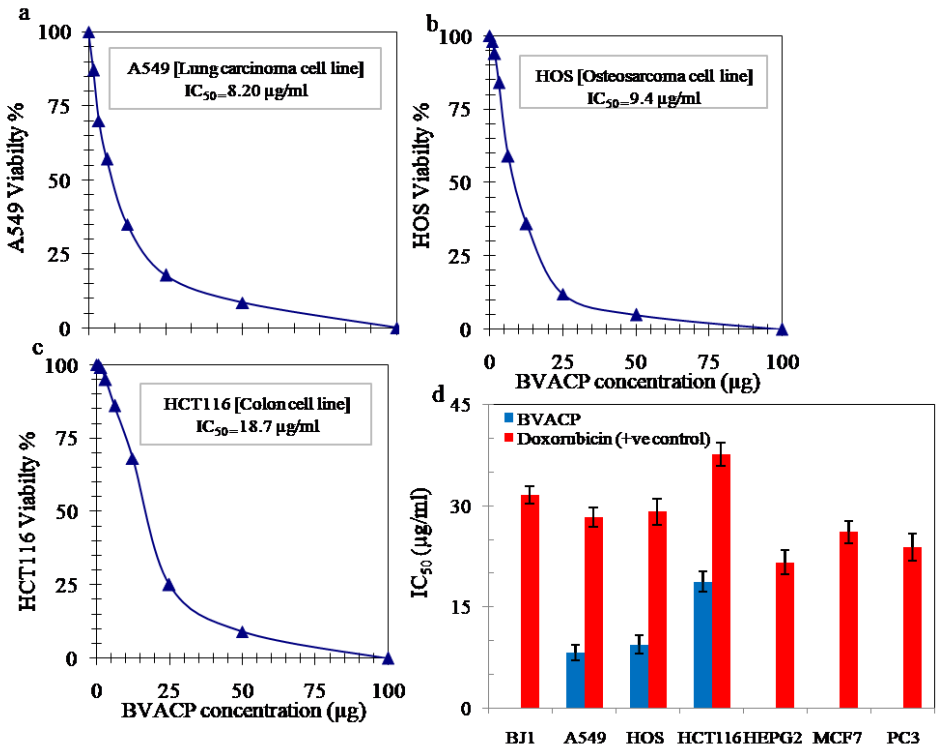

Fig. 7. (a) Antitumor inhibitory activity of varying concentrations of BVACP against A549 cancer cell line. (b) Antitumor inhibitory activity of varying concentrations of BVACP against HOS cancer cell line. (c) Antitumor inhibitory activity of varying concentrations of BVACP against HCT116 cancer cell line. (d): Comparison of IC_{50} of the BVACP against doxorubicin as a positive control. Data represent the mean ± SD of three independent experiments.

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عزل وتوصيف كيميائي حيوي للفوسفاتيز الحامضي من سم نحل العسل المصري *Apis Mellifera Lamarchii* ذات النشاط المضاد للبكتيريا والمضاد للسرطان

محمد مسعد عبد المنصف[1و2]، دعاء عبد الخالق درويش[1و2]، محمد صلاح الدين حلمى[1و2]، احمد عبد الغنى حامد[3]، هند عامر زيدان[4] و حسان محمد محمد مسعود [1و2]

[1] قسم البيولوجيا الجزيئية، المركز القومي للبحوث، شارع التحرير، الدقي، الجيزة، ص.ب. 12622، مصر.

[2]معمل أبحاث البروتيوم، المركز القومي للبحوث، شارع التحرير، الدقي، الجيزة، ص.ب. 12622، مصر.

[3].قسم الكيمياء الميكروبية، المركز القومي للبحوث، الدقي، الجيزة، ص.ب. 12622، مصر.

[4].معهد بحوث وقاية النبات، مركز البحوث الزراعية، الجيزة، مصر

الملخص

إن إنزيم الفوسفاتيز الحامضي هو أحد أهم الإنزيمات في سم النحل وله العديد من التطبيقات الطبية والعلاجية. وفي هذه الدراسة تم عزل إنزيم الفوسفاتيز الحامضي وتنقيته بشكل متجانس من سم النحل *Apis mellifera lamarckii* والذي أطلق عليه اسم BVACP. ولقد أجريت عملية التنقية باستخدام الفصل الكروماتوغرافي على عمود CM-cellulose و S-Sephacryl-300. وتم الحصول على نشاط نوعي لـ BVACP بمقدار 305.4 وحدة / ملى جرام مع استرداد 33.2٪ وتنقية 3.9 مرة. ولقد وجد ان بروتين BVACP يتكون من وحدة بنائية واحدة ولها وزن جزيئى 46 كيلو دالتون وذلك عن طريق الفصل الكهربى. ولقد أظهر BVACP أقصى نشاط له عند درجة حموضة 5.0، بقيمة Km 3.3 mM p-NPP و Vmax 0.45 Umg-1. وقد وجدا ان BVACP يتم تنشيطه باستخدام 2MnCl و 2MgCl و 2CoCl و 2NiCl و 2CaCl، بينما قام 2FeCl و 2CuCl و 2ZnCl بتثبيطه. ولقد قامت موليبدات الأمونيوم وفلوريد الصوديوم بتثبيط BVACP بشكل غير تنافسي بقيم Ki تبلغ 1.2 ميكرومولار و 0.37 ملي مولار على التوالي. ولقد كان لـ BVACP نسب تثبيط متنوعة ضد سلالات بكتيرية محددة ولم يكن لها تأثير على غيرها، مما يشير إلى طيف انتقائي من النشاط المضاد للبكتيريا. وايضا وجد ان BVACP يؤثر بشكل انتقائي وقوي على خلايا الورم 549A و HOS و 116HCT. يمكن أن تكون هذه الأنشطة القوية المضادة للأورام والمضادة للبكتيريا لـ BVACP مفيدة للعديد من التطبيقات العلاجية والطبية.

الكلمات الدالة: إنزيم الفوسفاتيز الحامضي ,سم نحل العسل المصري، تنقية ،دراسة خواص، مضاد للبكتيريا ومضاد للسرطان.