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Full Length Research Paper

Purification and biochemical characterization of alkaline protease from an Egyptian biopesticide- producing *Bacillus sphaericus* strain

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The objective of the current study was to purify and partially characterize an alkaline protease (AP) from a newly isolated Egyptian *Bacillus sphaericus* strain. The enzyme was subjected to a 3-step purification scheme involving i) ammonium sulfate [$(NH_4)_2SO_4$] fractionation, ii) acetone precipitation and iii) Sephadex G-200 gel permeation chromatography. Fractions precipitated with 30 to 60% [$(NH_4)_2SO_4$] saturation levels exhibited the highest enzyme activities, whereas acetone in the ranges between 50 to 75% (v/v). Gel permeation utilizing Sephadex G-200 column resulted in approx. 50 fold purification level, as compared to the original crude enzyme, with a yield recovery of 27%. The AP enzyme was successfully purified to homogeneity as a monomeric band with an estimated molecular mass of ~29 kDa, based on sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and zymogram activity staining analyses. While, the maximum enzymatic activity was recorded at pH 10, AP showed an optimal activity at incubation temperature range AP between 55 to 60°C. A thermal stability at temperatures ≥ 40°C for 15 min, using casein as a substrate, with a total loss of enzymatic activity upon heating at 70°C. Results for kinetic parameters indicated that the apparent K_m and V_{max} values for AP, with casein as substrate under optimal reaction conditions (pH 10 and 55°C), were found to be 230 µg min^{-1} ml^{-1} and 0.05% (w/v), respectively. Thus, the potentials of AP as an industrially important enzyme were assessed in the light of our current knowledge on microbial alkaline proteases.

Key words: *Bacillus sphaericus*, alkaline protease, biochemical characterization, fodder yeast, enzyme purification, biopesticide.

INTRODUCTION

Bacterial strains isolated from Egyptian soils have been shown to be effective larvicides for some species of mosquitoes and able to produce alkaline proteases (Afify et al., 2009). Production of enzymes by one organism, in the same fermentation process, is a favorable industrial application rendering the process cost-effective and highly applicable under local conditions (Afify et al., 2009). *Bacillus sphaericus* has been identified, as having

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both larvicidal toxicity to the blood-feeding mosquito *Culex pipiens* and potential to produce extra-cellular alkaline protease (AP) in the growth medium (Afify et al., 2009). Several types of proteases has been reported to be formed by bio-pesticides–producing bacilli with special reference to *B. thuringiensis* and *B. sphaericus*).

The Egyptian strain of *B. sphaericus* has been shown to produce a potent mosquitocidal toxin with a lethal concentration 50 (LC_{50}) value comparable to those produced by the international strain IN 2362 of *B. sphaericus* and other potent cultures (El-Bendary et al., 2002). Moreover, the Egyptian strain of *B. sphaericus* isolated was also able to produce extracellular AP when grown on a variety of commercial media as well as media composed of locally available agro-industrial by-products and powdered legumes seeds (Afify et al., 2009). Some bacilli, particularly those of *B. sphaericus,* have been advocated as safe and effective means of biological control of mosquitoes instead of hazardous chemical insecticides. *B. sphaericus* is known to include some strains that form parasporal bodies made of proteins that are toxic to mosquito larvae. Kellen et al. (1965) first described this group of bacilli. Subsequently more than 300 strains have been isolated and identified from all over the world (Poopathi and Tyagi, 2004).

The inability of plant and animal proteases to meet current world demands has led to an increased interest in those produced by microbes. In this context, microorganisms represent an excellent source of enzymes owing to their broad biochemical diversity and their susceptibility to genetic manipulation. Micro-organisms degrade proteins and utilize the degradation products as nutrients for their growth. *Bacillus* strains are recognized as sources of commercial proteases because of their ability to secrete large amounts enzymes with relatively great activities (Rao et al., 1998). Moreover, the significant role of proteases (peptide peptidohydrolases group, EC. 3.4.4.1 to 20) produced by *B. thuringiensis* and *B. sphaericus* in the physiology and regulation of sporulation and pathogenicity of those organisms is well-documented (Chen et al., 2003, 2004). In addition, their significance and importance in cell metabolism and endotoxin reactions are clear in terms of their roles in pro-toxin activation (Rukmini et al., 2000). Recent research trends have recognized these advantages and the potential for utilization of bacilli that produce biological insecticides in production of both industrially important proteases and bio-insecticides (Tyagi et al., 2002). This approach would reduce the production costs of bio-insecticides and useful protease enzymes with special reference to the developing countries.

Among proteases produced by some bacilli, alkaline proteases (AP) that work efficiently at alkaline pH range 8-10, have been successfully used for industrial applications including detergents, leather tanning and food industries (Kumar and Takagi, 2002). It has been reported that some bacilli that produce biological insecticides, such as *B. thuringiensis* and *B. sphaericus*, release enzymes into their growth media (Chen et al., 2004). Therefore, it is plausible to investigate utilization of a locally isolated Egyptian strains, that have been previously identified, such as and *B. sphaericus,* for the simultaneous production of biological insecticides and AP, with an aim to reduce production costs (Afify et al., 2009).

The objectives of the current study were to: 1) develop a better understanding of the protease-producing capacity of the entomopathogenic B. *sphaericus*, ii) purify and biochemically characterize the AP synthesized by those selected bacilli cultures and iii) investigate the physiological factors affecting the enzymatic activity.

MATERIALS AND METHODS

Isolation and cultivation of *B. sphaericus*

Isolation, identification and cultivation of *B. sphaericus* from Egyptian soil was done by the use of previously described method (Afify et al., 2009). The growth medium contained fodder yeast, a low-cost Egyptian agro-industrial Egyptian by-product, at a final concentration of 3% (w/v). These concentrations have been previously shown correlating with the production of the highest AP activity (Afify et al., 2009).

Enzyme purification from *B. sphaericus*

Step 1: Ammonium sulphate precipitation

B. sphaericus was grown for 48 h according to the methods of Afify et al. (2009). Cells were separated by centrifugation (10000 × g, 15 min), and the supernatant was fractionated by precipitation with $[(NH_4)_2SO_4]$ between 45 and 75% of saturation. All subsequent steps were conducted at 4°C. The protein was resuspended in 0.1 M Tris-HCl buffer, pH 7.8, and dialyzed against the same buffer. The detailed purification steps used for of AP from *B. sphaericus* are given (Tables 1 and 2).

Step 2: Acetone fractionation

Precooled acetone (-20°C) was slowly added to the combined supernatants from step 1 with continuous stirring to give a final concentration of 20% (v/v). After 30 min, the mixture was centrifuged at 7000 × g for 20 min. The resulting precipitate was discarded and cold acetone was added to the supernatant to give a final concentration of 50% (v/v). The precipitated protein was collected by centrifugation at 7000 × g for 10 min and dissolved in 20 ml 0.1 M Tris-HCl buffer, pH 7.8. The insoluble material was removed by centrifugation and the supernatant was dialyzed against the same buffer.

Step 3: Gel permeation chromatography

The dialyzed supernatant obtained from step 2 was directly loaded onto a column of Sephadex G-200 (1.5 × 24 cm) (Sigma-Aldrich, St Louis, MO) equilibrated with 0.1 M Tris-HCl buffer, pH 7.8. The column was eluted at a flow rate of 60 ml h^{-1} with a 1:1 volume gradient (0.1 M – 1.0 M NaCl) in the same buffer. Fractions (19 to

Table 1. Detailed purification steps of AP produced by *Bacillus sphaericus*.

Purification steps	Concentration (%)	Activity (U ml^{-1} min^{-1})	Total enzyme units	Total soluble protein (mg ml^{-1})	Specific activity (U ml^{-1})	Purification factor "X"	Yield recovery "YR" %
Crude enzyme	0	5777	28884	4236	236	100	10000
Protein fractionation by $[(NH_4)_2SO_4]$	0-15	6107	6107	2104	290	123	2114
	15-30	3036	3036	266	1142	484	1051
	30-45	9661	9661	535	1806	765	3345
	45-60	9862	9862	923	1068	453	3414
	60-75	4009	4009	977	410	174	1388
	75-90	1388	1388	796	174	074	481
Protein fractionation by acetone	0-25	165	83	000	00	000	029
	25-50	214	107	003	3502	1483	037
	50-75	7679	3839	036	10713	4538	1329
	75-90	188	94	053	176	074	032
Sephadex G-200 column fractions	Fraction no						
	1	089	49	1157	04	002	017
	14	11424	11424	107	10712	4538	3955
	16	12330	6782	064	10606	4493	2348
	17	11223	11223	088	12712	5385	3886
	18	10661	5330	038	14018	5938	1845
	25	1598	799	030	2689	1139	277
	30	210	105	029	364	154	036
	35	299	150	024	611	259	052
	40	004	02	020	11	005	001
	45	009	04	015	29	012	002

"X": Specific activity of purified enzyme / Specific activity of crude enzyme; **"YR"**: Total units of purified enzyme / Total units of crude enzyme * 100.

23) with greater protease activities were pooled, dialyzed, and concentrated by lyophilization (Lyophilizer, Vertisis, South Africa) and used for further studies.

Polyacrylamide gel electrophoresis and zymogram analysis

Analyses by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was conducted on a 3% stacking gel and a 12% running gel using Tris-glycine as a running buffer, according to a previously described protocol (Abdel-Megeed et al., 2010). After the end of the runs, protein bands were visualized by staining with Coomassie Brilliant Blue R250 (Merck, Darmstadt, Germany). Purity of AP and its subunit mass were determined by comparison to the migration of molecular weight protein standards. Zymography was conducted on SDS-PAGE according to a previous method (Deng et al., 2010) with some modifications. Polyacrylamide gels were copolymerized with 0.05% gelatin. Following electrophoresis, the gels were washed for 30 min in 50 mM Tris-HCl buffer (pH 7.6) containing 2.5% Triton X-100, with gentle agitation, in order to remove the excess of SDS. Gels were then incubated for an additional 4 hrs with several changes in a solution of 50 mM Tris-HCl (pH 7.6) containing 0.2 M NaCl and 5 mM $CaCl_2$. The zones of proteolysis were detected by overnight Coomassie blue staining.

AP activity

Activity of AP was determined by use of casein as a substrate at a concentration of 0.5% in 0.1 M glycine–NaOH buffer, pH 11.0, as previously described (Afify et al., 2009). Enzyme activity was

Table 2. Summarized purification steps and yield recovery (%) of AP produced by *B sphaericus*.

Purification steps	Concentration (%)/ Fraction	Total Enzyme Units	Total Soluble Protein (mg ml^{-1})	Specific Activity (U ml^{-1})	Purification Factor "X"	Yield Recovery "YR" %
Crude enzyme	0	2888	4236	24	100	100
Protein fractionation by ammonium sulfate	30-45	966	535	181	765	33
Protein fractionation by acetone	50-75	384	036	1071	4538	13
Protein fractionation by Sephadex G-200 column	13	516	040	1297	5494	18
	14	1142	107	1071	4538	40
	15	598	069	871	3689	21
	16	678	064	1061	4493	23
	17	1122	088	1271	5385	39
	18	533	038	1402	5938	19
	19	491	040	1222	5176	17
Pooled active fraction		726	064	1142	4959	27

expressed in term of enzyme units. One unit of enzyme activity is defined as the amount of enzyme resulting in the release of 1 µg of tyrosine per min at 60 °C under the standard assay conditions.

Protein determinations

Soluble protein concentrations were determined by use of the Bradford assay (Bradford, 1976) by use of the Bio-Rad Laboratories protein assay kit, with bovine serum albumin (BSA) as the standard. The A_{595} of 5-µl samples mixed with 200 µl of reagent was measured in 96-well micro plates with a Kinetic Micro plate Reader (Molecular Devices). The concentration of protein during purification studies was calculated from the absorbance at A_{280} nm.

Effect of temperature on the activity of protease

The effect of temperature on pure enzyme was studied by assaying aliquots of the enzyme at different temperatures in the range of 20 to 90°C, at pH 10 using casein as substrate (Afify et al., 2009).

Effect of pH on the activity of protease

The effect of pH on the protease activity was determined by incubating the reaction mixture at pH values ranging from 8.4 to 12.0, in the following buffer systems: 0.1 M Tris–HCl (pH 8.0- 9.0); 0.1 M glycine–NaOH (pH 9.5-12.0) at 55 °C (Afify et al., 2009).

RESULTS

AP purification from *B. sphaericus*

The greatest enzyme activities were obtained in $[(NH_4)_2SO_4]$ fractions between 30 to 45% saturation (Tables 1 and 2). The greatest enzyme levels obtained using acetone precipitation ranged between 50 to 75% v/v (Tables 1 and 2). The AP enzyme was purified 49.5 times, as compared to the original crude enzyme in culture supernatant, with a yield recovery of 27% (Table 2). From the gel permeation elution profile, it was observed that protease was eluted as a well-resolved single peak of caseinase activity coinciding with a single protein peak at a NaCl concentration of 0.6 M (Figure 1). Most of the AP activity units were successfully eluted within 7 (5 ml each) fractions 13 to 19, with a total eluate volume of 35 ml of purified enzyme (Figure 1). SDS-PAGE analysis of the eluate resulting from the application of the acetone-purified fraction on Sephadex G-200 column revealed that the protease is monomeric represented by one single gel band, indicating that the AP active band was successfully purified to homogeneity (Lane 2, Figure 2). The enzyme was shown to have a relative molecular mass of ~29 kD by SDS-PAGE (Lane 2, Figure 2). The zymogram activity staining indicated one clear zone of proteolytic activity. This zone was due to the degradation of gelatin used as a substrate in zymography (Lane 3, Figure 2).

Effect of pH on enzyme activity

Purified AP enzyme exhibited a maximum specific activity of 301 U mg^{-1} proteins at pH 10. It was observed that higher pH values resulted in a gradual decrease in the

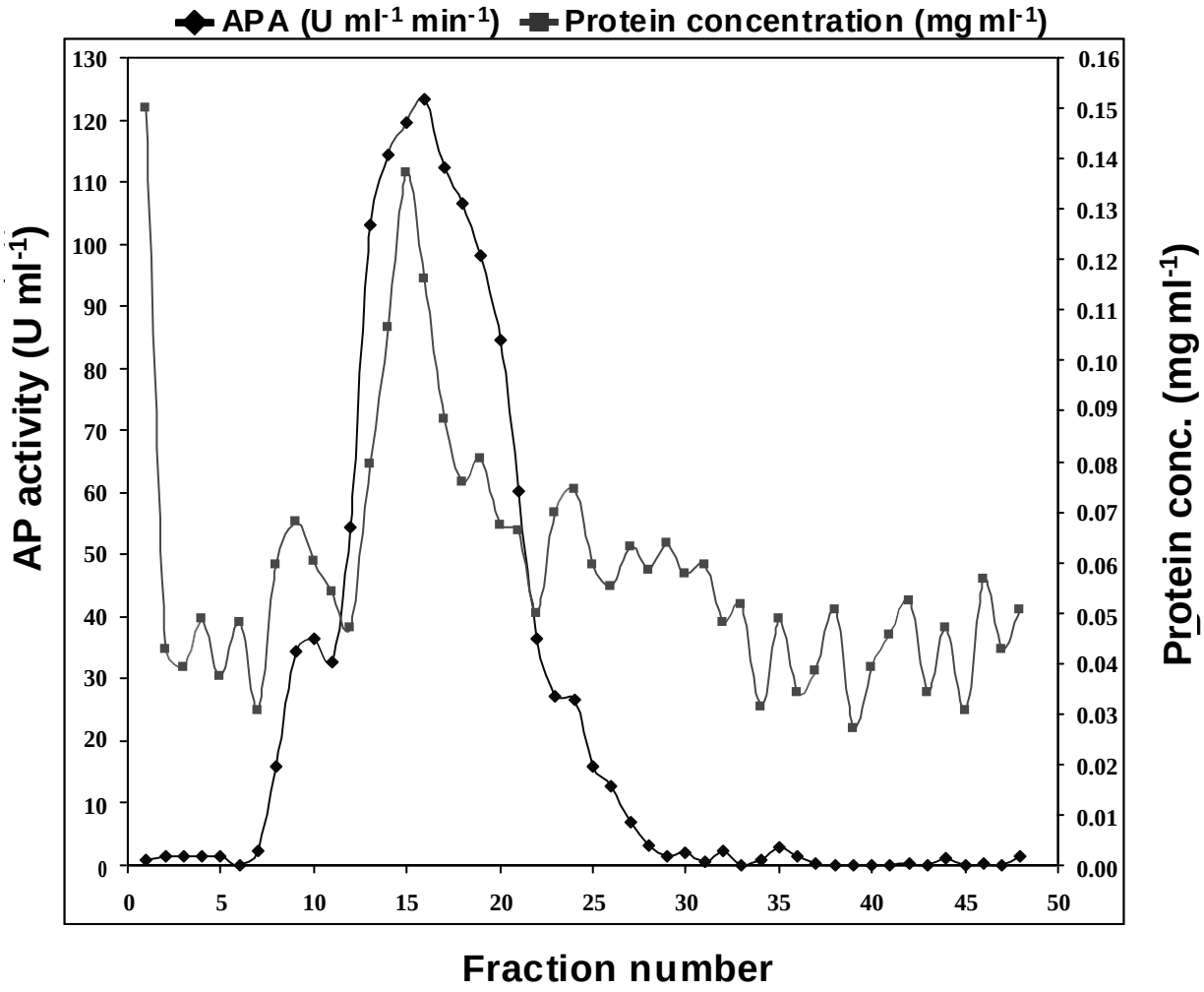

Figure 1. Elution profile of AP purified from *Bacillus sphaericus* by Sephadex G-200 chromatography; A total of 48 fractions (5 ml each) were collected and enzyme activity measured.

activity of the purified AP enzyme dropping to 266 U mg^{-1} protein at pH value of 12 (Figure 3a).

Effect of temperature on enzyme activity and stability

To test the effect of temperature on the activity of purified AP, standard reaction mixtures was incubated in water baths set at different temperatures ranging between 20 to 90°C for 10 min. Results indicated that the greatest activity of the purified AP enzyme was recorded at temperatures between 55 to 60°C with a fast decline at higher incubation temperatures (Figure 3b). In order to determine the thermal stability, aliquots of purified AP were heated at different temperatures for 15 min before assaying the remaining enzymatic activities (Figure 3c). In the absence of substrates, the enzyme was sensitive to temperatures greater than 40°C. Notably, a total loss of enzymatic activity was observed upon heating at 70°C for 15 min (Figure 3c).

Effect of enzyme concentration on activity

The effect of concentration on activity of purified AP is reported (Figure 3d). Increasing AP concentrations ranging from 0.004 to 0.008 mg protein ml^{-1} correlated with a steep increase in specific enzyme activity. A plateau of specific enzyme activity was attained with concentration range of 0.01 to 0.03 mg protein ml^{-1}. Greater concentrations led to a gradual decline in AP specific activity, recording a minimum activity of ~850 U min^{-1} mg^{-1} at enzyme concentration of 0.075 mg protein ml^{-1} (Figure 3d).

Effect of reaction time on enzyme activity

The progress in the reaction rate as a function of the incubation time is graphically illustrated (Figure 3e). A proportional increase in the enzymatic specific activity was obtained for the reaction times tested in the range of

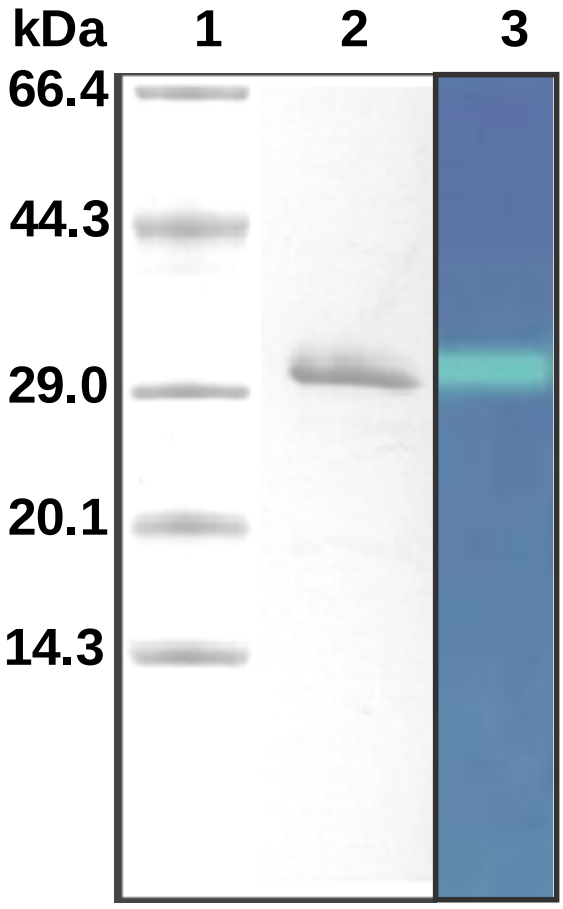

Figure 2. SDS-PAGE of purified AP protein sample (left) and zymogram analysis (right) Analysis was conducted on a 12% gel.

0 to 30 min, with a maximum activity of ~145 U min^{-1} mg^{-1} when the enzymatic reaction was allowed to proceed for 30 min (Figure 3e).

Determination of kinetic parameters for purified AP enzyme

Different concentrations of casein were used to calculate the kinetic parameters for the purified AP enzyme (Figure 3f). Kinetic parameters were determined after constructing a Line weaver-Burk plot with the reciprocal of substrate (casein) concentration (1/S) against the reciprocal of enzymatic reaction velocity (1/V). While a maximum velocity (V_{max}) was determined to be 230 U min^{-1} mg^{-1}, Michaelis-Menten (K_m) constant was calculated to be 0.05% (w/v).

DISCUSSION

Extracellular protease production in microorganisms was greatly influenced by components in media, especially carbon and nitrogen sources, metal ions, and physical factors such as pH, temperature, dissolved oxygen and incubation time (Afify et al., 2009; Moon and Parulekar, 1993; Razak et al., 1994; Takami et al., 1992). Hence, the purified AP fractions were pooled and used for the elucidation of the biochemical properties and enzyme kinetics studies. The purified enzyme exhibited optimum activity at pH value around 10 (Figure 3a). The optimum incubation temperature for the enzyme-catalyzed reaction was between 55 to 60°C (Figure 3b). The purified enzyme was sensitive to exposure to temperatures higher than 40°C (Figure 3c). The enzyme-catalyzed reaction exhibited linear response at low concentrations of the enzyme, incubation times (5 to 30 min) and lesser substrate (casein) concentrations (Figure 3d to f). Among the protein substrates tested casein gave greatest enzyme activity (V_{max}) of about 230 U min^{-1} mg^{-1} and Michaelis-Menten constant (K_m) of 0.05 % (w/v) of the casein substrate at pH 10 and 55°C incubation temperature.

Only a fewstudies have been reported on the purification

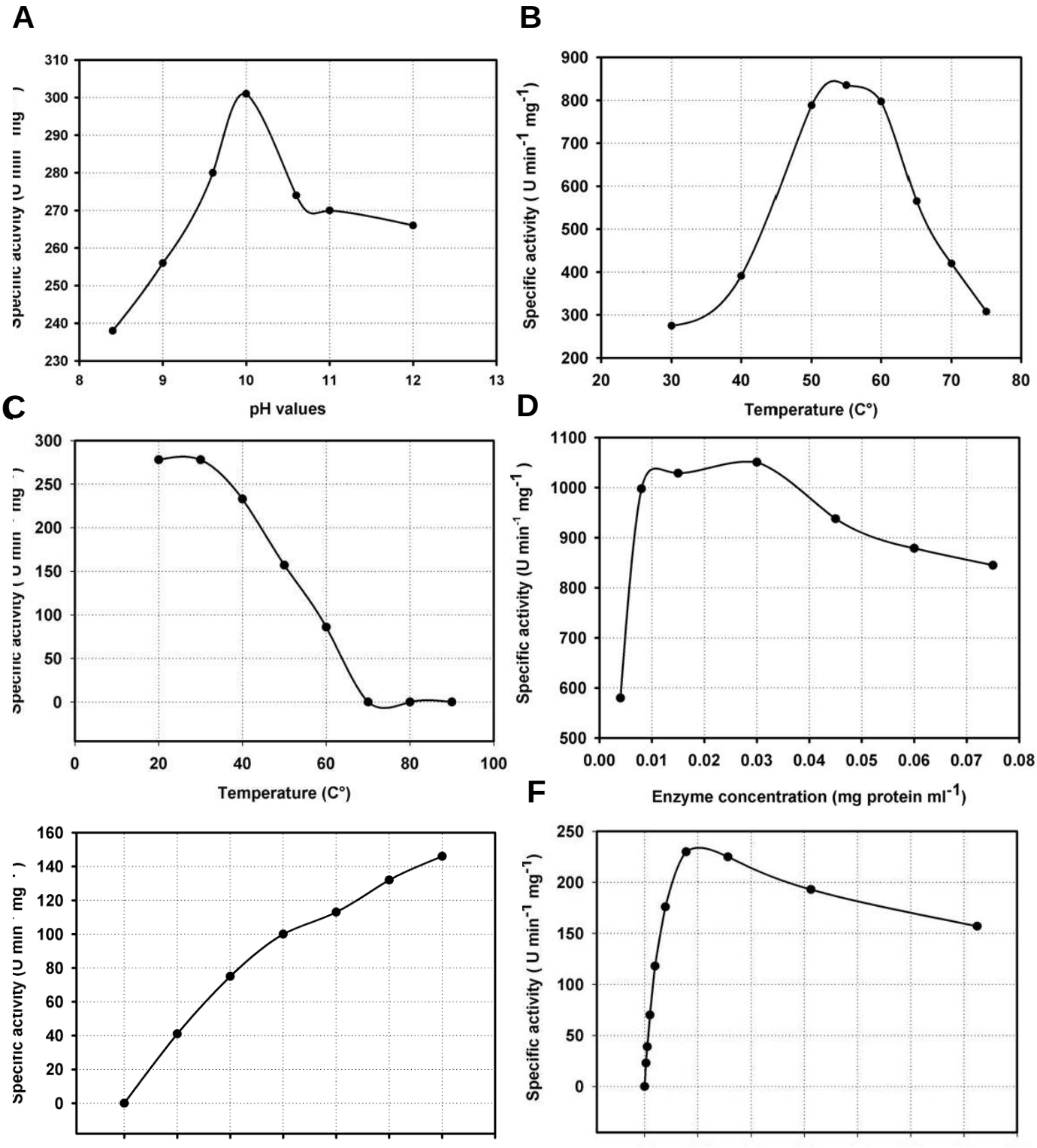

Figure 3. Reaction condition profiles for optimal purified AP activity (A) Effect of buffer pH on the specific activity of AP (B) Effect of temperature on the specific activity of AP The enzymatic reactions were carried out for 10 min in water-baths set at different temperatures (C) Thermal stability of the purified AP enzyme The enzymatic reactions were carried out for 5 min in a set of water baths at different temperatures (D) Effect of enzyme concentration on reaction rate of purified AP of *B sphaericus* (E) Effect of enzyme incubation time on reaction rate of purified AP of *B sphaericus* (F) Effect of substrate concentration on reaction rate of purified AP of *B sphaericus* Specific activity was expressed as U min^{-1} mg^{-1} protein Experiments were conducted twice with similar results.

and elucidation of enzymatic properties of the biological insecticides produced by bacilli, including *B. thuringiensis* and *B. sphaericus*. Screening of 78 *B. sphaericus* strains of different origins has been conducted and revealed that the majority of cultures produced extracellular AP to variable levels when grown in Nutrient broth - Yeast extract - Salt Medium (NYSM), under shake culture (El-Bendary et al., 2002). In that study, the enzyme exhibited maximum activity at 55°C incubation temperature and pH 8.5 suggesting an AP activity with retention of about 60 % of the activity at pH 10 for the enzymes of both bacterial strains. Enzyme stability at 60°C was markedly improved in the presence of calcium chloride. Inhibition studies showed that the AP activity was inhibited (72 to 79%) by an active-site inhibitor of serine protease, phenylmethylsulfonyl fluoride (PMSF) but not by soybean trypsin inhibitor or iodoacetamide. The authors suggested that the AP activity produced belongs to serine protease group of enzymes prevalent in some species of bacilli (El-Bendary et al., 2002). More recently, the same researchers (El-Bendary, 2004) have investigated the formation of extracellular milk clotting enzyme (MCE) by *B. sphaericus*. A screening study on 134 strains of *Bs* of different origins has revealed that the majority of cultures produced extracellular MCEs in variable levels. The most active MCEs were produced by the standard strain, *B. sphaericus* Ghar.I and the Egyptian isolate, *B. sphaericus* NRC 24. The ratios of milk clotting activity / proteolytic activity at pH 6 have been reported to be 14 and 13 for *B. sphaericus* NRC 24 and *B. sphaericus* Ghar.I, respectively. MCE exhibited maximum activity at 60°C incubation temperature and pH 5.7 to 6.0. The enzyme was thermo stable up to 50°C for 60 min. Preliminary tests for application of the enzyme for cheese manufacturing have indicated that MCE produced by *B. sphaericus* is able to produce white soft curd with good quality (El-Bendary, 2004).

Moharam et al. (2003) have conducted the most detailed study on the APs of B. sphaericus. The authors studied the alkaline serine proteases (EC 3.4.21) produced by two highly mosquitocidal *B. sphaericus* cultures, from local (*B. sphaericus* culture NRC-69 isolated from Egyptian soil) and international (IS-2362) origins. They subjected both enzymes to a purification scheme including dialysis, ammonium sulfate fractionation and gel filtration on Sephadex G-100 column in order to separate the enzyme from the culture supernatants (Moharam et al., 2003). Then, they carried out comparative studies between the biochemical characteristics of the purified enzymes. Both APs were purified to homogeneity and characterized. The two enzymes were purified 58 and 126 fold, as compared to the original activities of the crude enzyme preparations, with specific activity of 75 and 168 U min^{-1} mg^{-1} protein for strains IS-2362 and NRC-69, respectively (Moharam et al., 2003). The purified enzymes of the two sources exhibited high degree of similarity, both showing maximal activity at pH 8.5 and 60°C reaction temperature. Furthermore, those two reported enzymes were stable up to 50°C (Moharam et al., 2003). By comparison, our present study clearly indicates that the purified AP from *B. sphaericus* possesses different biochemical properties, having optimum activity at pH 10 and thermal stability up to 40°C (Figure 3).

It has been well documented that several species of bacilli produce AP extracellular in their growth media with a special reference to alkaliphilic strains (Horikoshi, 1999). In addition, the same researcher has described earlier the production of an extracellular alkaline serine protease from alkaliphilic *Bacillus sp.* strain 221 (Horikoshi et al., 1971). This strain, isolated from soil, produced large amounts of AP that differed from the subtilisin group. The optimum pH of the purified enzyme was 11.5, and 75% of the activity was maintained at pH 13 (Horikoshi et al., 1971). The molecular weight of the enzyme has been reported to be 30 kDa, which is similar to the estimated molecular mass of the AP purified from the current study (Figure 2). Some APs have been isolated from alkaliphilic microorganisms (Fujiwara and Yamamoto, 1987; Nomoto et al., 1984; Tsai et al., 1983, 1984, 1986; Tsuchida et al., 1986). A previous report described the purification of AP from a thermophilic alkaliphile, *Bacillus sp.* strain B18. The optimum pH and temperature for the hydrolysis of casein were pH = 12 to 13 and 85°C, respectively, both of which are greater than those generally recognized for APs (Fujiwara et al., 1993). AP has been isolated from a new alkalophilic *Bacillus* sp. strain AH-101 in which the enzyme was most active toward casein at pH 12 to 13 and stable for 10 min at 60°C and pH 5 to 13 (Takami et al., 1989). The optimum temperature was about 80°C in the presence of 5 mM calcium ions. The AP showed a greater hydrolyzing activity against insoluble fibrous natural proteins such as elastin and keratin than did subtilisins and proteinase K (Takami et al., 1990, 1992). A keratinase of a feather-degrading *Bacillus licheniformis* strain, PWD-1, has been reported (Cheng et al., 1995). This enzyme was stable from pH 5 to 12. The optimal reaction pH values for feather powder and casein were 8.5 and 10.5 to 11.5, respectively (Cheng et al., 1995).

In conclusion, the present studies have yielded very promising results that are potentially useful for future applications. This view is strengthened by the fact that two useful products could be produced simultaneously. Thus, the enzyme, which is extracellular, could be produced simultaneously in the same fermentation process, with production of the biological mosquitocidal toxins used in biological control of mosquitoes. These biological control agents produced by *Bacillus sphaericus* have been recently employed as novel trends of fermentation because they are safe for man, animal and environments. Moreover, the successful simultaneous production of bacterial biopesticides and AP has been reported in fermenters using wastewater sludge as the

main substrate in the growth media (Tyagiet al., 2002). These novel fermentation approaches would certainly increase the application feasibilities and reduce the production costs of those useful microbial products.

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