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Research Article

Purification Scheme and Biochemical Profile of Metallo-alkaline Protease Produced by *B.t. Dendrolimus* IP 4A/4B

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Abstract

Objective: Purify and classify alkaline protease (AP), produced by *Bacillus thuringiensis* (*B.t.*) *dendrolimus* 4A/4B.

Methods: Inoculum preparation, production media, enzymes assays, protein determination, ammonium sulfate precipitation, acetone fractionation, Sephadex Gel chromatography filtration.

Results: The purification scheme, (a) ammonium sulfate fractionation, the most active fraction with 1.93 purification fold was at 60-75% saturation; (b) acetone precipitation, where the most active fraction was at 30-45% with purification fold of about 3.92 times; (c) Column chromatography on G-100 Sephadex with final purification folds 5.36 times of the crude enzyme with final yield recovery 1.9%.

Purified AP showed stability against H_2O_2 1%, 5% and 10% (v/v), and retained 111%, 101% and 66% of its activity, respectively. AP retained 82%, 87% and 101% of its activity with 10, 5 and 1mM PMSF, respectively. Enzyme activity was completely inhibited by 10mM EDTA, while the enzyme retained about 125% of its activity with 10mM Mercapto-ethanol. $CaCl_2$ and $MgCl_2$ (10mM) showed activation effect, increasing the activity to 136% and 147% compared to the control, respectively. The optimum pH are 8.0 and 10.0. AP activity was increased through 30ºC to 50ºC. At 60˚C, the enzyme retained ~48% of its original activity. AP concentration 0.014mg protein/mL has maximum activity (6.750U/mL/min). The highest maximum velocity (Vmax) was 31.821µg tyrosine/mL/min. Km value 0.833%, w/v casein concentration.

Conclusion: Thus, this inhibition profile suggested that the AP produced belongs to the class of metalo-proteases.

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Keywords: bacillus thuringiensis, alkaline protease, AP purification, AP characterization, biochemical properties, metallo-protease

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1 INTRODUCTION

Bacillus thuringiensis (*B.t.*) is the most popular microbial biocidal in recent years, due to its crystal protein insect prototoxin; some strains have, in addition to the insecticidal toxin, certain enzymes that stimulate the biological activity against insects including alkaline protease (AP), which catalyzes the conversion of prototoxin to the active, low molecular weight form in different mechanisms[1].

Protease has a broad application base in various fields; protease enzymes represent two thirds of the enzymes of industrial application[2]. Proteases have various applications, such as photography, detergents, leather and food industries; such as fermentation of proteins, meat and dairy[3]. Enzymes are characterized as natural catalysts over their chemical counterparts in terms of sustainability and efficiency, while the role of catalytic enzymes in the commercial operations of the pharmaceutical, food and beverage industries, etc., has been reduced[4]. Developing bio-stimulants, improving industrial processes and increasing productivity with the maximum profit possible for the market on the one hand, and also reducing the environmental and material burden on the other hand[5].

Classifying the enzyme and knowing its biochemical specifications is an indispensable step in maximizing the efficiency of the enzyme and thus raising the efficiency of its applications. Therefore, this study aims to attempt an accurate and in-depth understanding of the optimal conditions for purifying an enzyme from the applied enzyme giants in the market (AP), in addition to studying its biochemical properties, to broaden the horizons for scientists and practitioners about the development and the direction of these industries while preserving the environment from the use of chemical catalysts that are extremely harmful to the environmental entire vital system.

2 MATERIALS AND METHODS

Bacterial strain, media used for growth and production of AP, AP activity assay and protein determination, it was also explained in detail in our previous article[6].

2.1 Experimental Chemicals

All used chemicals in this study were purchased from the Sigma Chemical Company.

2.2 Purification Steps of AP

2.2.1 Ammonium Sulfate Precipitate

Ammonium sulfate precipitation was carried out by using automatic equation program, cited from https://www.encorbio.com/protocols/AM-SO4.htm

2.2.2 Acetone Fractionation

This step of purification was conducted as mentioned by Chaykin[7]. The resulting precipitate was separated by cooling centrifugation. The precipitate was dissolved in 10mL distilled water and mixed well. The enzyme supernatant was taken to be re-precipitated by the subsequent addition of suitable milliliters of Acetone to bring the enzyme solution to the next saturation level.

2.2.3 Sephadex Gel Filtration Chromatography

Purification by Sephadex G-100 was carried out as described by Aboul-Soud et al[8].

2.2.4 Effect of Enzyme Inhibitors, Surfactants, Bleach, and Oxidizing Agents on the Enzyme Activity

The effects of Co^{2+}, Ag^{1+}, Mn^{2+}, Hg^{2+}, Ca^{2+} and Mg^{2+} as metallic ions, H_2O_2 as an oxidizing agent, Mercapto-ethanol, Phenyl Methyl Sulfonyl Fluoride, Ethylene Diamine Tetra Acetic acid as inhibitor on AP activity and Diisopropyl Fluorophosphate as sulfhydryl agents were investigated to further characterize the enzyme as detailed by Amira[3].

3 RESULTS AND DISCUSSION

3.1 Purification Steps of Crude AP Produced by *B.t. Dendrolimus* Ip 4a/4b Culture, Grown on Wheat Bran Under SSF Conditions

3.1.1 Ammonium Sulfate Fractionation of Crude AP Produced by *B.t. Dendrolimus* IP 4A/4B Culture under SSF (Solid State Fermentation) Conditions

The most active ammonium sulfate fraction with 1.93 purification factor, 645 units and 508U/mg, was obtained at 60-75% saturation as shown in Table 1. Ullah et al.[9] found that 70% ammonium sulfate saturation achieved total AP activity 890.368 units with 6.5 purification fold. While the purification efficiency was at 40-60%, ammonium sulfate salt yielded 2.3 fold and 1732.8 total units[10].

3.1.2. Acetone Precipitation of AP (Ammonium Sulfate Fractionated) Produced by *B.t. Dendrolimus* IP 4A/4B Culture under SSF Conditions

The highest purification factor reported in Table 2,

Table 1. Ammonium Sulfate Fractionation of Crude AP Produced by *B.t. Dendrolimus* IP 4A/4B Culture under SSF

Ammonium Sulfate Saturation (%)	APA (U/ml/min)	Total Enzyme Units	Total Soluble Protein (mg/ml)	Specific Activity (U/mg)	Purification Factor "X"	Yield Recovery "YR" %
Crude enzyme	240	12009	45.6	263	1.00	100.0
15	13	130	1.5	89	0.34	1.1
30	15	150	0.7	202	0.77	1.2
45	7	66	1.5	43	0.16	0.6
60	64	645	1.6	414	1.58	5.4
75	49	493	1.0	508	1.93	4.1
90	24	239	1.8	135	0.51	2.0

Table 2. Acetone Precipitation of AP (Ammonium Sulfate Fractionated) Produced by *B.t. Dendrolimus* IP 4A/4B Culture under SSF

Acetone Concentration (%)	APA (U/ml/min)	Total Enzyme Units	Total Soluble Protein (mg/ml)	Specific Activity (U/mg)	Purification Factor "X"	Yield Recovery "YR" %
15	140	1400	1.6	863	3.28	11.7
30	117	1168	1.2	993	3.78	9.7
45	100	996	1.0	1032	3.92	8.3
60	188	1879	3.7	505	1.92	15.6
75	80	804	2.1	382	1.45	6.7
90	165	1646	3.1	531	2.02	13.7

was 3.92 and total specific activity 1032U/mg at 30-45% acetone saturation. While the specific activity of Shaikh et al.[11] was 4975.942U/mg; Niyonzima and More[12] reported specific activity 13.6U/mg and 2.6 purification fold.

3.1.3 Elution Profile of AP from G-100 Sephadex Column of AP (Precipitated by Acetone) Produced by *B.t. Dendrolimus* IP 4A/4B Culture under SSF Conditions

Sephadex G-100 elution profile showed sharp AP activity peak at fraction No.11, as illustrated in Figure 1, achieved 234 total AP units and 1408U/mg specific activity with 5.36 purification factor. Shaikh et al.[11] found 31907.27 total enzyme activity, 8741.72U/mg specific activity and 3.12 purification fold; while Ullah et al.[9] achieved 208.591 total AP activity, 8902U/mg specific activity and 35.91 purification fold.

3.2 Characterization Profile of Column Purified AP of *B.t. Subspecies Dendrolimus* (IP 4A/4B)

3.2.1 Effect of Activators and Inhibitors on Column Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

3.2.1.1 Effects of Metallic Ions

$CaCl_2$ and $MgCl_2$ (10mM) increased the activity to 136% and 147% of the control, respectively. This can be explained that the enzyme required the presence of Ca^{2+} and Mg^{2+} ions to be active and stable. Furthermore, we found that Co^{2+}, Ag^{1+}, Mn^{2+} and Hg^{2+} ions inhibit the enzyme activity.

Shaikh et al.[11] reported significant increase of protease activity by adding Ca^{2+} while Co^{2+} and Hg^{2+} showed inhibition effect. Elgammal et al.[13] illustrated that Ag^{2+} inhibited protease activity. Ullah et al.[9] showed that Mg^{2+} and Ca^{2+} stimulated protease activity 125% and 140% respectively. Mahakhan et al.[14] reported that Mg^{2+} stimulated AP of Bacillus gibsonii 6BS15-4 slightly with relative activity 114% while retaining 84% and 97% of enzyme activity in the presence of Mn^{2+} and Ca^{2+}, respectively. Lemes et al.[15] illustrated protease inhibition by Mn^{2+} as the effect of the salts CaO, $MgSO_4$ and $CaCl_2$ was decreasing in activating the enzyme. Li et al.[10] found that 5mM Ca^{2+} activated protease with relative activity 164.2%; while Mg^{2+} and Mn^{2+} inhibited the enzyme by increasing directly with increasing concentration. Niyonzima and More[12] AP activated by Ca^{2+} and Mg^{2+}; while Co^{2+} did not affect activity; but Mn^{2+} caused mild inhibition. As for Hg^{2+}, 25% of enzymatic activity was lost.

3.2.1.2 Effects of H_2O_2 as an Oxidizing and Bleaching Agent

Results showed that the protease from strain *B.t. dendrolimus* Institute Pasteur (IP) 4A/4B showed high stability against all concentrations of H_2O_2 tested; 1%, 5% and 10% (v/v), and retained approximately 111%, 101%

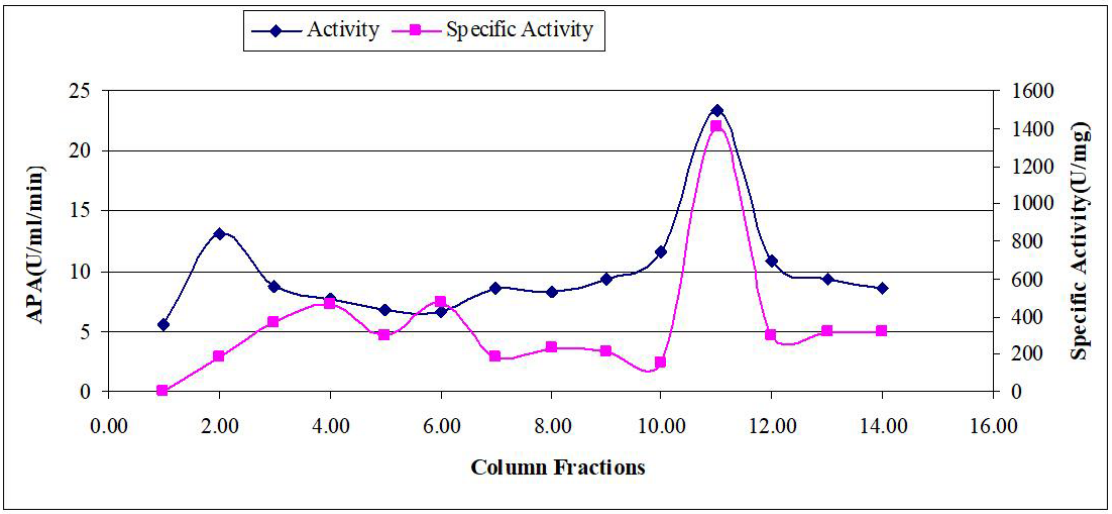

Figure 1. Elution Profile of AP from G-100 Sephadex Column of AP (Precipitated by Acetone) produced by *B.t. dendrolimus* IP 4A/4B culture under SSF.

and 66% of its activity, respectively.

Mahakhan et al.[14] noticed that the AP of Bacillus gibsonii 6BS15-4 did not affected by 0.1% (v/v) H_2O_2.

3.2.1.3 Mercapto-ethanol, PMSF and EDTA as Inhibitors; and DFP as Sulfhydryl Agents

We found that the AP enzyme under study retained 82%, 87% and 101% of its activity after reaction with 10, 5 and 1mM PMSF, respectively. Thus, it essentially inhibited the AP enzyme investigated at high doses, yet there was no inhibitory effect observed with the least PMSF concentration tested (1mM).

In the present study, the AP activity was completely inhibited by 10mM EDTA, while the enzyme retained about 125% of its activity by treatment with 10mM Mercapto-ethanol (as sulfhydryl agent).

Gençkal[16] characterized serine proteases by irreversible inhibition by PMSF. Hence, the AP enzyme under the current investigation was not a serine protease. According to the specificity of their action, metallo-proteases are inhibited by chelating agents such as EDTA but not by sulfhydryl agents or DFP[16]. Whereas, the belonging of the protease to the group of serine proteases was demonstrated by Shaikh et al.[11] through complete inhibition by PMSF and DFP; it strongly inhibits the enzymatic active site by sulfating essential serine residuals. Ullah et al.[9] showed that PMSF inhibited protease activity completely; while DFP inhibited 92% of protease activity. Mahakhan et al.[14] noticed that alkaline serine protease of *Bacillus gibsonii 6BS15-4* was not affected by EDTA but was inhibited significantly by PMSF. By indicating that the enzymatic activity is not affected by EDTA, while it was strongly inhibited in the presence of PMSF[12], it was proved that there was a tyrosine residue in the protease active site.

3.2.1.4 Effect of Acetone as Solvent

From results illustrated in Figure 2, it could be observed that the enzyme retained 39% of its activity when the acetone concentration in the reaction mixture was 50%. Lemes et al.[15] found that using Acetone at 50% concentration showed an inhibition percentage of 21.2%. They attributed the inhibitory effect of acetone to the withdrawal of water from the center of enzymatic activity and around the enzyme, which made it lose its catalytic properties.

Thus, this inhibition profile suggested that the protease produced from strain *B.t. subspecies dendrolimus* (IP 4A/4B) belonged to the class of metallo-proteases.

3.2.2 Effect of pH on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

Effect of pH on the purified AP was determined by assaying enzyme activity using glycine-NaOH buffer (at pH ranged from 8.4 to 11.0). Also, Tris-HCl buffer at pH range of (7.4-9.0) was used, and illustrated in Figure 3. While it retained about 44% of its activity at pH 11. It is clear from the bell-shaped curve that the enzyme has two optimum pH levels 8.0 and 10.0.

Blandine et al.[17] found that the optimum pH for the enzyme is at pH 2.79 and 9, and he explained that there are two types of proteases, one is acidic (aspartic protease) and the other is alkaline (serine protease). Whereas, Atrooz and Alomari[18] explained the existence of two optimum pH levels at 3 and 9pH by the presence of an isoenzyme of the molecular enzyme. As for Ma et al.[19], they explained the existence of more than one optimum pH for the enzyme for one of three theories, either for the degree of purity of the enzyme, for the presence of a different isozyme forms, or for the formation of two ionized enzyme-substrate complexes.

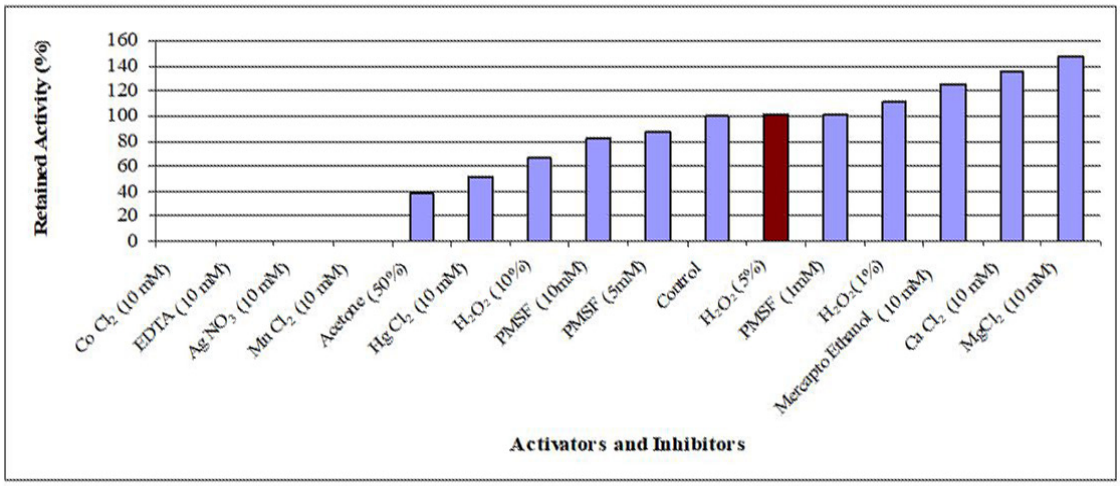

Figure 2. Effect of activators and inhibitors on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

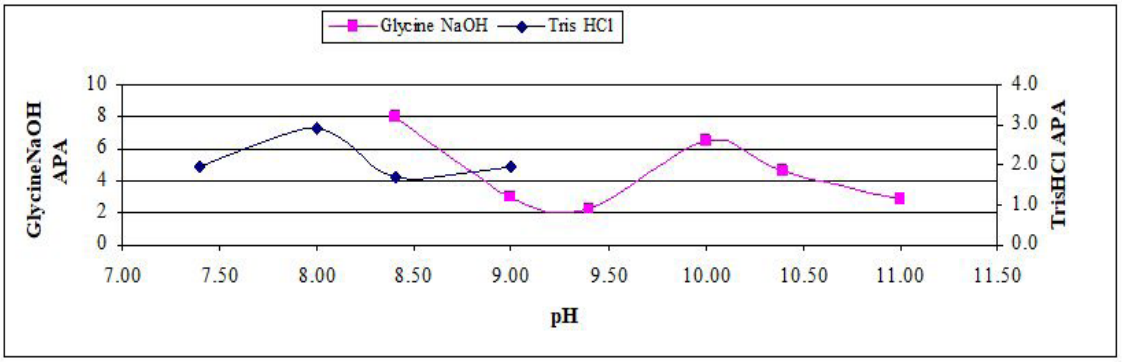

Figure 3. Effect of pH on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

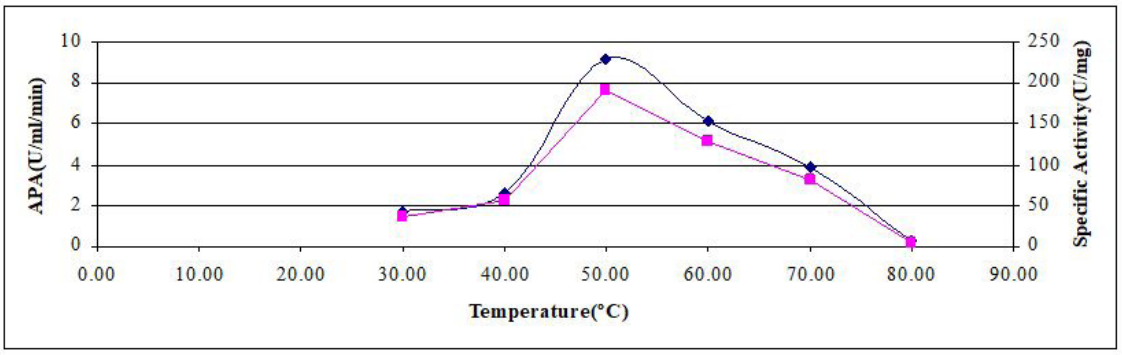

Figure 4. Effect of temperature on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

In any case, the presence of more than one optimum pH leads the enzyme to the best application; we referred to a summary of these applications in our previous research on this enzyme[3], in the application of silver recovery from used X-ray films and detergents.

3.2.3 Effect of Temperature on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

Temperature plays a vital role for activating and deactivating enzyme activity. AP activity in Figure 4, was increased from 30ºC to 50ºC showing maximum activity at 50ºC reaction temperature, and retained about 66% from protease activity at 60ºC and still stable until 70ºC.

Ullah et al.[9] reported AP optimum reaction temperature at 55ºC, and kept activity up to 60ºC[10,11,14]. While Elgammal et al.[13], Niyonzima and More[12] illustrated optimum temperature of AP activity at 50ºC, which retained almost all of its activity from 30ºC to 60ºC.

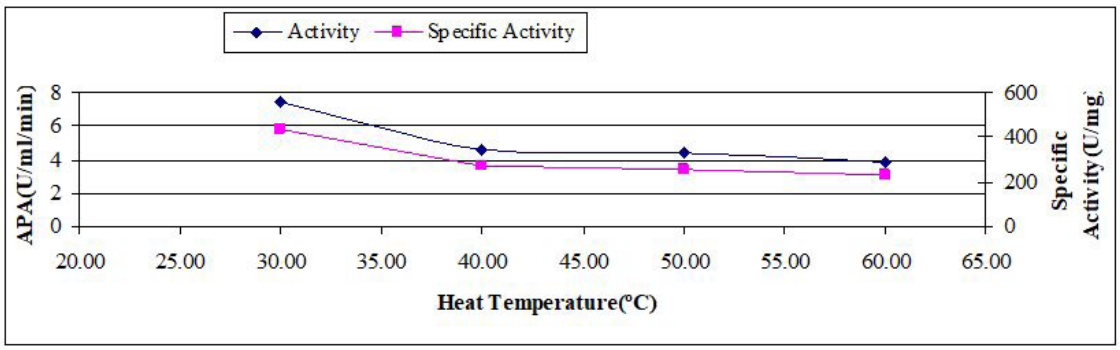

Figure 5. Effect of heat on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

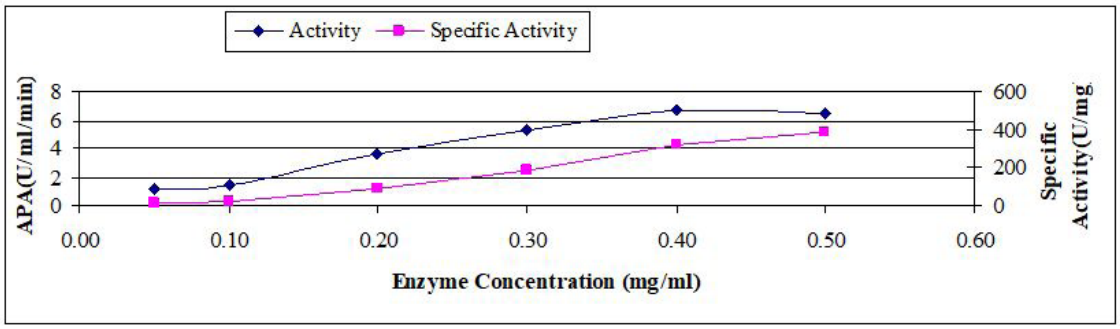

Figure 6. Effect of enzyme concentration on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

3.2.4 Effect of Heat on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

Thermostability of AP was illustrated in Figure 5 and checked by heating enzyme solution at 30, 40, 50 and 60ºC for 15min, then incubating at 50ºC. At 60˚C, the enzyme retained ~48% of its original activity. While purified protease retained 46% of control activity at 60ºC[9]. *B. gibsonii* 6BS15-4 AP keeped activity between 30-55ºC for 60 min reaction period[14]. As AP lost 1.3% relative activity at 50ºC graduated to 19.6% at 80ºC[12].

3.2.5. Effect of Enzyme Concentration on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

In Figure 6, the enzyme concentrations in the reaction mixtures varied between 0.002-0.017mg protein/mL of the reaction mixture. The enzyme activity was determined under standard conditions. The reaction rate exhibited approximately linear response with enzyme protein concentration at least up to 0.014mg/mL of the reaction mixture followed by leveling off with no further increase in rate of reaction at higher enzyme concentrations.

Among the different enzyme concentrations (0.002-0.017mg protein/mL) studied, 0.014mg protein/mL of enzyme produced maximum protease activity (6.750U/mL/min).

Huang et al.[20] revealed that 0.34mg protein/ml of crude protease for obtaining maximum activity. While Aboul-Soud et al.[8] reported purified AP activity about 850U/min/mg at 0.075mg enzyme protein/ml.

3.2.6 Effect of Reaction Time on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

The effect of reaction time on the reaction rate of AP was carried out with periodic sampling for measuring the reaction progress with the reaction intervals shown in Figure 7. The slope of the first period (up to 5 minutes reaction time) exhibited a steeper slope indicating reaction velocity near the theoretical initial velocity (V_{max}) that has decreased in value as the reaction was extended up to 60min.

Ullah et al.[9] found maximum activity of AP at 35min. While Aboul-Soud et al.[8] illustrated a maximum at 30min.

3.2.7 Effect of Substrate Concentration on Purified AP Activity Produced by *B.t. Dendrolimus* IP 4A/4B

The effect of different substrate concentrations on the rate of the AP catalyzed reactions was studied and illustrated in Figure 8. The highest maximum velocity (V_{max}) was obtained upon using casein as a substrate amounting to 31.821µg tyrosine/mL/ min. Determination of Michaelis constant (K_m) using the hyperbolic function of Michaelis-Menten Plot of substrate concentration vs. reaction velocity as well as from Lineweaver-Burk

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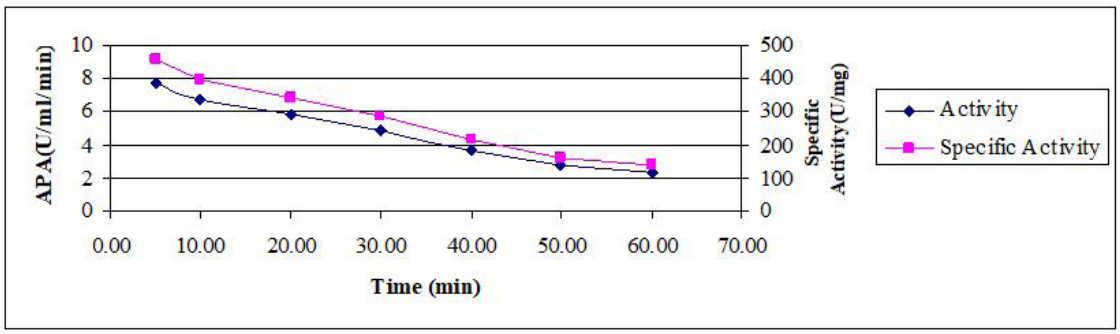

Figure 7. Effect of time on purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

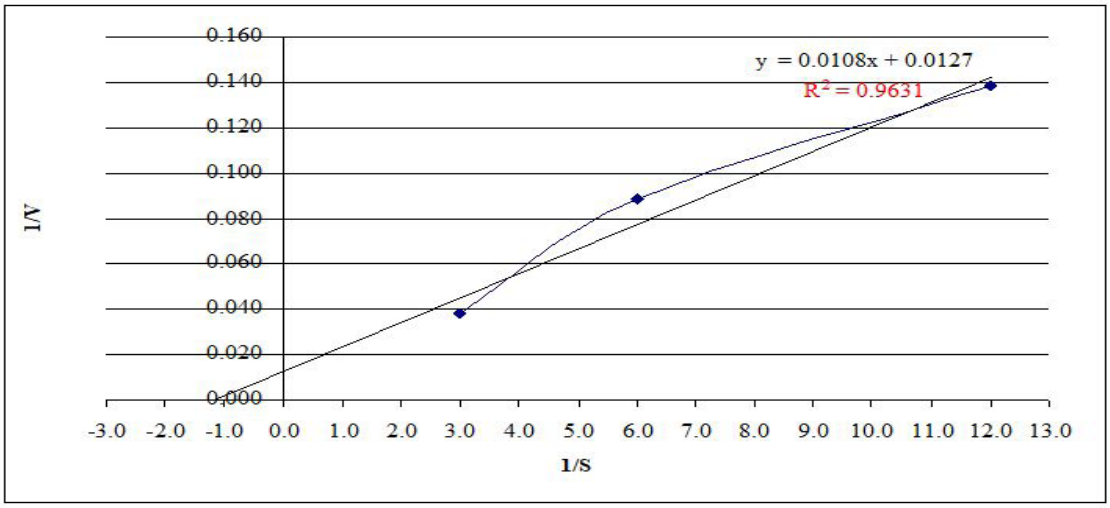

Figure 8. Lineweaver-Burk Plot of reciprocal of enzyme substrate (casein) concentration (1/S) against reciprocal of enzymatic reaction velocity (1/V) for purified AP activity produced by *B.t. dendrolimus* IP 4A/4B.

reciprocal plot revealed Km values of 0.833%, w/v casein concentration.

Niyonzima and More[12] reported K_m and V_{max} of purified AP were 5.4mg/ml and 12.8U/ml respectively. K_m and V_{max} of Bt AP were 0.072% (w/v) and 7.952U/ml respectively[21].

4 CONCLUSION

The results in this paper showed that the AP enzyme produced from the bacterium strain *B.t. dendrolimus* IP 4A/4B, subjected to a purification scheme by ammonium sulfate fractionation, acetone precipitation, and Column chromatography on G-100 Sephadex; and by testing the purified enzyme; with conventional activation and inhibition factors to characterize the enzyme, purified AP showed stability against H_2O_2 and PMSF. While enzyme activity was completely inhibited by 10mM EDTA, the enzyme retained about 125% of its activity with 10mM Mercapto-ethanol. $CaCl_2$ and $MgCl_2$ (10mM) showed activation effects. The optimum pH are 8.0 and 10.0. AP activity was increased through 30ºC to 50ºC. Thus, this characterization profile suggests that the AP produced belongs to the class of metallo-proteases.

4.1 Recommendations

It can be applied in industries suitable for alkaline metalo-protease.

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Conflicts of Interest

The authors declared no conflict of interest.

Author Contribution

Roshdy AM studied, wrote, reviewed and corrected this article; Shata HM, Ali SM, Youssef AM, Foda MS and Kahil T studied and wrote this article.

Abbreviation List

AP, Alkaline protease
APA, Alkaline protease activity
Bt, Bacillus thuringiensis
IP, Institut Pasteur
SSF, Solid state fermentation

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