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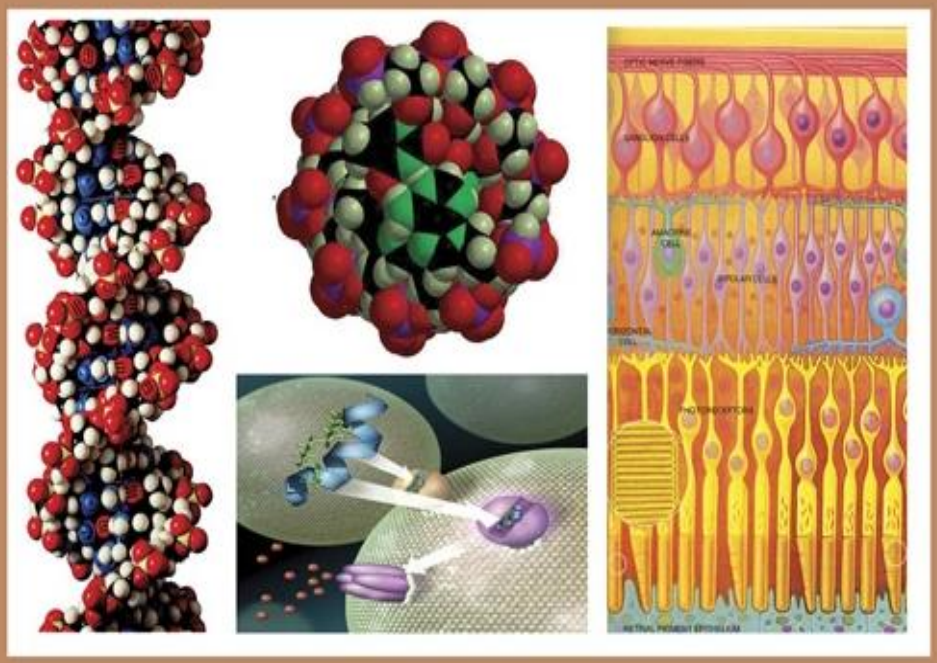

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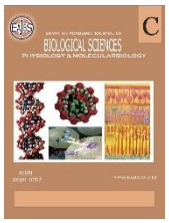

Purification and Characterization of Alpha Toxin *Clostridium perfringens* Type A

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ABSTRACT

Clostridium perfringens is a toxin-producing anaerobe with the ability to cause multiple diseases in humans and animals. One of the toxins produced by all strains of this bacterium is an alpha toxin (phospholipase C). This study aimed to purify and characterize alpha-toxin. *C. perfringens* type A was grown in an optimized medium, the culture supernatant was separated, and ammonium sulfate precipitated protein was subjected to ion exchange and gel filtration chromatography methods. The purification steps were monitored by SDS-PAGE and hemolysin activity for each sample and performed by the hemolysin assay method. The purified alpha-toxin appeared in a single band in 43 KDa. The enzymatic efficiency yield was about 88% with a specific activity of 69170 and 654 fold of purification. According to the results, alpha-toxin is Zn and Mg metal-enzyme dependent on the temperature and pH optimum of 40 °C and 6, respectively. The enzyme K_m and V_{max} values of 3.8 mg/ml and 0.19 were calculated as the enzyme in the presence of lecithin. The partially purified alpha toxin with specific characters can be used for different applications such as antibody production and determination of the anti-toxin rate in animal serum.

INTRODUCTION

Clostridium perfringens is an anaerobe residing in water, sewage, soil, and intestinal contents of humans and animals. The bacterium has five strains according to the production of different toxins. All types of *C. perfringens* produce alpha toxin, but the highest production is reported in *C. perfringens* type A (Zückert *et al.*, 1998). Alpha toxin is dangerous to human and animal health. This bacterium causes gas gangrene, food born poisoning, necrosis, and enterotoxaemia. The gas gangrene, caused by alpha toxin leads to bradycardia, shock, and eventually death(Cavalcanti *et al.*, 2004).

Alpha toxin is a phospholipase C with lecithinase activity. Hydrolysis of lecithin in egg yolk medium and red blood cells (RBC) hemolysis tests are commonly used in the detection of its activity and presence (Skariyachan *et al.*, 2010). In general, the alpha toxin has a molecular weight of 43 KDa and a pI of about 5.4; it is a metalloenzyme that is dependent on zinc ion. Conventional and classical purification such as salt precipitation, organic solvents, electrophoresis, column chromatography, ion-exchange chromatography, and affinity chromatography. Mainly combination of four methods consisting of salting out, cation exchange chromatography, anion exchange chromatography, and finally gel filtration resulted in purify toxins of *C.perfringense*(Zayerzadeh *et al.*, 2014).

Using dialysis and salting out with ammonium soleplate and finally, anion chromatography was managed to purify epsilon-toxin of *C. perfringens* type D (Najafi, 2012). The BoNT/A3 neurotoxin of *C. botulinum* bacterium was purified by ion-exchange chromatography (Johnson et al., 2016). Purified botulin neurotoxin of *C. botulinum* was reported with applying affinity chromatography (Nickel column) (Sayadmanesh *et al.*, 2013). The precipitation method (polyethylene glycol) could successfully be used to purify alpha toxin from *C.novyi* (Fathi Najafi *et al.,* 2011). Alpha toxin purification of *C. perfringens* type A was performed by ultrafiltration and gel chromatography (Odendaal, 1987). And alpha toxin of *C. perfringens* was purified by treatment with DEAE-Sephadex in a batch-wise process, Sephadex G-75 chromatography, and isoelectric focusing (Möllby *et al.,* 1976). The present study aimed to produce and purify alpha toxin from *C. perfringense*, and in order to analyze its physiochemical properties.

MATERAILS AND METHODS

Microorganism and Growth:

C. perfringens type A was taken from Dr. Fathi Najafi form Razi Vaccine and Serum Research Institute, Mashhad. The bacterium was grown in the liver extract medium for 24 hours. The bacterium stock culture was used for other work such as growth and toxin production.

Alpha Toxin Assay and Lecithinase Activity Detection:

Hemolysis Assay:

Alpha toxin is a phospholipase C by acting on cell membrane phospholipids in red blood cells (RBC). After cell disruption of RBCs, the hemoglobin pigments releases. Released hemoglobin from RBC has a direct relation with alpha toxin activity. The hemolysis assay was performed according to Möllby and Wadstrom with some modifications. The reaction was performed by adding 0.2 ml of test samples into 1 ml of buffer washed RBC. The mixture was incubated at 37ºC for proper time incubation(Möllby *et al.,* 1976). Then, the mixture was centrifuged for 5 min at 3000 RPM and the clear supernatant absorption was measured at 570 nm.A unit of Hemolysin enzyme is the amount of the alpha toxin that catalyzes the lysis of one percent of the substrate (Red Blood Cells in this case) per minute under the optimized condition.

Lecithinase Assay:

Alpha toxin has lecithinase activity that results in hydrolyzing phospholipids and releasing phosphorous, which can be measured by using different methods. Here we used a phosphorous colorimetric quantification kit (Darman Faraz Kave Medical Equipment Company) to determine and quantify released phosphorous. The substrate was prepared (0.5% lecithin dissolved in a 50 ml of Tris-HCl buffer 50mM at pH 8), added to sample tests, and incubated for 30 min at 37 °C. The negative control was the same as the test reaction without activity. The reaction color made in the reaction mixture was absorbed at 640 nm.

A unit of lecithinase activity is the amount of the alpha toxin that catalyzes the lysis of one percent of the substrate per minute under the optimized conditions.

Protein Detection:

Protein concentration was measured with the Bradford method (Bradford, 1976) using bovine serum albumin as standard. For rapid detection and estimation of protein direct absorption of the protein according to Layan. (Layne, 1957) was performed.

Microorganism and Growth:

Clostridium perfringens type A, was grown in optimized medium containing 3% (w/v) meat peptone, 0.5%(w/v) maltose, 0.5%(w/v) glucose ,1%(w/v) yeast extract , 0.5% (w/v) sodium chloride, 0.25% (w/v) disodium phosphate in pH 7 under anaerobic condition at 37°C for 25 hours. Samples were taken at different times and used for cell density by measuring the optical density (O.D) of the cell suspension at 600nm and alpha toxin was measured by hemolysis activity. The extracellular broth was centrifuged (10,000 rpm 5 min) and used for

further studies.

Purification of Alpha Toxin:

Ammonium Sulfate Precipitation:

Solid ammonium sulfate (up to 70 % of saturation) was added to the culture supernatant and kept at 4^{o}C for 16h for better protein precipitation. The precipitated protein was collected by centrifugation (10000 rpm, 20 min) at 4^{o}C and the pellet was dissolved in 20 mM Tris buffer at pH 6.

DEAE-Cellulose Chromatography:

The protein collected from salting-out was subjected to ion-exchange chromatography. The sample was loaded onto an equilibrated DEAE-Cellulose column (10cm×2cm- equilibration buffer: 100 mM Tris-HCl buffer pH 7), at room temperature. An eluted fraction from the column subjected to a second DEAE column (prepared at Tris buffer pH 9, 100mM).

Sephadex G100:

The sample obtained from the previous step (DEAE-Cellulose) was loaded on a Sephadex G-100 column (1.2cm×130cm, the flow rate of 25ml/h),), eluted fractions (with the volume of 4.9 ml) were collected and used for the next steps.

SDS-PAGE:

To qualitatively assess and monitor the purification steps, the samples from each purification step were electrophoresed, using 12.5% polyacrylamide gel SDS-PAGE and stained with silver nitrate. (J.M, Walker).

Characterization of Alpha Toxin:

The partially purified alpha toxin was used for physicochemical analysis such as temperature and pH optimum and stability, the effect of metal ion and EDTA and K_m calculation.

Effect of pH:

To determine optimum pH for alpha toxin activity, the lecithinase activity of alpha toxin in different buffers in a range of pH values 3.5-9 was analyzed (3.5-5 with citrate buffer and 6-9 with Tris-HCL). 1 ml of 0.5% lecithin solution as a substrate was dissolved in a 100 mM of each buffer at different pHs of (3.5, 5, 6, 7, 8 and 9) at separate tubes. 0.2 ml of the enzyme was added to each tube and incubated at 37°C for 60 minutes. The lecithinase activity was measured as above. The pH stability of alpha toxin was carried out by pre-incubation of the toxin with 100 mM concentration of different buffers at 4oC for 24 hours. The residual activity was determined under standard assay conditions.

Effect of Temperature:

The effect of temperature on the activity of the toxin was determined by carrying out the alpha toxin assay at different temperatures (30, 35, 40, 45, and 50 ^{o}C) for 60 min in 100 mM of the optimized buffer using 0.1% lecithin as a substrate. To determine thermal stability, the samples stored at the considered temperature for 24h and then stopped, and the residual activity of the toxin was analyzed.

Effect of metal ions:

The effect of metal ions on the toxin activity was tested by pre-incubation of the toxin with different metal ions (5mM of :(Cu, Zn, Hg, Ca, Pb, Mg, Ni, Cs, Mn, Ba, Mg, Fe, Co, Ca) at room temperature for 60 min and then determining the residual activity by assaying under optimum conditions.

Effect of EDTA:

The effect of EDTA on α-toxin activity was investigated by the addition of different concentrations of EDTA to the reaction mixture. All the experimental samples were assayed (lecithinase test) under optimum conditions.

Study State Kinetic:

The kinetic parameters such as K_m and V_{max} were obtained from the Lineweaver-Burk plot for the lecithinase activity at different concentrations of lecithin.

RESULTS

Purification:

Elutions from ion-exchange chromatography were loaded on gel filtration chromatography (Sephadex G100). The resultant chromatogram is shown in Figure 1. In 60ml volume, the picks of 280 nm and 570 are coincident. The pick point is a purified active alpha toxin that can be seen as a single band at 43KDa in SDS-PAGE (Fig. 2).

The toxin was concentrated in the salting-out step with a 1 % increase in the rate of purification. However, more purification

happened in the first step in ion-exchange chromatography with a rate of 230 and 92% yield. Finally, the protein was purified with a specific activity of 69170 (U/mg) and a purification rate of 654 with a final yield of 88%. The purification results are summarized in Table 1.

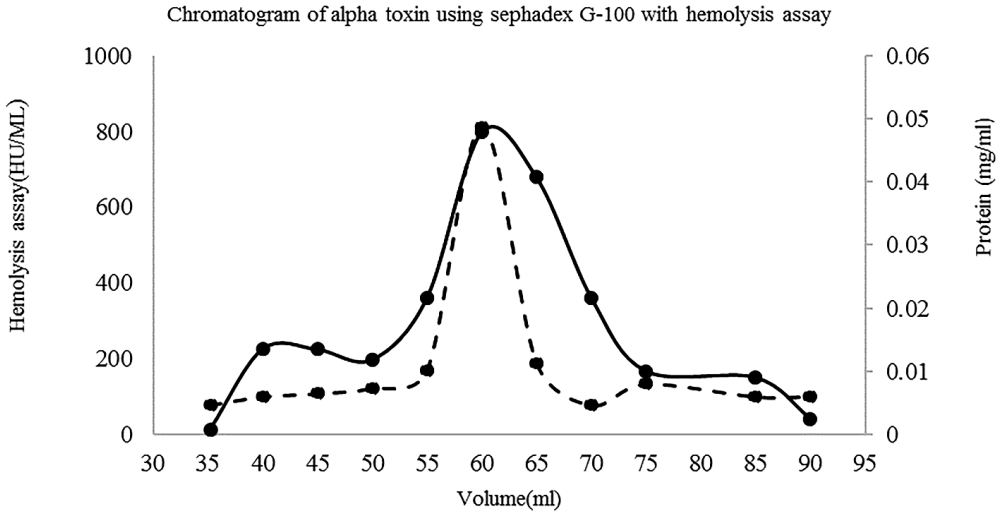

Fig.1: Purification of alpha toxin with column chromatography sephadex (G100) ((Hemolysis▬), (Protein …))

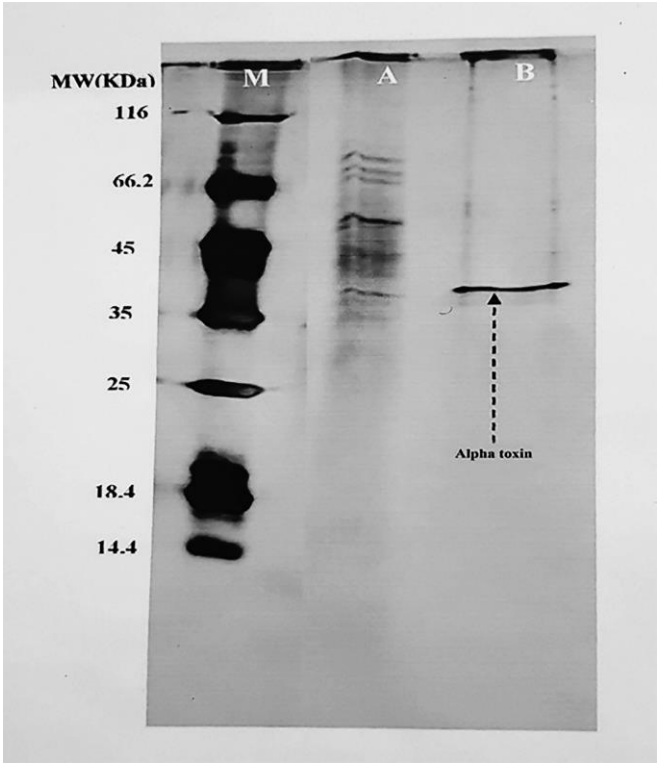

Fig.2: Gel 12.5% analysis of purified alpha-toxin, lane M, marker (KDa), lane A culture supernatant, lane B, ammonium sulfate fraction, lane C, ion –exchange pH 9 fraction, lane D. Column chromatography fraction**.**

Table1. Summary of purification of alpha toxin *Clostridium Perfringense* type A from culture

Step	Total protein (mg/ml)	Total activity U(HU/ml)	Specific activity (U/mg)	rate	yeild.enzyme (%)
Culture super	560	59200	105.71	1	100
Ammonium sulephate,70%	546	58596	107.1	1.01	98.97
Ion.exchange (pH7)	2.25	54700	24311	230	92.4
Ion.exchange (pH9)	2.2	53103	24138	228.3	89.71
Gel filtration Chromatography	0.72	51970	69170	654	88

Effect of Temperature:

The alpha toxin activity was investigated at different temperatures and the results are shown in Figure. 3. A temperature of 40 °C was found to be. The enzyme showed approximately 80% of its residual activity at 45 °C, after, which it decreased rapidly with increasing temperature.

The toxin activity was measured for samples incubated at different temperatures for 24 h. According to the results obtained from the relation of enzyme activity for 24 h pre-incubation (Figure.3), the toxin was 100% stable up to 45 ^{o}C, but the residual activity decreased by nearly 80% at 50^{o}C.

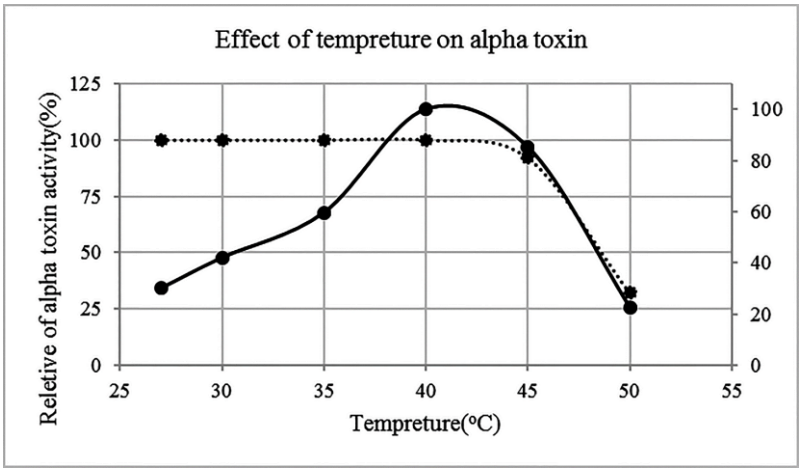

Fig3. Effect temperature on the activity and stability of alpha toxin with lecithinase assay (activity (▬) stability (…))

Effect of pH:

The alpha toxin activity of the partially purified fraction was investigated at different pH levels in the range of 3.5_ 9 and the results are shown in Figure.4. An optimum pH to be 6 was recorded for alpha toxin. The enzyme activity rose from 3.5 points to the optimum pH and had a sharp decrease from 6 to 7.

The activity of alpha toxin at different pH levels after a 24 h pre-incubation time in the different range pH. The results were obtained from the relation of enzyme activity for 24h pre-incubation was measured and the results are summarized in fig.4. According to results represented in Figure.4, the toxin was 100% stable at a pH of 6, but the residual activity decreased by nearly 90% at a pH of 7.

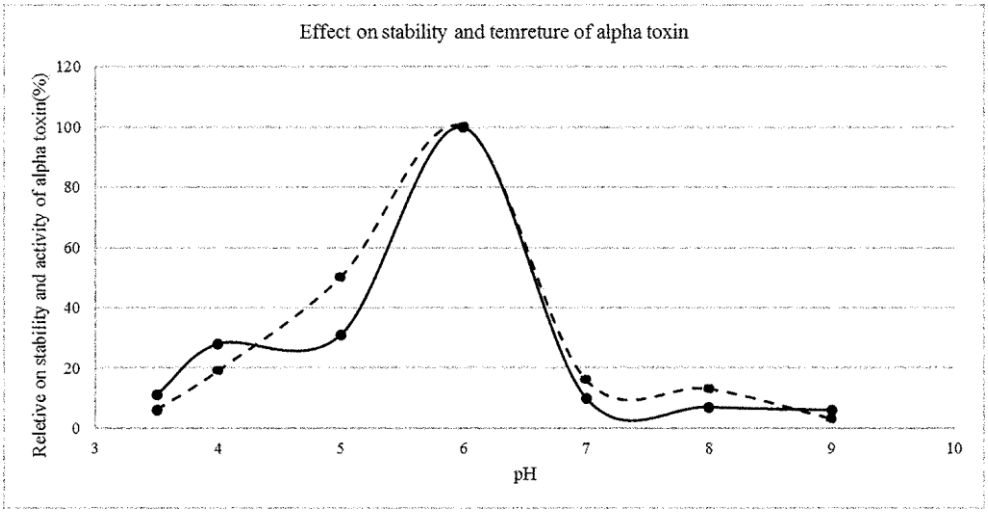

Fig.4. Effect pH on the activity and stability of alpha toxin with lecithinase assay (activity (▬) stability (…)).

Effect of EDTA on the Enzyme Activity:

EDTA is a chelating agent and, taking away the metal ions from the enzyme reaction system, affects the enzyme activity. EDTA at a low concentration of 0.5 mM increases the activity by up to 200 %. The residual activity decreased by increasing EDTA concentration.

Effect of Metals:

Summarizes the influence of different metals on the enzyme activity. Cobalt and barium ions have a 100% inhibitory effect and inhibit the activity of alpha toxin. Heavy metals, such as lead and mercury reduce toxin activity. Calcium, manganese, and copper inhibited nearly 70% of the total activity. In contrast, zinc and magnesium made induction on the toxin activity. Zinc and magnesium have a synergistic effect on enzyme activity.

Determination of K_m and V_{max}:

According to different V_0 values for different concentrations of lecithin, the values of kinetic constants Km and V_{max} are 0.19 mg/ml and 3.8 respectively. For the partially purified toxin calculated from a Lineweaver-Burk plot

DISCUSSION

Alpha toxin is a secreted enzyme toxin of *Clostridium perfringens* type A. Due to the effect of toxins on humans and animals (injury and death caused by the toxin), more documentation of their purification and characterization are of high importance. Alpha toxin is from the family of phospholipase C enzymes (PLases C) that hydrolyzes glycophospholipids and generates diacylglycerol and a water-soluble molecule linked to phosphate(Layne, 1957). Alpha-toxin has two conformational states, an open active form, and closes the inactive form, which was revealed by crystallographic studies. The accessibility of active sites in the open form is confirmed by three Zn^{2+} ions bonded to the active site while only two Zn^{2+} ions are bounded in the closed-form (Titball *et al.,* 1999). Mac Farlen et al, purified alpha toxin by combining salting out and column chromatography methods(Sundqvist and Macfarlane, 1985). In 1973, purification of alpha toxin from *C. perfringens* using three methods of DEAE-Sephadex in a batch-wise process, Sephadex G-75 chromatography and isoelectric focusing that resulted in a 200-fold purified alpha toxin with 10.4 percent recovery(Möllby *et al.,* 1976).In this study, purification of alpha toxin was performed by a combined method consisting of salting out, ion exchange, and column chromatography (the purification scheme is shown in Table 1). The results by SDS-PAGE revealed the presence of alpha toxin as a single banded 43 KDa protein with a yield of 88% and a specific activity of 69170.

According to the results of temperature and pH optimization (Figs. 3 and 4 for), the optimal condition for alpha toxin activity was obtained at 40 °C and pH 6 (in

Tris-HCL). As shown in figure 3, the toxin was 100% active (and stable, therefore) up to 45 ^{o}C, but its activity decreased by increasing the temperature. This activity reduction happened in pH changing from 6 to lower and higher levels.

According to metal ion results, alpha toxin is dependent on zinc ion: also, a combination of magnesium and zinc together can increase the toxin activity more than 50% when adding Zn. This induction may be due to the increased electronegativity of the reaction system.

The inhibition of alpha toxin by Co^{2+} and Hg^{2+} may be due to the decreased electronegativity of the reaction system. This suggests that these metal ions might act as a competitive antagonist at the active site and/ or acting on the structural site changing the conformational structure of the active site thereby decreasing the toxin activity.

The enzyme was inactivated by EDTA and phenanthroline and the inactivation was reversed by the addition of Zn^{2+} but not by Ca^{2+}(Möllby et al., 1976). Alpha toxin is a metalloenzyme that needs metal ions for its activity or stimulation. Alpha toxin from *C. perfringens* strains has been reported to show wide variations in metal ion requirement and sensitivity to chelating agents. At low concentrations of EDTA (0.5mM) led to the increased alpha toxin was increased in contrast to higher concentrations making the enzyme inactive. It interacts and forms a complex with them. By increasing its concentration, EDTA affects all metal ions present in the environment, causing inactivation of alpha toxin *C.perfringense*.

Möllby explained some properties of purified alpha toxin that are of importance for biological studies. A more extensive published study on the properties of alpha toxin and its substrate specificity mentions that purified alpha toxin degrades lecithin and sphingomyelin(Möllby et al., 1976). In the current study, lecithin was used to investigate the effect of the substrate and evaluate the effect of the purified alpha toxin on the degradation at different concentrations. The calculated V_{max} and K_m for alpha toxin were 0.19 and 3.8 respectively. According to the results, the partially purified alpha toxin with specific characters can be used for different applications such as antibody production and determination of the rate of anti-toxin in animal serum.

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Compliance with Ethical Standards:

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Conflict of interest: The authors consisting: Heshmati, Fathi Najafi, Ronaghi declare that they have no conflict of interest.

Animal studies: This article does not contain any studies with animal subjects performed by any of the authors

Human studies: This article does not contain any studies with human subjects performed by any of the authors

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