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Purification and Characterization of Nattokinase Produced by Local Isolate of *Bacillus* sp. B24

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Abstract: The results presented in this study were based on purifying and characterizing the nattokinase produced from local isolate of *Bacillus* sp. B24. Nattokinase was purified by two steps including the precipitation by ammonium sulfate at 50% saturation and ion exchange chromatography using DEAE-cellulose. The final purification folds were 16.46 time with an enzyme yield of 30%. The enzyme was stable in temperatures range 25-45°C and pH values of 8.0–9.5, with maximal activity was defined at 60°C and pH value of 8.0, whereas was retained 94% of its activity at 60˚C for 20 min. The presence of KCl, $CaCl_2$, $MnCl_2$, NaCl and $MgCl_2$ was observed to enhance enzyme activity to levels above their original activity, whereas $CoCl_2$, $HgCl_2$ and $FeCl_3$ decreased the enzyme activity. Furthermore, EDTA and PMSF strongly inhibited the enzyme activity suggesting that it is a metalloprotease and serine protease enzyme. The enzyme molecular weight as determined by gel filtration using Sephadex G-150 was found to be 63 kDa. Fibrinolytic activity of the partial purified nattokinase was studied *in vitro* conditions and it was found that the enzyme has degradation effect.

Key words: Nattokinase, Purification, Characterization, Fibrinolytic activity.

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Introduction

Nattokinase (NK) is a fibrinolytic enzyme (previously designated Subtilisin BSP) that belongs to the second wide family of serine protease (1). The nattokinase is a fibrinolytic enzyme, intent that it hydrolyzes fibrin. Fibrin is the major protein constituent of blood clots, an insoluble white protein formed by the conversion of fibrinogen (a protein in the blood plasma for clotting) via thrombin (a blood clotting enzyme) (2). Nattokinases firstly discovered and extracted from Japanese conventional fermented soybean foods were of certain importance due to their efficient biological thrombolysis of fibrin and clots of blood in blood vessels (3). Previous studies observed that these enzymes could also be purified from brewing rice wine (4) and Indonesian fermented soybean (tempeh) (5). The most well-known nattokinases are secreted by different *Bacillus* spp. including *B. subtilis, B. amyloliquefaciens, B. amylosacchariticus* and *B. licheniformis* (6). It is an extracellular enzyme with 27 to 90 kilo Dalton alkaline serine protease from *Bacillus subtilis* (natto). It has an isoelectric point of 3.9, in its

mature form, it has an isoelectric point of 8.6, and is stable in pH 7-12, at a temperature less than 50°C (7, 8).
Nattokinase enzyme has been mentioned to have effective fibrinolytic activity and it has amenities over other commercially used medicines in preventative and prolonged effects, stability in the gastrointestinal tract, and convenient oral administration, and the oral administration of it produced a mild and repeated enhancement of the fibrinolytic activity in the plasma (9) make NK to be a potential clot-dissolving agent for the treating of cardiovascular disease. The aim of this study was to purify and characterize the enzyme, and then evaluate its fibrinolytic activity at *in vitro* conditions.

Materials and Methods

Bacterial Culture and Production Medium

Local isolate of *Bacillus* sp. B24 was cultured in a brain heart infusion broth medium and incubated for 24 hrs at 37°C. The isolate was cultured in the fermentation medium consisting of 10g wheat bran humidified with 10 ml of distilled water as moisturizing solution with initial pH of 7.0. Then fermentation medium was inoculated with 1.0 ml of 24 hrs old culture isolate with bacterial concentration of 5×10^{7} cell/ml, the flasks were vigorously shaken to distribute the inoculum uniformly in the medium and were incubated at 37◦C. After 72 hrs incubation, enzyme was extracted by addition of 100 ml of distilled water, and the mixture was placed in the rotary shaking incubator (190 rpm) for 1 hr. The crude extracts were centrifuged at 10.000 rpm for 30 min., and then the supernatant was filtered through Whatman filter paper No.1. The clear supernatant was considered as a crude enzyme and it was assayed for nattokinase activity by casein digestion method.

Determination of Nattokinase Activity

Enzyme activity was measured by the method of Senior (10) using casein as substrate. The reaction mixture was prepared by mixing 0.2 ml of the enzyme solution with 1.8 ml of 0.5% casein solution prepared in 0.1M potassium phosphate buffer (pH 7.0) and incubated in a water bath at 37◦C for 20 min. After that, 3 ml of ice cold trichloroacetic acid (10%) reagent was added and then centrifuged at 10.000 rpm for 10 min. The blank was prepared by using the same step except the addition of TCA into casein substrate before the addition of enzyme solution. The amount of TCA-soluble products (supernatant) formed was measured by reading the absorbency at 275 nm. using UV-VIS spectrophotometer.
Unit of enzyme activity was defined as the amount of enzyme that enhanced 0.01 in the absorbance at 275 nm. within 1 min. under the experimental conditions and according to the following equation:

$$\text{Enzyme activity (U/ml)} = \frac{\text{Absorbance at 275 nm.}}{0.01 \times 20 \times 0.2}$$

Where:
0.01: Constant
20 : Reaction time (min.)
0.2 : Enzyme volume (ml)

Determination of Protein Concentration

The concentration of protein was determined according to the method

described by Bradford (11) using bovine serum albumin (BSA) as standard. The absorbance for concentration was read at a wave length 595 nm using spectrophotometer.

Purification of Nattokinase

The nattokinase was purified from the bacterium *Bacillus* sp. B24 isolate using ammonium sulfate precipitation and ion exchange chromatography.

Ammonium Sulfate Precipitation

Solid ammonium sulfate was gradually added in different saturation ratio to the crude enzyme at 4°C, the components were mixed gently for 45 min. to reach saturation of 50, 60, 70 and 80%, centrifuged at 10.000 rpm for 20 min., the supernatant was discarded and the precipitate was dissolved in 10 ml of 0.2 M potassium phosphate buffer.

Separation of Enzyme through Ion Exchange Resin (DEAE-Cellulose)

According to the method suggested in (1997) by Schutte *et al*. (12) the ion exchange (DEAE-Cellulose) was prepared.
A quantity of ten milliliters of the concentrated enzyme in the ammonium sulfate precipitation step was loaded on DEAE–Cellulose column (23×1.7 cm) using a clean Pasteur pipette in a circular motion on the column wall. The column was washed with equilibrating Tris-HCL buffer (0.02 M, pH 8.0) at flow rate of 18 ml/hr, 3 ml for each fraction; until the optical density at 280 nm. read zero. The elution was done with a gradient concentration of NaCl (0-1.0 M) by using Tris-HCl buffer prepared according to Tris-HCL buffer (0.02 M, pH 8.0) and Tris-HCL buffer 0.02 M, pH 8.0-NaCl solution 1.0 M, at a flow rate of 18 ml/hr. The protein fractions was estimated at the wave length 280 nm. of both washed and eluted fractions, and the parts of protein peaks were assayed for nattokinase activity and the peaks containing enzymatic activity were collected. Then enzyme activity and protein concentration of collected fractions were measured.

Characterization of Nattokinase

Optimal pH for Nattokinase Activity

In order to determine the effect of pH on nattokinase activity, solutions of substrate (casein 0.5%) was prepared at pH ranged from 5.5-9.5. The enzyme activity was assayed and the relation between enzyme activity toward pH values was plotted to determine the optimum pH of nattokinase activity.

Stability of Nattokinase at Different pH Values

Equal volume from partially purified enzyme was mixed with the buffers at pH ranged from 5.5-9.5 at a ratio of (1:1) and the mixture was incubated in a water bath for 15 min. at 37˚C, then the samples were transferred directly to ice bath. The enzyme activity was measured, and then the relation between remaining activity % toward pH values was plotted to determine the optimum pH of nattokinase stability.

Optimal Temperature for Nattokinase Activity

Nattokinase activity was estimated using different range of temperatures

(25-80)˚C, and the relation between enzyme activity toward temperatures was plotted to determine the optimal temperature of enzymatic activity.

Stability of Nattokinase at Different Temperatures

Partially purified nattokinase was incubated at different temperatures ranged between 25-80˚C for 30 min. followed by incubation in ice bath. Then the relation between remaining activity % toward temperatures was plotted to determine the optimum temperature of nattokinase stability.

Stability of Nattokinase at 60˚C using Different Incubation Times

The thermal stability of nattokinase was investigated by incubating the enzyme solution at 60˚C for different periods (0-120 min.) followed by incubation in ice bath. Nattokinase activity was assayed at 60˚C, and then the relation between remaining activity % toward incubation times was plotted to determine the optimum operational time for nattokinase stability.

Effect of some Metal Ions and Inhibitors on Nattokinase Activity

The enzyme solution was incubated with different solutions (Chloride solutions and EDTA at 5 and 25mM concentrations, and PMSF at 2, 3, 4, 5, 25mM) in a ratio of 1:1 (v/v) at 37˚C for 30 min., the enzymatic activity was assayed and compared with the control that represents the untreated enzyme, and then the remaining activity % was calculated.

Determination of the Molecular Weight of Nattokinase

The nattokinase molecular weight was estimated by Sephadex G-150 gel filtration chromatography depending on the standard curve. The column (33×1.3cm) was prepared and packed according to the instructions of the manufacturing company (Pharmacia-Sweden), and was equilibrated with 0.2 M phosphate buffer (pH 7.0). The standard proteins and the enzyme were eluted at 24 ml/hrs flow rate, and 3ml per fraction using the same phosphate buffer. The four standard proteins used were: Ovotransferrin (76000 Da.), BSA (67000 Da.), Trypsin (23000 Da.) and Insulin (6000 Da.). The standardization was done by plotting the elution volume (V_e) of each standard protein to the void volume (V_o) of the blue dextran-2000 (V_e/V_o) versus the log values of the standard proteins molecular weight (13).

Fibrinolytic Activity of Nattokinase

The fibrinolytic activity of *Bacillus* sp. B24 NK was assayed *in vitro* conditions using two different methods:

Fibrinolytic Activity Assay

The fibrinolytic activity of NK was performed by artificial fibrin plate assay. Fibrin plate medium was prepared according to Chang *et al*. (14) with some modifications. The crude enzyme and partially purified nattokinase were applied separately in a plate well with different units of enzyme: 0.175U, 0.35U, 0.525U or 0.7U, and incubated at 37°C for 48 hrs. For control, phosphate buffer (50µl) at pH 7.0 was used. Colorless zone of fibrin hydrolysis was observed directly

and the radius of the formed zone was measured.

Blood Clot Degradation Tests

The blood clot lytic activity of NK was assayed by artificial blood clot degradation using two different methods:
Human blood was withdrawn without anticoagulant and placed on a slide. A drop of blood (100µl) was left for 45 min. at room temperature for clot formation, followed by dropping 100µl enzyme (0.7U) on clot drop. As a control phosphate buffer (100µl) at pH 7.0 was dropped on a clot drop. The clot degradation was monitored at 37°C for 90 min. (15). Another blood clot destruction test was performed according to the method of Vijayaraghavan *et al*. (16) with slight modification. The blood clot was made spontaneously by placing the blood sample (100µl) in a glass test tubes and left at room temperature for 60 min. After that, the artificial blood clot was treated with partially purified enzyme using different units of enzyme: 0.175U, 0.35U, 0.525U or 0.7U or phosphate buffer (50µl) pH 7.0 as a negative control at 37°C. After 60 min. incubation, blood clot destruction was analyzed.

Results and Discussion

Purification of Nattokinase

Ammonium Sulfate Precipitation

The crude extract produced was initially subjected to precipitation with ammonium sulfate. In the present study, four ammonium sulfate saturation ratios (50, 60, 70 and 80%) were selected to determine the best ratio for nattokinase precipitation. It has been observed that 60.3% of NK enzyme was precipitated with purification fold 3.05 in the saturation ratio of 50% as shown in table (1).
Solid ammonium sulfate was selected to precipitate the nattokinase due to it's high solubility, availability, low cost and proteins stabilization that occurs. In addition, high salt concentration prevents proteolysis and it is useful to have a time in purification where the sample can be remained overnight (17).

Ion Exchange Chromatography

The enzyme solution that concentrated by ammonium sulfate step was passed through the ion exchange column (DEAE-Cellulose). Results in (Figure1) showed one peak of protein and the activity was concentrated in wash fractions and these results indicated that nattokinase carries a positive charge similar to the charge of the resin in the experimental conditions. In this step the specific activity was 319.02U/mg protein with a purification fold 16.46 and yield reached to 30% as shown in table (1).
Whereas the elution step (Figure 1) shows three peaks of protein when it eluted by gradient salt and one peak for activity which has very low activity (1.4 U/ml), therefore it was ignored. The presence of activity peak in elution step indicated that the nattokinase from *Bacillus* sp. B24 isolate had isoenzyme form differs in charge and pI; in another word, the nattokinase purified from elution steps had a negative charge adverse to the charge of ion exchange resin.

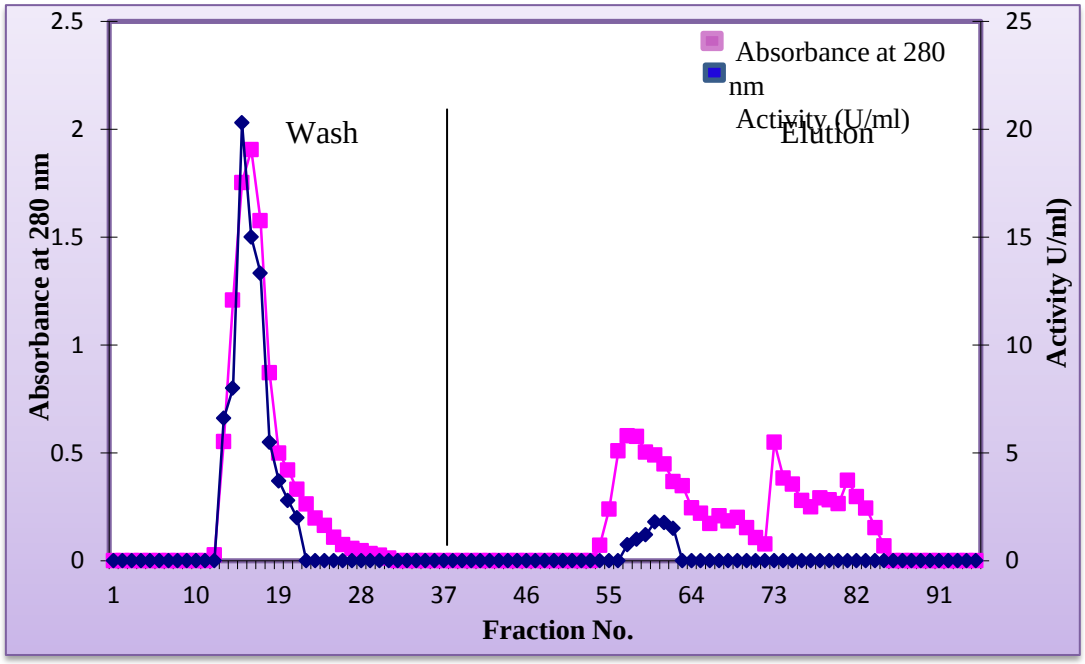


Figure (1): Ion exchange chromatography for nattokinase purification from *Bacillus* sp. B24 by using DEAE-Cellulose column (23×1.7) cm equilibrated with Tris-HCl buffer (0.02 M, pH 8.0), eluted with Tris-HCl buffer with NaCl gradient (0-1.0) M in flow rate 18ml/hr. 3ml for each fraction.

DEAE-Cellulose resin has many advantages including: good separation, high resolution power, easy handling, high capacity, possibility of reactivation for using many times and the simplicity of separation principle which based on charge differences (18). Therefore it is used to purify the fibrinolytic enzyme from various types of bacteria such as the fibrinolytic enzyme from *Pseudoalteromonas* spp., IND11, on which the purification fold was 6.75 and a yield of 20% (16).

Table (1): The purification schedule of nattokinase from *Bacillus* sp. B24 isolate

Purification steps	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg protein)	Total activity (U)	Purification fold	Yield (%)
Crude enzyme	50	15.7	0.81	19.38	785	1	100
Ammonium sulfate precipitation (50%)	10	47.25	0.8	59.06	473	3.05	60.3
Ion exchange chromatography (DEAE-Cellulose)(Wash)	18	13.08	0.041	319.02	235.44	16.46	30

Characterization of Nattokinase

Optimal pH for Nattokinase Activity

The effect of pH on purified nattokinase of *Bacillus* sp. B24 was evaluated in a pH range of 5.5-9.5 as presented in (Figure 2).
It was inferred that nattokinase has the best activity in pH ranged between (7.0 to 8.5), with maximum enzyme activity at pH 8.0 on which enzyme activity was 15.75 U/ml. Whereas reduced activity was observed at nearly acidic pH (pH 5.5, 6 and 6.5) and at alkaline pH (pH 9 and 9.5). The results of this study were compatible with the result mentioned by Lin *et al*. (2015) (19).

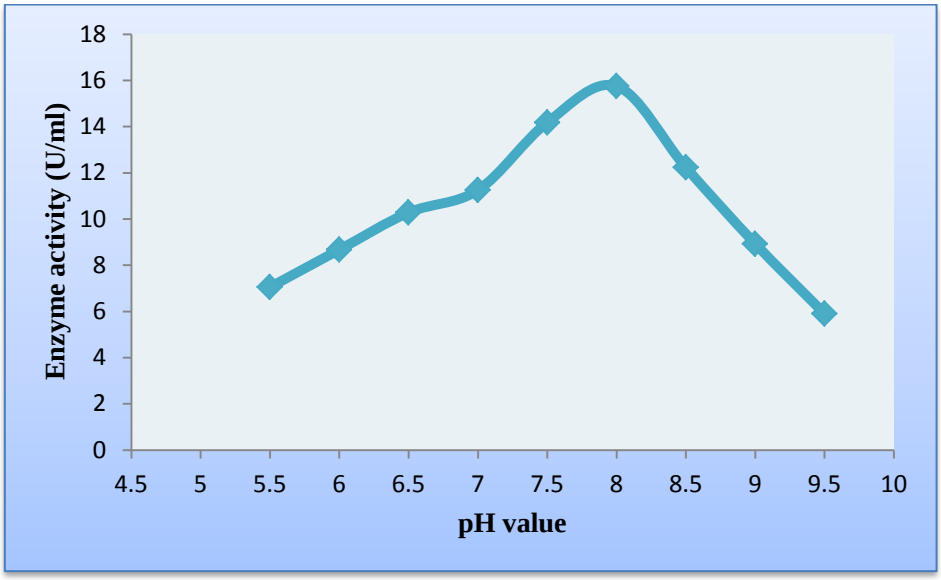


Figure (2): Effect of different pH values (5.5-9.0) on *Bacillus* sp. B24 nattokinase activity using casein as a substrate.

Stability of nattokinase at different pH values

From the results (Figure 3), it was noticed that pH ranged between 8.0-9.5 was the an optimum pH for nattokinase stability, the enzyme was retained 87% of its activity in pH 8.0 while retained 89% of its activity in pH 9.0, about 78% for pH 9.5. The activity decreased either side of the optimum pH values, the residual activities were above 70% for pH 7.0 and pH 7.5. The enzyme activity was very low at nearly acidic pH, approximately half of activity was lost at pH 5.5, 6.0 and 6.5, below 50% residual activity was recorded. The results may donate a conclusion that nattokinase of *Bacillus* sp. B24 is more stable in nearly alkaline pH.

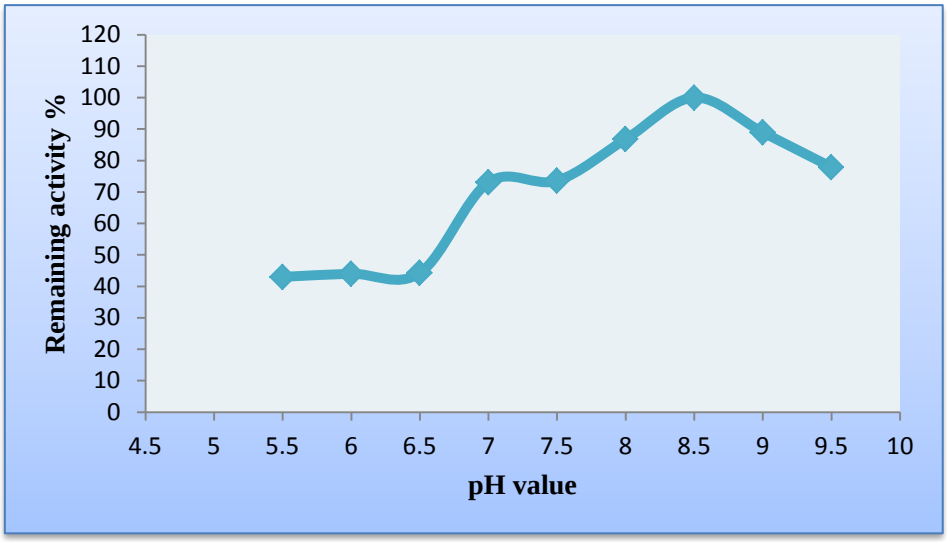


Figure (3): Effect of different pH values (5.5-9.0) on *Bacillus* sp. B24 nattokinase stability using casein as a substrate.

In general, this lowering in enzymatic activity at pH values away from the optimum condition may be due to the effect of pH in enzyme structure which leads to denaturate enzyme molecules or to a change in the ionic state of the enzyme active site, as well as its effect on secondary and tripartite structure of the enzyme which led to lose the activity in buffers solution that far away from optimal pH (20).

Lin *et al*. (19) found that the optimum pH of nattokinase stability purified from *Bacillus subtilis* N1 ranged between 5.0-9.0 .

Optimal Temperature for Nattokinase Activity

Results in Fig. 4 indicated that increasing in nattokinase activity by increasing the temperature reached to highest value at 60˚C; nattokinase activity was 58.8U/ml, then it began to decrease with increasing temperature till it reached 22.7U/ml at 80˚C. The results showed an increase in reactions speed until it reached 60˚C then began to decline over 65C°, this may due to the increase of the clash between the enzymatic molecules sharing in the reaction with the substrate as a result of increasing the movement energy of the molecules, whereas the decline in the enzymatic activity by temperatures over 65C° is a result of the denaturation of protein structure and changes in the active sites which leads to loss of the enzyme activity (21).

The optimum temperature of purified nattokinase was lower than of Nguyen *et al*., where they found that the highest activity of nattokinase purified from *Bacillus subtilis* VTCC-DVN-12-01 was 65 ºC (6).

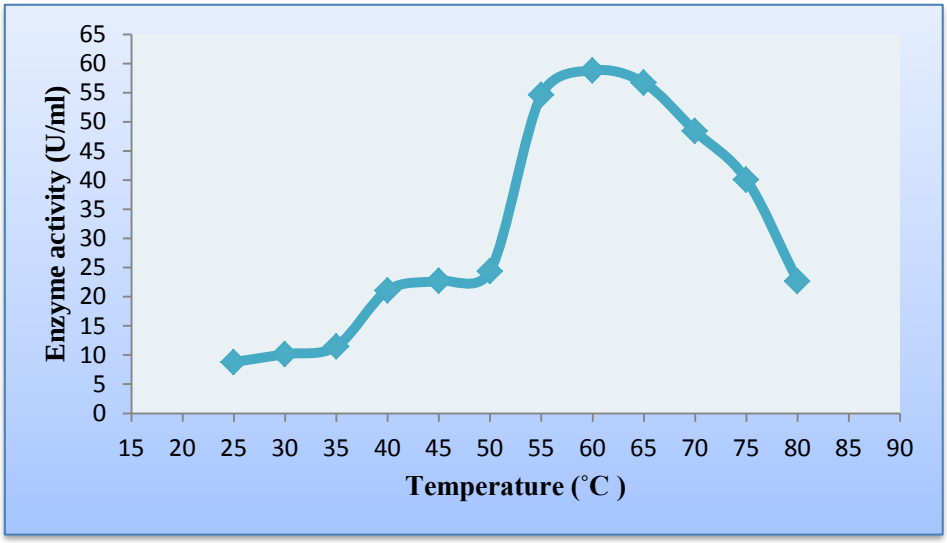


Figure (4): Effect of different temperatures (25-80)˚C on *Bacillus* sp. B24 nattokinase activity at pH 8 using casein as a substrate

Stability of Nattokinase at Different Temperatures

From the results presented in fig. 5, it was inferred that the enzyme was maintained its activity at temperatures ranged between 25-45°C, then the activity began to decrease with increasing temperature although at 50°C about 92% of the activity was remained. Higher temperatures showed sharp decrease in the stability, the enzyme retained 52% of the initial activity at 65°C, whereas, at 80°C there was no remaining activity indicating loss of enzyme activity.

Most enzymes are more stable at low temperatures therefore, they are stored at low temperatures. The decline in nattokinase activity at a temperature more than 60˚C belongs to its sensitivity against high temperature, reflecting the temperature effect on the 3D structure of the protein by damaging R-groups of amino acids which results to denaturation of protein and losing its activity (22).

Nguyen *et al*. (6) found that the nattokinase from *Bacillus subtilis* VTCC-DVN-12-01 showed thermophilic properties and retained more than 85% of its initial activity at a temperature up to 50°C for 1 hr .

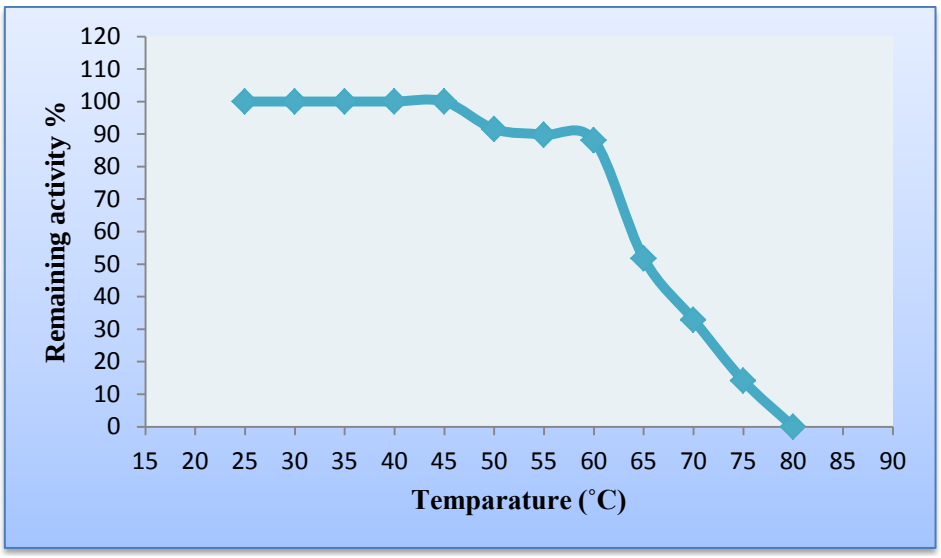


Figure (5): Effect of different temperature (25-80)˚C on *Bacillus* sp. B24 nattokinase stability at pH 8 using casein as a substrate

Stability of Nattokinase at 60˚C using Different Periods of Incubation

Results presented in (Figure 6), indicate that 94% of the enzyme activity was retained at 60°C for 20 min., then the enzyme activity began to decrease with time. The enzyme retained 50% of its activity after 40 min., however, remarkable loss of activity was observed after 60 min. with nattokinase activity became 27% after 2 hrs of treatment.

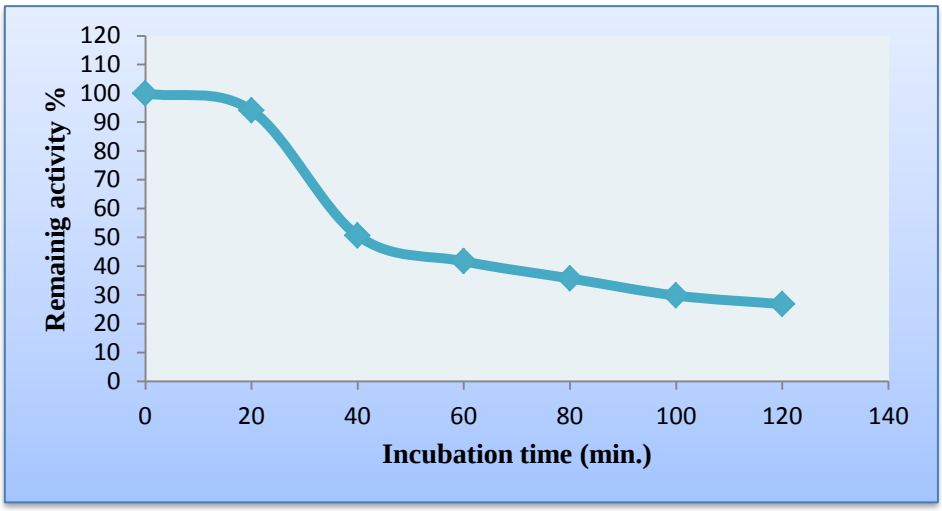


Figure (6): Effect of incubation time (0-120) min. on *Bacillus* sp. B24 nattokinase stability at 60˚C using casein as a substrate

Some of the previous studies on the thermostability of nattokinase observed that the enzyme was lost overall initial activity after 10 min. of incubation at 60°C (Chang *et al.*) (14). Wang *et al.* (8), found that nearly 70% of the nattokinase activity was preserved at 60°C for 40 min., 80% of activity was preserved at 50°C for 1 hr, and the all initial activity was lost after 60 min. of incubation at 60°C .

Effect of Some Metal Ions and Inhibitors on Nattokinase Activity

Nattokinase of *Bacillus* sp. B24 treated with some of chemicals. (Figure 7) showed the influence of some metallic chlorides and EDTA on nattokinase activity after incubation with the solutions of these ions for 30 min at 37˚C.

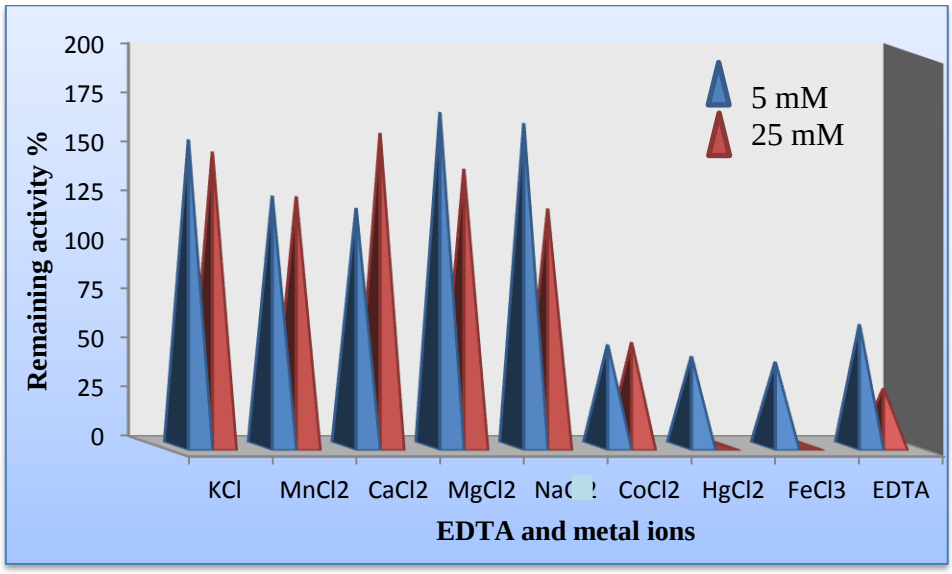


Figure (7): Effect of some metallic chlorides and EDTA on nattokinase activity purified from *Bacillus* sp. B24.

The effect of metal ions on the fibrinolytic activity depends on the origin of the enzyme, in general the presence of KCl, $CaCl_2$, $MnCl_2$, $MgCl_2$ and NaCl enhanced enzyme activity to levels above their original activity (control value) at 5 and 25mM. These results agree with the most researches that studied the effect of these materials on fibrinolytic enzyme activity. Many cations such as Ca^{+2} enhance the activity of some enzymes due to their role in safeguard of enzyme structure and hence creating a suitable reaction state (23).

Other chemicals ($CoCl_2$, $HgCl_2$ and $FeCl_3$) decreased the enzyme activity, whereas complete inhibition of enzyme caused by $HgCl_2$.

It was also observed in the presence of EDTA, the nattokinase showed a residual activity of 62.2% at 5mM which decreased to 29.5% when the concentration was increased to 25mM, which confirmed that the enzyme was from metalloenzymes that the divalent ions formed important part from

induced activity, the EDTA works as chelating agent and draw the divalent ions existing in the active site and form complexes leading to inhibit enzyme activity (24).

Also the phenylmethysulfonyl fluoride (PMSF) showed an inhibition effect of the enzyme and the inhibitory activity that enhances with the increasing concentration of this reagent, until the enzyme lost complete activity when incubated with 25mM from PMSF (Figure 8).

The inhibition of the enzyme by PMSF suggests that the purified enzyme is a serine protease. PMSF is well-known to sulphonate the essential serine residue in the protease active site, resulting in a total loss of enzyme activity (25).

Vigayaraghavan and co workers found that fibrinolytic enzyme was activated by 10mM NaCl and KCl (16).

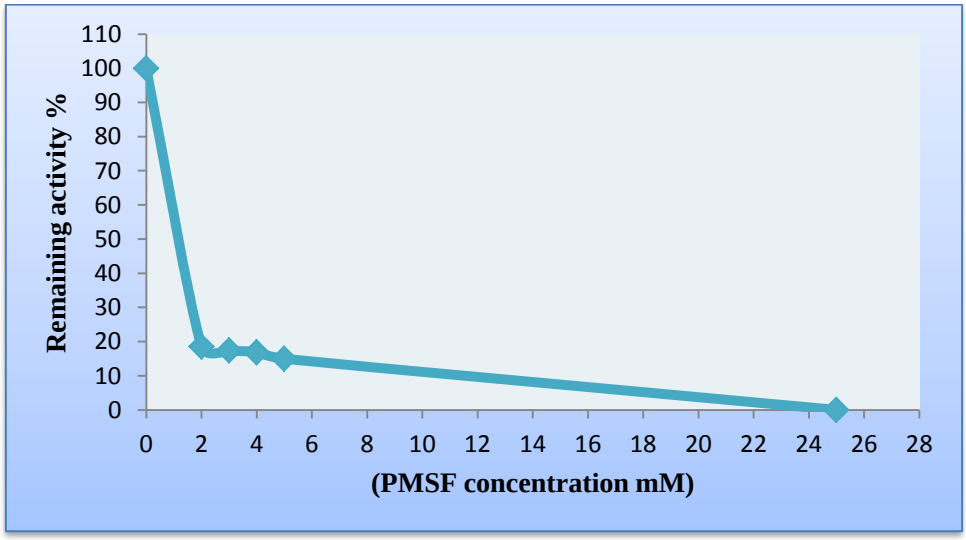


Figure (8): Effect of PMSF at different concentrations on *Bacillus* sp. B24 nattokinase activity.

Molecular Weight of Nattokinase

Molecular weight of the purified nattokinase produced by *Bacillus* sp. B24 was estimated by gel filtration chromatography using Sephadex G-150 in the presence of four different standard proteins which they were Ovotransferrin (76000 Da.), BSA (67000 Da.), Trypsin (23000 Da.) and Insulin (6000 Da.). Nattokinase and each standard protein were applied and eluted individually. Results showed that nattokinase has a molecular weight of 63000 Da.

Lin *et al*. (19) found that the molecular weight of *Bacillus subtilis* N1 nattokinase was about 46500 Da. by using SDS-PAGE and 46000 Da. using gel filtration on Sephadex G-50 .

Fibrinolytic Activity of Nattokinase

The fibrinolytic activity of *Bacillus* sp. B24 NK was studied using artificial fibrin plate assay and artificial blood clot degradation. The effect of partially

purified nattokinase on the *in vitro* fibrin digestion was investigated in comparison with crude enzyme (Fig. 9). Partially purified nattokinase displayed stronger fibrinolytic activity than crude enzyme, notably, the effect of partially purified nattokinase and crude enzyme increased with enzyme concentration and time (table 2).

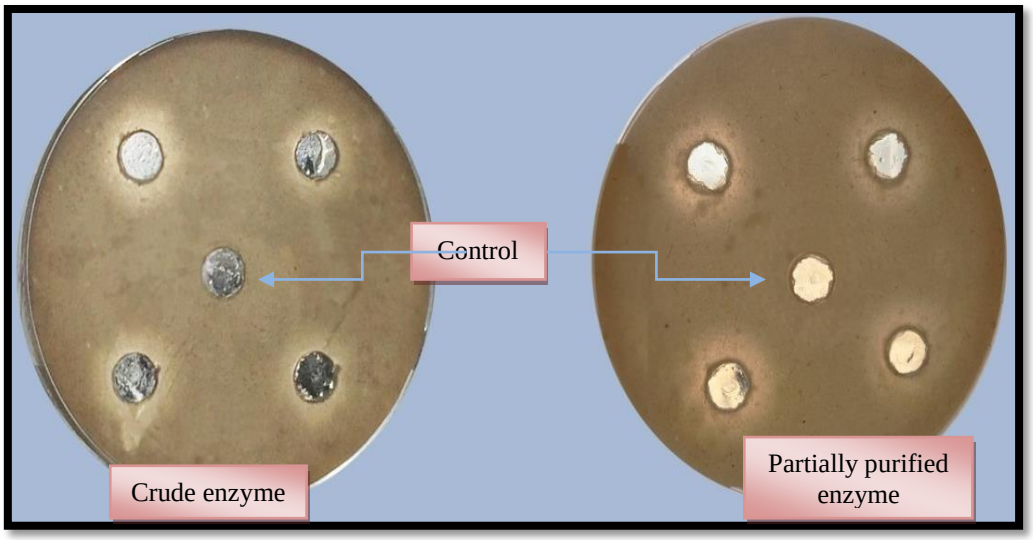


Figure (9): Fibrinolytic activity of crude enzyme and partially purified nattokinase on fibrin plate

Table (2): The clearance zone radius of crude enzyme and partially purified nattokinase on fibrin plate in mm

Time	Crude enzyme radius (mm)				Purified enzyme radius (mm)			
	0.175U	0.35U	0.525U	0.7U	0.175 U	0.35 U	0.525 U	0.7U
24 hr	12	13	16	17.5	15	17	18	19
48hr	13	15	18	20	19	19	19.5	20

Blood clot degradation by nattokinase was determined by dissolving human blood clot. Coagulated drop was digested by 100µl of the partially purified nattokinase at 37°C temperature. (Figure 10) A demonstrated the digestion of blood clot using 0.7U/100µl enzyme after 90 min., in contrast to coagulated drop of phosphate buffer as a control, clot lysis was not observed.

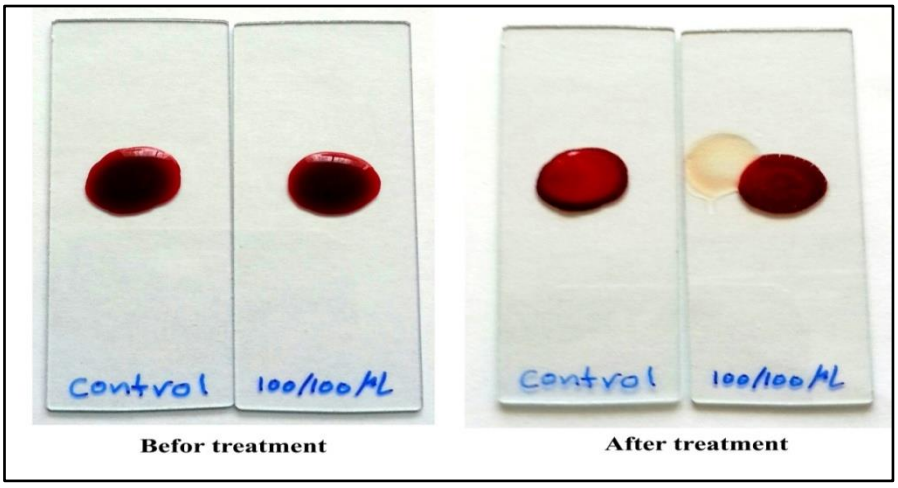
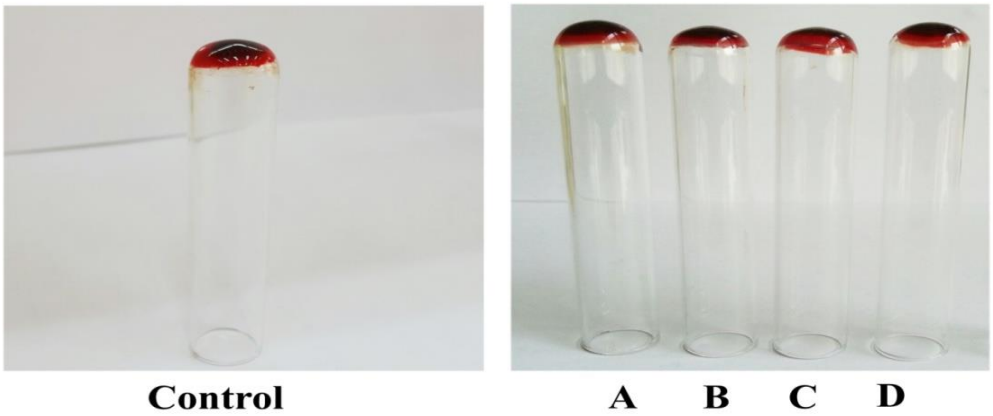


Figure (10 A): Effect of *Bacillus* sp. B24 enzyme on human blood clot

The blood clot lytic was also assayed in the glass test tubes. Human blood clot was incubated with the enzyme, and clot degradation was analyzed. Treatment of 0.175U, 0.35U, 0.525U and 0.7U nattokinase digested the clot, clot lysis was not shown in the control. In addition, nattokinase at higher doses digested blood clot effectively and are dose dependent (Fig. 10 B).

However, digestion of fibrin using fibrin plate medium and dissolution of the blood clot based on blood clot degradation tests by enzyme indicates the degradation of the fibrin net (16).

It can be concluded that, the nattokinase purified from *Bacillus* sp. B24 using wheat bran as substrate displays excellent fibrinolytic activities *in vitro*. Similar results were also reported with other microorganisms like *Pseudoalteromonas* sp., IND11 (16).



Before treatment

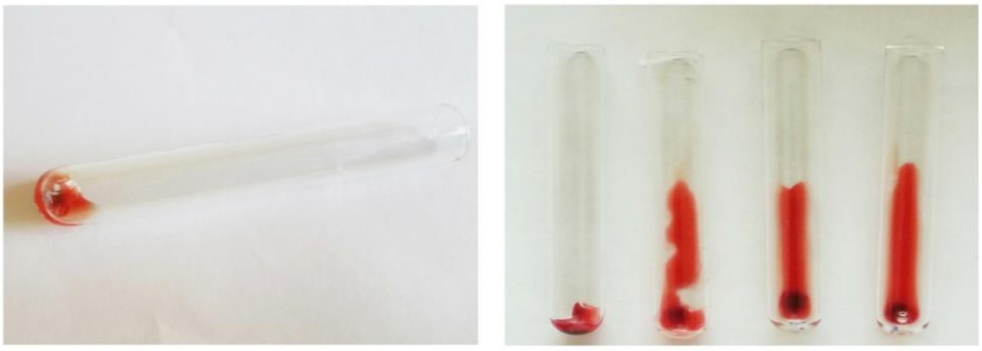


Figure (10 B): Effect of *Bacillus* sp. B24 enzyme on human blood clot using different enzyme concentrations (A: 0.175U, B: 0.35U, C: 0.525U, D: 0.7U).

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