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Purification and characterization of a caseinolytic enzyme from Bacillus subtilis.

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Abstract: Nattokinase (EC 3.4.21.62) is a serine protease and profibrinolytic enzyme with a strong fibrin-degrading activity, and many host strains can produce it. By its comparison with other fibrinolytic enzymes such as (streprokinase, urokinase and t-PA), nattokinase showed many advantages of having long life-time, no side effects, costless and it owns the ability to be a drug candidate for treatment of heart diseases and used as an effective food additive. *Bacillus subtilis natto* discovered in natto, a typical Japanese fermented soybean food. After the purification of Nattokinase from the supernatant of *B. subtilis* culture media, it showed a strong fibrinolytic activity. The enzyme was refined to a 4.4-fold purity level, with 61.8 percent of activity recovered. SDS-PAGE showed that nattokinase is homogenous and identified it as a monomer of 29000 Da in its original state. The optimum temperature and pH were 65°c and 9.0 respectively. Purified nattokinase is thermally stable at temperatures ranging from 35 to 55 °C and alkaline stable at pH 7.0 to 10.0. Skim milk agar showed clear zone of crude enzyme. Mg^{2+} increased the activity of nattokinase, but Zn^{2+} and Fe^{3+} decreased the activity. Triton x-100 as detergent significantly increased activity of nattokinase on the contrast to SDS inhibit nattokinase activity.

keywords: Nattokinase, *Bacillus subtilis,* Skim milk, TritonX-100.

1.Introduction

Cardiovascular diseases killed almost 18 million people and impacted 520 million people worldwide in 2019 [1] .CVD is becoming more common in young adults, which is a cause for concern. CVD can result in a variety of disabilities and even death in those who are affected, putting a socioeconomic load on humans [2, 3]

Nattokinase was discovered in natto, a classic Japanese fermented food made primarily from *Bacillus subtilis natto* [4]. The aprN gene encodes nattokinase, which is biosynthesized in the precursor form. The mature polypeptide's N-terminus is coupled to the signal peptide and propeptide [5]. Nattokinase is a protein which have 275 residue of amino acids, a 27.7 kDa molecular weight, and a pH of 8.6. The catalytic core is made up of Ser-His-Asp (D32, H64, S 221).

Sumi et al. [4] were the first to name a novel fibrinolytic protease enzyme, NK, that was discovered in natto, a traditional Japanese soybean food fermentation. *Bacillus subtilis natto* produces the most significant extracellular enzyme, NK [6]. NK has been shown to have significant fibrinolytic activity in both in vitro and in vivo experiments [7]. As a result, NK is now regarded as an effective, safe, and cost-effective enzyme in thrombolytic drug research [8-11]. Furthermore, NK is utilised to treat Alzheimer's disease, vitreoretinal diseases, and some tumours [12, 13]. Increased nattokinase intake has also been shown to be effective in preventing and treating hypertension in clinical trials [14].

Nattokinase was dissolving blood clots by degrading fibrin and plasmin substrate directly, also increasing tissue plasminogen activator (t-

PA) through degrading PAI-1 (plasminogen activator inhibitor-1) and converting internal prourokinase to urokinase (uPA),and all of that promoting fibrinolytic protease activity [15]. NK has few to no adverse effects, unlike typical fibrinolytic proteases such as uPA and t-PA, which have a lot of side effects such as bleeding and haemorrhage. The digestive system has been proven to absorb oral NK therapy [16, 17]. NK has a significant fibrinolytic activity after absorption in the duodenum. Because of these properties, NK is a powerful fibrinolytic enzyme that can help to prevent blood clots .

When healthy human volunteers orally received NK with dose (10 mg/kg) daily for 28 days in human clinical investigations, a no-adverse-effect-level (NOAEL) was discovered [18]. The urine, blood pressure, and pulse of the trial participants did not alter significantly. To both regulatory agencies and pharmaceutical businesses, the combined data from toxicity studies has offered a vigorous safety assessment for NK use. NK is currently indicated to be taken twice a day in the form of two capsules (100 mg/capsule). Based on previously published safety studies, this dose of NK has caused very minor toxicological concerns [19]

Bacillus, as well as *Pseudomonas sp.* and marine organisms, are thought to be excellent nattokinase producers [6]. Nattokinase-producing bacteria were mostly identified from fermented foods. Chinese soybean paste was used to isolate *B. subtilis LSSE-22* ,chickpeas were employed as substrates and the nattokinase specific activity increased to 356.25 FU/g [20]. *B. amyloliquefaciens DC-4* and *B. subtilis DC33* were isolated from a Chinese fermented food douche [21,22]. *Bacillus sp. CK 11-4*, *Bacillus subtilis WRL101,* and *Bacillus sp. CK 11-4* were isolated from the traditional Korean fermented cuisine Chungkook-Jang [23]. This study was aimed to the "extraction, purification and characterization of a caseinolytic

enzyme from Bacillus subtilis for its possible use as fibrinolytic agent in clinical applications.

2. Materials and methods

Optimization media for production nattokinase from *Bacillus subtilis (Ehrenberg 1835) Cohn 1872*^{AL}

Wheat bran media pH 7.0: 3% wheat bran, 2% glucose, , 0.4% K_2HPO_4, 0.1% KH_2PO_4, 0.5% NaCl and 0.05% $MgSO_4.7H_2O$, , in 500 mL flasks, and the flasks were incubated on a shaker at 30 °C and 150 rpm for 60 h.

Soybean media: 3% soybean meal, 2% glucose, 0.1% KH_2PO_4, 0.4% K_2HPO_4, 0.5% NaCl and 0.05% $MgSO_4$. $7H_2O$, pH 7.0, in 500 mL flasks, and also the flasks were incubated on shaker at 30 °C and 150 rpm for 60 h.

Yeast extract media: (g per liter): (10 g) of tryptone, (10 g) yeast extract, (1 g) $K_2HPO_4.3H_2O$, (0.5) $MgSO_4.7H_2O$, and (0.5) $CaCl_2.2H_2O$ and glucose was added with a concentration of 20 g per liter [24].

Crude enzyme was precipitated with ammonium sulphate to reach 70 % saturation. Then collecting crude extract and centrifuged at 12,000 rpm for 20 min at4°C. The precipitate was then dissolved into 20 mM Tris–HCl (pH 7), and then dialyzed against the same buffer overnight. The enzyme dialyzed (5 mL) was packed onto a DEAE-Cellulose column, then equilibrated with 20 mM of Sodium phosphate buffer (pH 7). The enzyme was washed and eluted from the column by using different concentration of salt ranging from 0.05 M to 0.5 M NaCl. The fractions were collected, protein concentration and activity were measured.

3.1 Skim milk agar plate

Initially, skim milk agar (2.0 % agar, 0.02 % sodium azide, and 1 % skim milk) was used to investigate protease activity. This required 10 microlitres of each crude enzyme [25]

On skim milk, after that, the plates were incubated at 37 Celsius degrees overnight, proteolytic activity was determined by the presence of clear zones.

3.2 Casein Digestion Assay

The casein digestion method was used to calculate NK activity. The amount of enzyme librating 1 µg of tyrosine equivalent/min was established as one unit of caseinolytic activity (U).**Enzyme Stability Studies**

3.3 pH Effect

To measure the optimal pH, NK activity was determined at 37°C at various pH values (2.0–13.0) of potassium phosphate buffer [26] .

3.4 Temperature effect

The enzyme activity was determined at different temperature (10 - 80°C). Stability of the NK was measured by incubating nattokinase with buffer at different temperatures and then the residual activity was measured.

3.5 Metal Ions, Inhibitors and Surfactants effects on nattokinase Activity

The metal ions effects with concentration (5 and 10 mM) were measured on nattokinase activity. The effects of inhibitors on NK activity were studied using ethylene diamine tetra acetic acid (EDTA) and phenyl methyl sulfonyl fluoride (PMSF) also. The surfactants effects were studied using 0.1 % of the surfactants Triton X-100, Tween-20, and 1 mM SDS. Purified enzyme was incubated with different metal ions, inhibitors or surfactants in 50 mM potassium phosphate buffer (pH7.5) for 30 min at 25°C then the remaining NK activity was measured [27].

3. Results and Discussion

Because of the medicinal interest in B. subtilis fibrinolytic enzymes (e.g., Nk), it was determined to look for more strong thrombolytic enzymes as alternatives to currently licenced plasminogen activators that lack direct action mechanisms over the substrate fibrin. The AprE127 encoding gene has a lot of similarities to Bacillus subtilis' Nk gene [28], and Bacillus subtilis' subtilisin E [13]Bacillus amyloliquefaciens' subtilisin BPN [35], , but it varies from the TK, Pb92 subtilisin families and Carlsberg.

Skim milk agar

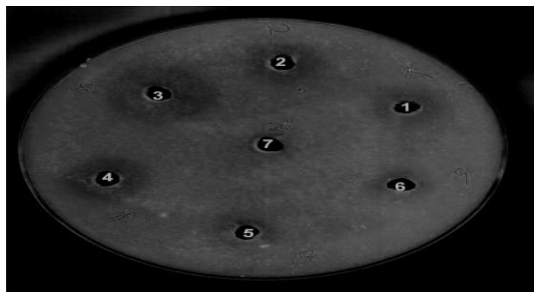

Figure 1 show the skim milk; 1: crude enzyme; 2: ammonium sulphate purification; 3: dialyzed enzyme; 4: purified enzyme; 5: soybean media; 6: wheat bran media; 7: yeast extract media.

4.1 pH effect on nattokinase activity

Table 1: show the pH effect on nattokinase activity.

pH	Activity U/mL
2	1.533 ± 0.1528
3	2.333 ± 0.3512
4	5.533 ± 0.4163
5	14.80 ± 1.058
6	22.27 ± 1.617
7	73.53 ± 1.286
8	82.23 ± 2.250
9	87.10 ± 0.8544
10	83.95 ± 0.8047
11	48.93 ± 1.102
12	42.20 ± 2.706
13	6.967 ± 1.704

The optimum pH of different kinds of nattokinase from B. subtilis strains varies greatly. Nattokinase from strains DC-2 [29], DC27 [30], WTC016 [31], and S127e all have an optimum pH of around 7 [32]. Others, such as nattokinase from strains, B-12 [35], BK-17 [34] and CK 11–4 [33] are active at acidic or alkaline pH [36].

Nattokinase from *B. subtilis C10*, like NatTH9, shows significant fibrinolytic activity at 37°C [37]. Nguyen, Quyen, and Le [38] discovered a nattokinase with an optimum temperature of 65°C.

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