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PURIFICATION AND CHARACTERIZATION OF RECOMBINANT NATTOKINASE FROM *BACILLUS SUBTILIS* R0H1

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SUMMARY

Nattokinase (NK) is a fibrinolytic enzyme with the potential for fighting cardiovascular diseases (CVD) thanks to its antithrombotic, antihypertensive, anticoagulant, anti-atherosclerotic, and neuroprotective effects. Nattokinase was first discovered and purified from soybean fermented food by *Bacillus subtilis* natto. To enhance NK's activity and simplify downstream processes, production of recombinant NK using several microbial expression systems such as *Escherichia coli*, *B. subtilis*, and *Lactococcus lactic* has been studied. Among all of them, *B. subtilis* is a prominent host for overproduction of functional proteins which can be secreted directly into the culture medium. In this study, recombinant NK from *B. subtilis* R0H1 was purified using two-step membrane filtration. Results showed 3.2-fold increase in activity and a recovery rate of more than 80%. Molecular weight of NK was approximately 28 kDa and its fibrinolytic degradation capacity was proved according to SDS-PAGE. The optimal pH and temperature of this NK were 8.5 and 55°C, respectively. The enzyme activity was boosted by Mg^{2+}, Ca^{2+} and obviously inhibited by Co^{2+}, Zn^{+2}, Fe^{2+}, and SDS. The apparent Km and Vmax with fibrin as the substrate were 3.08 mM and 6.7 nmol/min, respectively. The results suggested that membrane filtration is a useful method for purification of recombinant NK from *B. subtilis* R0H1. Therefore, application of membrane system is proposed to purify NK at the pilot scale. In addition, our findings indicated that recombinant NK produced in *B. subtilis* R0H1 showed high and stable proteolytic activity in slightly alkaline pH and at high temperature. It also exhibited strong fibrinolytic activity again both substrates: fibrinogen and fibrin.

Keywords: *Bacillus subtilis*, characterization, nattokinase, purification, recombinant

INTRODUCTION

Nattokinase (NK) is a serine protease which belongs to the subtilisin family. Historically, it is extracted and purified from a Japanese food called natto (Sumi *et al.*, 1987). It can dissolve fibrin fibers in blood clots that is known as the main cause of cardiovascular diseases (CVD) (Chen *et al.*, 2018). The fibrinolytic activity of NK was shown to be more stable and effective than that of plasmin (Sumi *et al.*, 1990; Fujita *et al.*, 1995). In 2016, around 17.9 million people died from CVD, accounting for 31% of all registered premature deaths (Kaptoge *et al*., 2019). This number may reach 23.6 million annually by 2030, mostly due to stroke and heart disease (Deepak *et al.*, 2010). In this context, NK might be a brilliant potential product for prevention and treatment of CVD.

Downstream processing is the key and bottle-neck step in the production of NK due to the presence of impurities proteins and high viscosity of culture broth. In order to reduce the

complexity of the purification process, NK was over-expressed in different hosts such as *Bacillus subtilis* (Cui *et al.*, 2018; Liu *et al.*, 2019; Tian *et al.*, 2019), *Escherichia coli* (Bora *et al.*, 2018), *Lactococcus lactis* (Liang *et al.*, 2007). From previous works, salt precipitation was frequently used for purification of both wild type and recombinant NK. This was combined with gel filtration chromatography (GFC) (Tuan *et al.*, 2015; Hu *et al.*, 2019) and/or ultrafiltration (Tian *et al.*, 2019; Xin *et al.*, 2019). Recovery yield of recombinant NK may reach up to 80% (Tuan *et al.*, 2015; Tian *et al.*, 2019), which is significantly higher than that of from wild enzymes. Other methods for purification of recombinant NK such as Ni-NTA and GFC was used but its final recovery yield attained only 16.8% (Bora *et al.*, 2018). From these publications, it seems that the selection of purification methods strongly determines the efficiency of the NK recovery process. Ultrafiltration may be an alternative method to achieve high purification efficiency when maintaining a high recovery yield.

In the present study, the purification of recombinant NK from *B. subtilis* R0H1 using a two-steps filtration was determined. Biochemical and kinetics properties of purified enzyme were then investigated.

MATERIALS AND METHODS

Microorganism and media

Recombinant strain R0H1 (*B. subtilis* 3NA carrying *apr*N gene with inducible promoter P_{veg}) was maintained in 50% glycerol and stored at -80°C. The bacterium was activated on Luria-Bertani skim milk (LBS) agar ($g.L^{-1}$) (yeast extract 5, tryptone 10, NaCl 5, skim milk 10 and agar 15) supplemented with 5 $\mu g.mL^{-1}$ chloramphenicol at 37°C. Colony with clear halo was selected for cultivation.

Chemicals and reagents

Fibrin bovine blood was provided by MP Biomedicals (France). Analytical chemicals and reagents were purchased from Sigma Aldrich (USA). Media nutrients were purchased from Himedia (India) and Oxoid (England).

Enzyme production and purification

One colony was grown overnight in Luria-Bertani (LB) medium containing chloramphenicol (5 $\mu g.mL^{-1}$) at 37°C and 150 rpm until its $OD_{600\ nm}$ reached 4.5. Cells from the seed culture was then transferred into fermentation medium ($g.L^{-1}$) (yeast extract 5, $CaCl_2$ 0.15, tryptone 35 and NaCl 5) at initial $OD_{600\ nm}$ value of 0.2. Shake flask cultures were carried out at 37°C and 150 rpm. After 14 hours of fermentation, the crude enzyme was obtained by centrifugation the culture broth at 10,000 rpm and 4°C for 15 min.

To purify the crude enzyme, a two-steps-filtration was applied. The supernatant obtained from centrifugation was first filtered through a 0.2 µm cut-off to eliminate cells and large impurities. The permeate obtained from 0.2 µm filtration was then subjected to a 10 kDa cut-off and the purified enzyme was remained in the retentate. In each purification step, protein concentration was determined by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as standard protein.

The yield and purification fold were calculated as the follow:

$$\text{Yield (\%)} = \frac{\text{Total activity in purified sample x 100}}{\text{Total initial activity}}$$

$$\text{Purification (fold)} = \frac{\text{Specific activity of purified sample } \frac{FU}{mg}}{\text{Specific activity of initial sample } \frac{FU}{mg}}$$

SDS-PAGE analysis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis was carried out using 12% polyacrylamide gel in the separating gel and 4% polyacrylamide gel in the stacking gel (Laemmli, 1970). A fixed volume

(10 µL) of samples was loaded and the electrophoresis was carried out at 15 mA. Protein bands were visualized by staining with Coomassie brilliant blue R 250. Gangnam stain protein ladder (Amersham Biosciences CAT.NO.24052) was used as standard.

Enzyme assay

The fibrinolytic activity was determined according to the method of Lin *et al*. (2015) with minor modifications. Firstly, 150 µL of fibrin solution (4 $g.L^{-1}$) was added to 420 µL 0.1 M Tris-HCl buffer (containing $CaCl_2$ 0.01 M, pH 7.4) and kept at 37°C for 5 min. Then, 30 µL of enzyme was added and the reaction mixture was incubated at 37°C for 30 min after which, the reaction was ceased by 300 µL of trichloroacetic acid (TCA). The samples were kept at 37°C for 20 min and then centrifuged at 10,000 rpm for 15 min. The absorbance of the supernatant was then measured at 275 nm.

One unit of fibrinolytic activity (FU) is defined as the amount of enzyme required to produce an increase in absorbance at 275 nm equal to 0.01 in 1 min.

Fibrinogen degradation by nattokinase

The degradation of fibrinogen by NK from the recombinant strain R0H1 was investigated using fibrinogen 10 $g.L^{-1}$ as a substrate and incubated at 37°C for different reaction times (up to 60 min). Hydrolysis products were visualized on SDS-PAGE.

Effect of pH and temperature on enzyme activity

The effect of pH on NK activity was determined at 37°C and in various pH (from 6 to 10). A various of buffers (0.1 M Tris-HCl buffer for pH 6 - 9, and 0.05 M Na_2CO_3 - $NaHCO_3$ buffer for pH 9 - 10) were used accordingly.

To determine the effect of temperature on NK activity, the enzyme activity was measured in 0.1 M Tris-HCl buffer (pH 7.4) at temperatures ranging from 30 - 75°C.

The relative activity of NK was measured, and the highest activity was defined as 100%.

Effect of pH and temperature on enzyme stability

The effect of pH on enzyme stability was evaluated based on its residual activity after pre-incubating the enzyme at pH 2.5, 7.4 and 8.5 for 30 to 300 min at 37°C. Whereas residual activity of NK as measured, the highest activity was defined as 100%.

The thermal stability of NK was examined by measuring the residual activity after incubating enzyme at various temperatures (30 - 80°C) for 60 to 420 min.

Effect of metal ions and inhibitors on enzyme activity

The effect of metal ions (Na^+, Mg^{2+}, Mn^{2+}, K^{2+}, Zn^{2+}, Co^{2+}, Fe^{2+}, Ca^{2+}, and Cu^{2+}) and inhibitors (EDTA and SDS) at concentrations of 1 and 5 mM on enzyme activity were examined by performing the enzyme assay in the presence of these ions or inhibitors. The relative activity of enzyme was calculated as the percentage of the treated enzyme activity compared with that of the untreated enzyme.

Enzyme kinetics

Enzymatic reactions were performed by using purified NK and different concentrations of fibrin (0.25 - 4 $g.L^{-1}$). The Lineaver-Burk reciprocal plot was generated for 1/S versus 1/V. The Michaelis-Menten constant (K_m) and maximum velocity (V_{max}) were calculated based on the intercept value and slop of this plot (Lineweaver and Burk, 1934).

All measurements were carried out in duplicate with the resulting values being the mean of the cumulative data obtained.

RESULTS AND DISCUSSION

Purification of nattokinase

Nattokinase produced by *B. subtilis* R0H1 was purified by two-step membrane filtration (Table 1). It has been observed that the specific

activity in the retentate of 10 kDa increased more than 3-fold with a yield of 89.7 ± 8% based on the crude enzyme. It suggested that the membrane filtration is one of the useful methods to obtain the high recovery yield for recombinant NK. Recently, Tian *et al*. (2019) reported a yield of 80% when purifying of NK from *B. subtilis* WB800N/pHT43-pro-*apr*N via salt precipitation and ultrafiltration. Tuan *et al*. (2015) also achieved a similar recover yield of 79% when purifying NK from *B. subtilis* pBG01-*apr*N/BD104 using ammonium sulfate precipitation combined with gel filtration chromatography. While Xiao-Lan *et al*. (2005) and Xin *et al*. (2019) only obtained recovery yields of 42.6 and 48.3%, respectively after purifying NK from wild type NK producing strains using ammonium sulfate precipitation and gel filtration chromatography. It is noted that the recovery rate of purification process is strongly dependent on initial state of the crude enzyme. The high recovery yield obtained in this study might related to the original characteristic of host strain *B. subtilis* 3NA, which was known as low protease producing strain (Reuß *et al*., 2015).

Table 1. Purification of Nattokinase from *B. subtilis* R0H1.

Sample	Total activity (FU)	Total protein (mg)	Specific activity (FU/mg)	Purification (fold)	Yield (%)
Crude enzyme	585.0 ± 5.0	0.911 ± 0.050	642.2 ± 41.07	1 ± 0	100 ± 1
Permeate 0.2 µm	556.9 ± 26.3	0.816 ± 0.007	682.5 ± 38.0	1.10 ± 0.02	95.2 ± 5.0
Retentate10 KDa	524.6 ± 39.7	0.252 ± 0.005	2082.2 ± 36.4	3.20 ± 0.19	89.7 ± 8.0

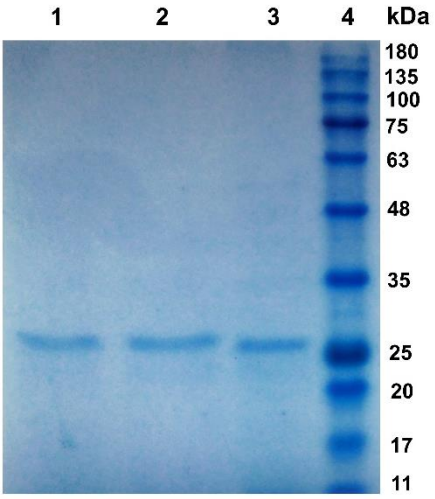

Figure 1. SDS-PAGE analysis of Nattokinase produce by *B. subtilis* R0H1. 1: Crude enzyme; 2: Permeate of 0.2 µm cut-off; 3: Retentate of 10 kDa cut-off; 4: Gangnam stain protein ladder.

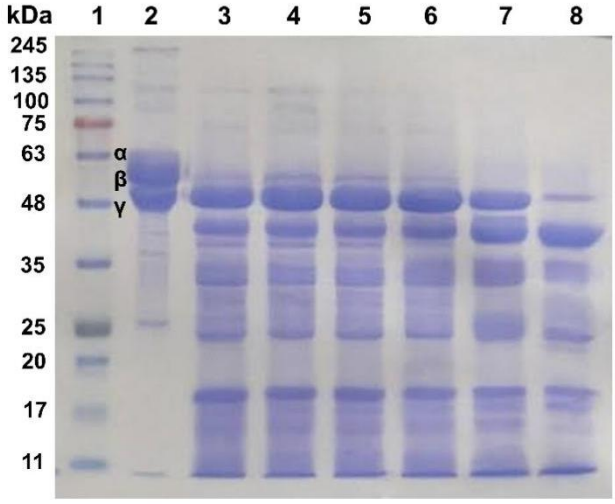

Figure 2. Degradation of fibrinogen by Nattokinase. 1: Gangnam stain protein ladder; 2: Fibrinogen control without enzyme; 3-8: Degradation products after 5, 10, 30 sec and 1, 10, 60 min incubation at 37ºC, respectively. α, β, and γ denotes the alpha, beta and gamma fragments of fibrinogen from bovine plasma, respectively.

SDS-PAGE analysis indicated single band with similar molecular weight for both crude and purified enzyme (Figure 1). The molecular weight of NK from strain R0H1 was estimated at approximately 28 kDa, which showed good correlation with reported the molecular weight of NK expressed in recombinant strains such as *E. coli* BL21 (DE3) (Yongjun *et al*., 2011), *B. subtilis* pBG01-*apr*N/BD104 (Tuan *et al*., 2015), *B. subtilis* BSN01 (Cui *et al*., 2018), and *B. subtilis* WB800N/pHT43-pro-*apr*N (Tian *et al*., 2019).

Fibrinogen degradation by nattokinase

Fibrinogen is a 340-kDa soluble plasma protein consisting of three pairs of disulfide bonded α-, β-, and γ-chains (Walker, Nesheim, 1999). These chains of fibrinogen from bovine plasma have molecular weight of 63.5, 56, and 47 kDa, respectively. The degradation of fibrinogen into several lower molecular weight fragments and the profile of the hydrolysis products were strongly dependent on the reaction time (Figure 2). After 5 sec (lane 3), bands corresponding to α- and β-chains were clearly broken, and several bands appeared between 11 and 48 kDa. It showed that the α-chain was completely degraded within 5 sec, the β-chain was degraded within 10 min, and most of γ-chain was hydrolyzed in 60 min. These results were consistent with previous reports of the degradation of fibrinogen by NK from *B. subtilis* BD104 (Tuan *et al*., 2015) and *B. subtilis* (Zen *et al*., 2018). It suggested that NK degraded α-chain first, and then followed by the β-chain and γ-chain of fibrinogen to smaller products (Tuan *et al*., 2015; Ren *et al.*, 2018).

Effect of pH and temperature on enzyme activity

The effects of pH and temperature on NK activity were illustrated in Figure 3. Nattokinase from strain R0H1 retained above 60% of its activity at pH values ranging from 7 to 9 and tended to rapidly lose its activity when further decrease pH (Figure 3a). The pH for optimal activity of this enzyme was 8.5 which was close to the values report for recombinant NK from *B. subtilis* BD104 (Tuan *et al*., 2015), and wild type NK from *B. subtilis* natto B-12 (Wang *et al*., 2009), *B. subtilis* TKU007 (Wang *et al*., 2011), and *Bacillus* sp. B24 (Hmood, Aziz, 2016).

Nattokinase activity was significantly enhanced by increasing reaction temperature from 30 to 55°C. At temperature above 55°C, enzyme activity showed a strong decreasing trend, and it was completely inactivated at 75°C (Figure 3b). The optimum temperature of this enzyme was 55ºC that was similar with those mentioned by Wu *et al*. (2009); Tuan *et al*. (2015) and was higher than those determined (40ºC) by Wang *et al*. (2009). However, its optimum temperature was lower than those recovered from some other *Bacillus* strains, i.e., 60ºC (*Bacillus* sp. B24) (Hmood, Aziz, 2018), 65ºC (*B. subtilis* VTCC-DVN-12-01) (Thao *et al.*, 2013).

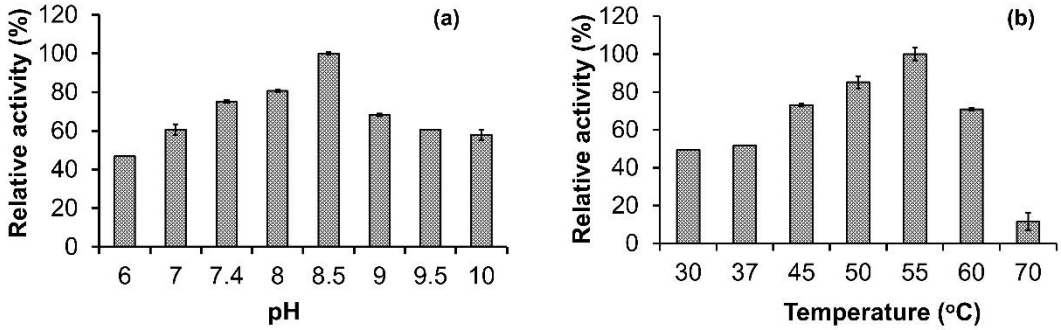

Figure 3. Effect of pH (a) and temperature (b) on Nattokinase activity (the highest activity was taken as 100%).

Effect of pH and temperature on enzyme stability

To investigate the potential use of NK for CVD treatment, enzyme stability at pH of gastric (2.5), blood (7.4) and gut (8.5) was evaluated. Figure 4a indicated the rapid inactivation of NK from strain R0H1 at pH 2.5 after only 0.5 h. The complete loss of NK activity at low pH (2 - 4) was also reported for NK from *Rhizopus chinensis* 12 (Xiao-Lan *et al*., 2004), *B. subtilis* natto B-12 (Wang *et al*., 2009), *B. subtilis* natto (Chang *et al*., 2012). However, the loss of enzyme activity at pHs 7.4 and 8.5 was negligible after 5 h at 37°C. Our results suggested that NK from strain R0H1 may perform the best action in human blood or gut.

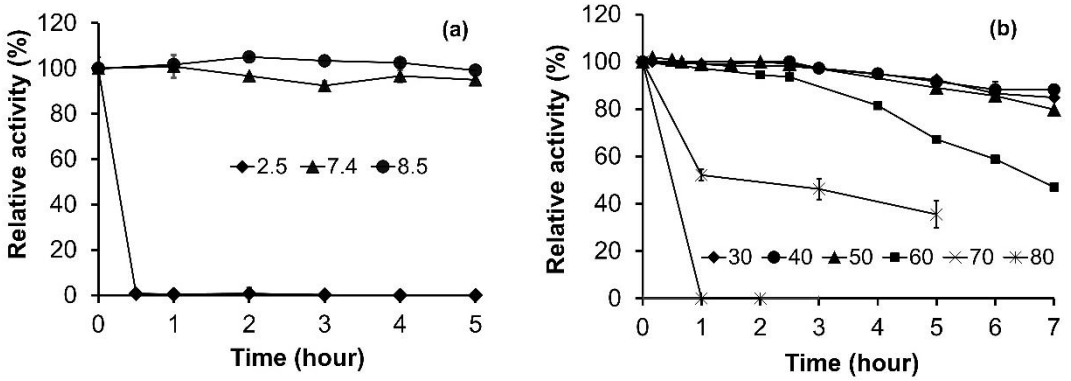

Figure 4. pH (a) and thermal stability (b) of Nattokinase from *B. subtilis* R0H1 (the highest activity was illustrated as 100%).

The NK from strain R0H1 was stable up to 60°C and retained more than 80% of its activity after incubation at this temperature for 4 hours. Further increase in temperature negatively affects enzyme activity. At 70°C, less than 40% of the enzyme activity was remained after 5 h incubation and the enzyme was completely inactivated after 1 h at 80°C (Figure 4b). Lin *et al*. (2015) reported that NK from *B. subtilis* N1 incubated at 55°C for 2 h remained a relative activity of higher than 40% while its relative activity dropped down to less than 30% at 65°C within 20 min. As for NK from *B. subtilis natto* B-12, the enzyme was almost inactivated after 60 min at 60°C (Wang *et al*., 2009). Besides, NK from *B. subtilis* BSN1 showed 52% of its initial activity at 70°C (Wang *et al*., 2011). Obtained results suggested that NK from strain R0H1 may be considered a thermophilic protease.

Effect of metal ions and protease inhibitors on NK activity

The enzyme activity was boosted up to 109, 115 and 116% in the presence of 1 mM Na^+, Ca^{2+} and Mg^{2+}, respectively. However, further increase of the ion's concentrations to 5 mM did not improve NK activity. Other agents such as Mn^{2+} (1 mM), K^+ (1 & 5 mM), EDTA (1 & 5 mM) and Na^+ (5 mM) showed no significant effect on NK activity. On the contrary, NK from strain R0H1 was inhibited by Co^{2+}, Zn^{2+}, Fe^{2+}, Cu^{+2}, and SDS. The presences of these ions or inhibitors led to a drop of enzyme activity by 13 – 78%, depending on effector's concentrations (Table 2). The effects of ions and inhibitors on NK activity were partially consistent with previous works. Xin *et al*. (2018) reported 86% of residual activity of NK from *Bacillus tequilensis* (No. 11462) in the presence of 5 mM Cu^{2+}. Chang *et al*. (2012) also claimed no significant effects of K^+, Na^+, Ca^{2+}, Mg^{2+} and Zn^{2+} on the activity of NK from *B. subtillis* fermented red bean. Across the literature, it seems that effects of different ions on NK activity were not unified. For example, Fe^{2+} at 5 mM was a booster for NK from *B. subtilis* TKU007 (Wang *et al*., 2011) but opposite conclusion was witnessed from others works (Wang *et al*., 2009; Hu *et al*., 2019). Similar

contradiction statement about effect of Zn^{2+} and Cu^{2+} on enzyme activity was reported. Garg and Thorat (2014) observed an 8% increase in the activity of purified NK from *B. natto* (NRRL B-3666) in presence of 5 mM Zn^{2+} or Cu^{2+}. In the other hand, a loss of more than 15% activity in the presence of neither Zn^{2+} or Cu^{2+} at 5 mM was reported for NK from *Bacillus tequilensis* (No. 11462) (Xin *et al*., 2018). For NK from *B. subtilis* BD104, Zn^{2+} (5 mM) boosted the NK activity by 1.72-fold while Cu^{2+} (5 mM) led to a 20% activity loss (Tuan *et al*., 2015).

Table 2. Effect of metal ions and inhibitors on the activity of Nattokinase.

Metal ions and inhibitor	Relative activity (%)	
	Concentration (1 mM)	Concentration (5 mM)
None	100 ± 1	100 ± 1
Na^{+}	109 ± 6	98 ± 6
Mg^{2+}	116 ± 4	103 ± 4
Mn^{2+}	100 ± 2	53 ± 2
K^{+}	100 ± 0	95 ± 0
Zn^{2+}	74 ± 5	38 ± 5
Co^{2+}	73 ± 2	0
Fe^{2+}	46 ± 0	0
Ca^{2+}	115 ± 4	113 ± 4
Cu^{2+}	87 ± 2	85 ± 1
EDTA	101 ± 4	88 ± 4
SDS	49 ± 6	22 ± 0

Kinetics of nattokinase

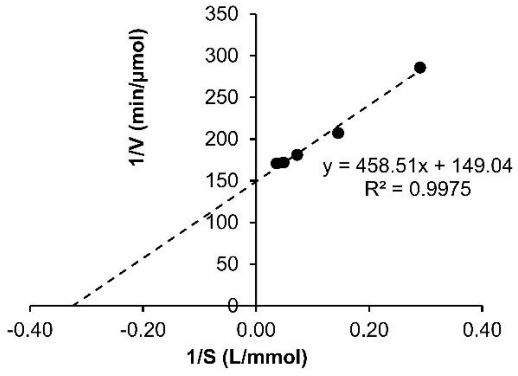

Figure 5. Lineweaver-Burk plot for fibrin hydrolysis by Nattokinase.

Kinetic parameters of NK from strain R0H1 were determined from initial velocity at various substrate concentrations. The Lineweaver-Burk plot showed that the y-intercept at 1/V was 149.04 (min/µmol) and the x-intercept at 1/S was -0.33 (L/mmol) (Figure 5). The values of K_m and V_{max} devised from this data were 3.08 mM and 6.7 nmol/min, respectively. As the obtained Km was low, it showed that the NK had high affinity for fibrin. Garg and Thorat (2014) reported that the values of Km and Vmax of NK from *Bacillus natto* NRRL B-366 for the substrate N-Succinyl-Ala-Ala-Pro-Phe-p-nitro-anilide (S-7388) were 3.5 mM and 1250 nmol/min, respectively.

CONCLUSION

It was found that NK from the recombinant strain *B. subtilis* R0H1 could be purified using two-steps membrane filtration with yield of more than 80% and purification fold of 3.2. The purified NK showed a single protein band of approximately 28 kDa which possessed strong fibrinolytic degradation activity according to SDS-PAGE analysis. The recombinant enzyme exhibited significant stabilities for pH and

temperature. Its maximum activities were reported at pH of 8.5 and temperature of 55°C. This NK's activity was fairly increased in presence of Mg^{2+}, Ca^{2+} but strongly inhibited by Co^{2+}, Fe^{2+} and SDS. Further study should be focused on the fermentation strategy to improve NK production and the application of membrane system to purify NK at the pilot scale.

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