Production of nattokinase from *Bacillus* sp. by solid state fermentation

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Abstract

Nattokinase is a fibrinolytic enzyme produced in fermented soybean food by *Bacillus* sp. In this study, we have produced nattokinase under solid state fermentation of soybeans with minimum amount of water (30% v/w). Milk powder (1% w/w) along with soybean was found to increase the production of nattokinase. Nattokinase showed maximum fibrinolytic activity (29 mm zone) when it was extracted with Tris buffer at pH 9. Crude enzyme was purified by ion –exchange chromatography and its molecular weight was estimated to be around 28 kDa. The activity of the purified enzyme increased to 50% as compared to the crude enzyme.

KEYWORDS: Nattokinase, solid state fermentation, soybean, *Bacillus subtilis*

Introduction

Natto is a traditional fermented soybean food in Japan, prepared with Gram positive bacterium *Bacillus subtilis* natto. It contains an extracellular serine protease, nattokinase, produced during fermentation by the *Bacilli* [1, 2]. This enzyme have been studied by many researchers and shown to possess a high fibrinolytic activity than plasmin [3]. It has several advantages over the commercially used fibrinolytics like ease of oral administration and stability along the gastrointestinal tract [4, 5]. Nattokinase has also shown to be well tolerated in adults and effective in reducing blood pressure in combination with low cholesterol diet [6]. Intravenous fibrinolytic agents like urokinase and tissue plasminogen activator have been currently in use but they are expensive and have several side effects including haemorrhage [7]. Because of disadvantages of these agents, microbial enzymes have attained much attention as the alternatives in the last few years. Some other examples of microbial enzymes used for fibrinolytic therapy include streptokinase,staphylokinase, subtilisin DFE, BK-17 and many more [8]. Various research groups have focussed on optimizing the fermentation medium components for enzyme production towards higher fibrinolytic activity and purificationof the enzyme [3]. In the present study, we have produced nattokinase under solid state fermentation of soybean s, evaluated its fibrinolytic activity and purified the enzyme.

Materials and Methods

Materials

Bacillus subtilis NCIM 2708 was procured from National Collection of Industrial Microorganism, Pune, India; maintained on nutrient slant at 4°C and sub cultured at every 30-days interval. Soybean variety SL 525 was obtained from Pulse Laboratory of Genetic division of IARI (Indian Agricultural Research Institute), PUSA, Delhi, India. Fibrin was purchased from Himedia, India. All other chemicals and microbiological media used were of analytical grade.

Solid state fermentation

Production of fibrinolytic enzyme was carried out in a soybean based medium containing 14 g of dehulled and pre-soaked soybean (100g of soybean was soaked overnight in 500 ml water at room temperature)s and 6 ml of water. Rice husk and milk powder, alone and in combination, at different concentrations (0%. 0.5% and 1%) were also added to the media. The production medium was placed in 100 ml Erlenmeyer flasks and sterilized using autoclave at 121°C for 15 min. After the medium was cooled to room temperature, freshly prepared seed culture (5% v/v) of *Bacillus subtilis* NCIM 2708 was added and incubated at 37°C for 24 h.

Extraction of enzyme from the fermentation media

The cultures after fermentation were mixed individually with saline or Tris-HCl (pH 8, 9 and 10) in the ratio of 2:1 and stirred for 30 min at 4°C, to extract the enzyme. After this, the mixture was centrifuged at 8000 rpm for 30 min. The supernatant containing nattokinase was collected and assayed for fibrinolytic activity.

Fibrin plate assay

The method described by Chang et al [9] was slightly modified. For determination of fibrinolytic activity of enzyme, fibrin plates were prepared by adding 0.75% agarose and 1.5% fibrin in NaOH solution at pH 7. The agarose was dissolved by heating the mixture at 60°C. The heated mixture was poured into Petri plates and allowed to solidify. Antibiotic powder (1%) was sprayed onto the solidified agar and holes of 6 mm were punched. The extracted enzyme was poured in the wells and plates were incubated at 37°C for 24 hrs. Colourless zones around the wells were measured.

Purification of nattokinase

All the steps were carried out at 4°C. The extracted enzyme was precipitated with ammonium sulphate. For this the ammonium sulphate was added to culture supernatant and stirred overnight. The sample was again centrifuged at 15000 rpm for 15 min and the pellet obtained was dissolved in 50 mM phosphate buffer pH 6. After dialysis, active fraction of the enzyme was loaded onto the CM cellulose column pre equilibrated with 50 mM phosphate buffer at pH 6. Gradient elution was carried out with 0 to 1 M NaCl. Finally, active and purified fractions were collected and dialysed [10]. SDS-PAGE was carried out to determine the purity of the enzyme as described by Laemmli using 6 and 12% stacking and resolving gels, respectively [11].

Results and discussions

Production of enzyme under solid state fermentation

Nattokinase was produced under solid state fermentation by employing soybean s with little amount of water (30% v/w). Along with soybean s, rice husk and milk powder were supplied alone or in combination, to study their influence on the production of

nattokinase. The production of fibrinolytic enzyme was assessed by quantifying the fibrinolytic activity using fibrin plate assay. Effect of different fermentation substrates on production of nattokinase based on zone of lysis had been summarized in Table 1. The maximum zone of lysis had been obtained when the fermentation medium contained soybeans and milk powder while the least zone of lysis was observed with soybean and rice husk as medium components. Milk powder enhanced the production of enzyme while rice husk at both 0.5% and 1% concentration had a negative effect on the enzyme production. Soybeans supplied with milk powder (1% w/w) produced more amount of nattokinase compared to 0.5% w/w

Effect of extraction medium on enzyme activity

The enzyme was extracted in saline as well as in Tris-HCl buffer and the fibrinolytic activity of nattokinase in different extraction medium has been shown in Table 2. The enzyme showed more activity in Tris buffer of all pH 8, 9, 10 than saline (pH 7). The enzyme produced is an alkaline protease which can be related to its better activity at high pH. The enzyme displayed higher activity as the pH of the buffer was increased. Although the difference in the activity was not much but maximum activity of the enzyme was obtained at pH 10.

Purification of nattokinase

With each step of protein concentration and purification, specific activity of nattokinase increased. The specific fibrinolytic activity of the purified enzyme increased up to 50% as compared to the crude extracted enzyme. Crude enzyme yielded specific activity (in terms of zone of fibrinolysis) of 14 mm/mg while the specific activity increased to 30 mm/mg with the purified protein. The SDS-PAGE analysis showed one major band and its molecular weight was estimated to be 31 kDa (Figure 1).

Conclusions

Nattokinase, an oral and a potent fibrinolytic enzyme was produced under solid state fermentation. *Bacillus subtilis* NCIM 2708 can be a microbe of choice for bulk production of nattokinaase. Milk powder as a nutritional component (along with soybean) positively influenced the production of nattokinase. The enzyme showed better fibrinolytic activity when extracted with Tris buffer at pH 10. Partial purification of nattokinase has resulted in almost 50% increase in the activity. Molecular weight of the nattokinase produced was found to be approximately 31 kDa. This soybean food containing nattokinase needs to be evaluated in animal model before its use as therapy for thrombosis prevention.

References

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Table 1 Zone of lysis by nattokinase produced with different medium components

	Fermentation substrates					
	Soybean seeds	Soybeans supplemented with				
		Rice husk (0.5% w/w)	Rice husk (1% w/w)	Milk powder (0.5% w/w)	Milk powder (1% w/w)	Rice husk (0.5% w/w) & Milk powder (1% w/w)
Zone of lysis (mm)	19	17	17	23	29	21

*(No zone of fibrinolysis was found with water and un-inoculated soybean seeds.)

Table 2 Effect of extraction medium on fibrinolytic activity

Extraction medium	Zone of lysis (mm)
Saline pH 7	19
Tris buffer pH 8	24
Tris buffer pH 9	26
Tris buffer pH 10	29

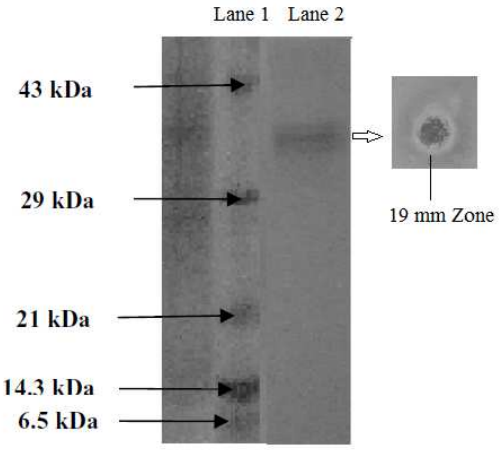

Figure 1. SDS PAGE of purified NK (Lane 1: protein markers, Lane 2:purified NK) and fibrinolytic activity of purified enzyme.