

Study on the enzymatic properties of Nattokinase

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Abstract. The fractionation by $(\text{NH}_4)_2\text{SO}_4$ of nattokinase was studied,the $(\text{NH}_4)_2\text{SO}_4$ saturation of 30% was selected to remove impurity protein, $(\text{NH}_4)_2\text{SO}_4$ saturation of 60% was selected to precipitate nattokinase.The nattokinase was purified by salting out method to obtain the crude enzyme solution,the enzymatic properties of nattokinase were studied.The nattokinase was long time no inactivation under the temperature of 40°C ,nattokinase was preserved for 5 hours at the temperature of 37°C ,the enzyme activity was not affected.The activity of nattokinase was stable in the range of pH6-9,when the pH is 3,the activity of nattokinase was very low,but when pH returned to 7,enzyme activity recovery.

Introduction

In 1987,Japanese scholar H Sumi found a high fibrinolytic enzyme activity from the Japanese traditional fermented soybean food:natto,and named nattokinase(nattokinase,referred to as NK)[1-3].Compared with the commonly used drugs for the treatment of cardiovascular and cerebrovascular diseases such as streptokinase,the nattokinase has good safety,the nattokinase was easy to be absorbed by human body,the nattokinase has the advantage of low cost,the nattokinase has long duration of drug action[4].The crude enzyme liquid of nattokinase were as the research object,the enzymatic properties of nattokinase were explored,nattokinase is laid the foundation for the future development of new thrombolytic drugs.

Materials and methods

Reagents used in the study are shown as follow. Na_2HPO_4 AR Wuxi City Yasheng Chemical Co.Ltd., NaH_2PO_4 AR Shanghai Zhanyun Chemical Co.Ltd.,fibrinogen BR Sigma,Urokinase 10KU CALBIOCHEM, $(\text{NH}_4)_2\text{SO}_4$ AR Xilong Chemical Co.Ltd., NaCl AR Shanghai Guangnuo Chemical Co.Ltd., K_2HPO_4 AR Shanghai Lingfeng Chemical Co.Ltd., KH_2PO_4 AR Sinopharm Chemical Reagent Co.Ltd.

Apparatus used in the study are shown as follow.Refrigerated centrifuge DL7M-12L Yancheng City Kate Experimental Co.Ltd.,Constant temperature water bath XMTD-204 Shanghai Boxun Co.Ltd.,Biochemical culture box MJ-300BS Shanghai Weicheng Instrument Co.Ltd.,Super clean worktable SW-CJ-2F Suzhou purification equipment limited company,High pressure steam sterilization pot YXQ-SG46-280SA Shanghai Boxun Co.Ltd., Spectrophotometer 756 Shanghai optical instrument factory

Natto was fermented according to the method.Small , plump fresh soybean were selected,soybean were thoroughly cleaned,and then soaked for 24 h with 3 times amount of water.400 g wet soybeans were packed in 1000ml beaker,thickness is 4.65cm,8 layers of gauze covered the beaker, 1×10^5 Pa 30min sterilization.

Strain was first activated,and then activated strain was inoculated in 150ml liquid beef extract peptone medium,cultured for 36h at 37°C .

Soybeans were first boiled,and then was cooled to 60°C , strains were inoculated by 2ml bacteria liquid/100g wet soybean ratio.At the temperature of 37°C ,soybean was fermented for 24 h,the

fermented natto was preserved in refrigerator at 4°C for 24 h.

Crude enzyme liquid was prepared according to the method. Fresh fermented natto, adding 2 times volume of sterilized saline, mixed, extracted 24h, 7000r/min 15min frozen centrifugation, the supernatant was extracted.

100ml extracted solution was measured, $(\text{NH}_4)_2\text{SO}_4$ was added slowly to extracted solution. The saturation is respectively 0%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%. The extracted solution was mixed and placed at 4°C for 12 h. 20min 7000r/min frozen centrifugation. The supernatant and precipitate were collected respectively. The precipitate was deposited in phosphate buffer solution. The activity of nattokinase in liquid supernatant and precipitation dissolution fluid were determined respectively. According to the experimental results, the appropriate saturation of $(\text{NH}_4)_2\text{SO}_4$ was selected to remove miscellaneous protein and deposit nattokinase.

Effect of temperature on the activity of nattokinase was studied. The crude enzyme liquid of nattokinase were placed at 20、25、30、35、40、45、50、55、60、65、70°C for 2 h, the enzyme activity was determined, the enzyme activity retention rate was calculated.

Heat resistance of nattokinase was studied. Under the temperature of 37°C, nattokinase was preserved for 1h, 3h, 5h, 7h, 9h respectively, the enzyme activity was determined, the enzyme activity retention rate was calculated.

Effect of pH on the activity of nattokinase was studied. Nattokinase were placed in the pH 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0, 11.0, 12.0 for 4h, the enzyme activity was determined, the enzyme activity retention rate was calculated.

The acid resistance of nattokinase was studied. The crude enzyme liquid of nattokinase were adjusted to pH 3, preserved for 0.5 h, 1h, 1.5h, 2h, 2.5h, 3h respectively. Then pH were transferred to 7. The enzyme activity was determined, the enzyme activity retention rate was calculated.

The activity of nattokinase was determined by fibrin plate method. Fibrin plate method: the fibrinogen plate was prepared, 9mm oxford cup was placed on the fibrinogen plate. The sample was added to in the oxford cup. Then cultured for 24h at 37°C. The transparent circle diameter was determined, the area was calculated. Enzyme activity was calculated according to urokinase standard curve.

The enzyme activity retention rate calculation: the enzyme activity retention rate = (enzyme activity treated after / enzyme activity treated before) \times 100%

Protein concentration was determined by Ultraviolet absorption method[5]. Protein concentration (mg/ml) = $1.45A_{280\text{nm}} - 0.74A_{260\text{nm}}$

Results

Effect fractionation of $(\text{NH}_4)_2\text{SO}_4$ on extraction of nattokinase was studied. The result is shown in figure 1. With the increase of $(\text{NH}_4)_2\text{SO}_4$ saturation, the nattokinase in supernatant gradually reduce, the nattokinase in precipitation gradually increased. $(\text{NH}_4)_2\text{SO}_4$ saturation is lower than 30%, nattokinase mainly existed in the supernatant, precipitation mainly for mixed proteins. $(\text{NH}_4)_2\text{SO}_4$ saturation is higher than 30%, the nattokinase in supernatant was gradually reduced, the nattokinase in precipitation increased. $(\text{NH}_4)_2\text{SO}_4$ saturation is higher than 60%, the nattokinase in precipitation no longer increase. So, the $(\text{NH}_4)_2\text{SO}_4$ saturation of 30% was selected to remove impurity protein, $(\text{NH}_4)_2\text{SO}_4$ saturation of 60% was selected to precipitate nattokinase.

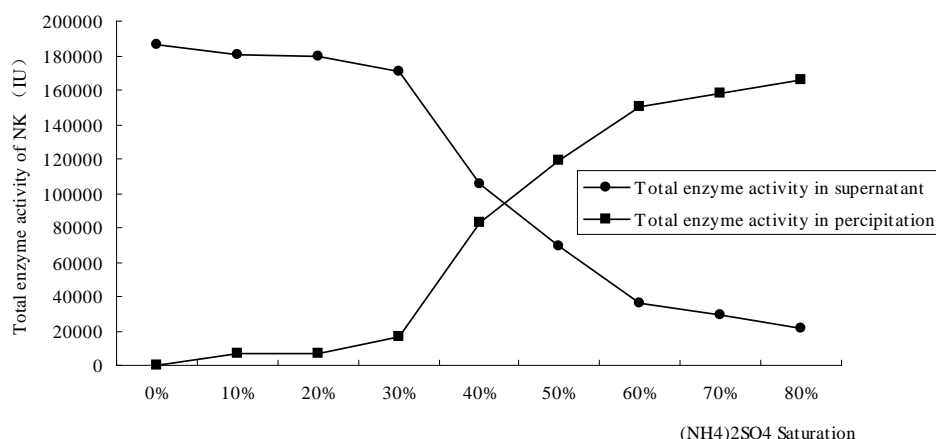


Fig.1 Effect of (NH₄)₂SO₄ saturation on the activity of nattokinase

Recovery of nattokinase enzyme activity and total protein by salting out method was studied. 100ml extracting solution is measured, solid (NH₄)₂SO₄ was added to 30% saturation, 7000r/min 15min frozen centrifugation. Precipitation is removed, solid (NH₄)₂SO₄ was added to 60% saturation, 7000r/min 15min frozen centrifugation. The supernatant was removed, the precipitation was dissolved in phosphate solution of 10ml 10mmol/L pH 6.4. The result is shown in table 1. The purification fold of nattokinase was 1.8 and recovery was 80.6%.

Table 1 Recovery of nattokinase enzyme activity and total protein by salting out method

Tests	Extrating solution	30%(NH ₄) ₂ SO ₄ supernatant	60%(NH ₄) ₂ SO ₄ precipitation dissolved solution
Enzyme activity of Nk/(IU/ml)	1867	1706	15048
Protein concentration/(mg/ml)	0.61	0.43	2.8
volum/ml	100	100	10
Total enzyme activity of Nk /IU	186700	170600	150480
Total protein/mg	61	43	28
NK recovery /%	100	91.37	80.6
Protein recovety/%	100	70.49	45.9
Specific activity/(IU/mg)	3060	3967	5374
Purification fold	1	1.3	1.8

Effect of temperature on the activity of nattokinas was studied. The result is shown in figure 2. When the temperature is lower than 40°C, the activity of nattokinase keep well for long time. When the temperature is 37°C, the activity of nattokinase keep very well. When the temperature is higher than 40°C, the activity of nattokinase inactivates rapidly. When the temperature exceeds 55°C, the activity of nattokinase was very low.

Heat resistance of nattokinase was studied. The result is shown in figure 3. When the temperature is 37°C, the nattokinase was preserved for 5h, the enzyme activity was not affected. When the time exceeds 5h, the activity of nattokinase inactivates rapidly.

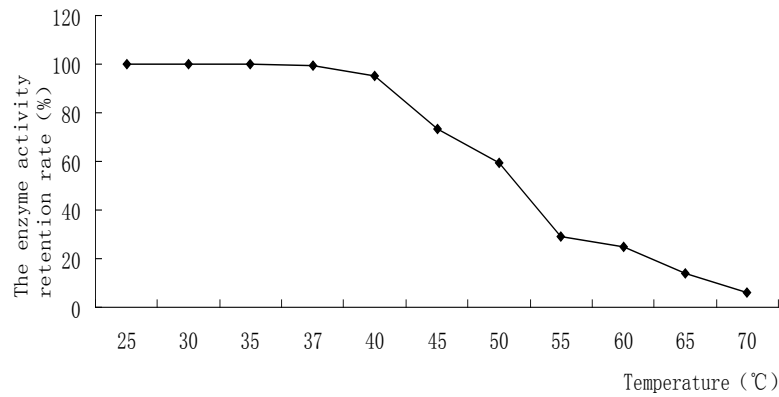


Fig.2 Effect of temperature on the activity of nattokinase

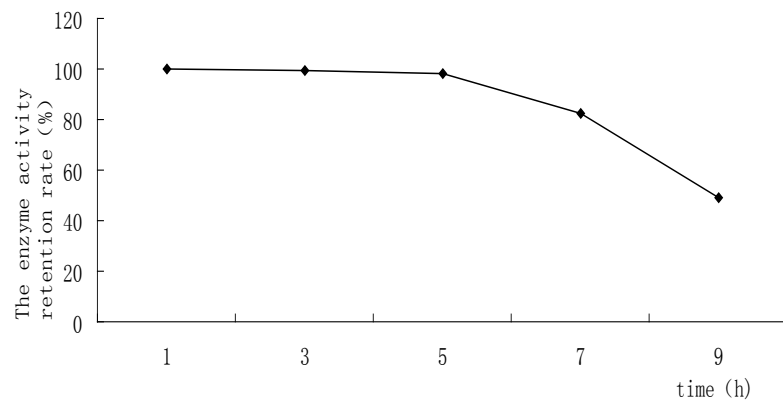


Fig.3 The stability of nattokinase under 37°C

Effect of pH on the activity of nattokinase was studied. The result is shown in figure 4. The activity of nattokinase was stable in the range of pH 6-9, the optimum pH was 7. Below pH 5 and above pH 10, the activity of nattokinase was rapidly lost.

The acid resistance of nattokinase was studied. The result is shown in figure 5. While at pH 3, the activity of nattokinase was very low. But pH is back to 7 in 1h, the activity of nattokinase can still be restored. More than 1h, the activity of nattokinase can not be return for permanent denaturation.

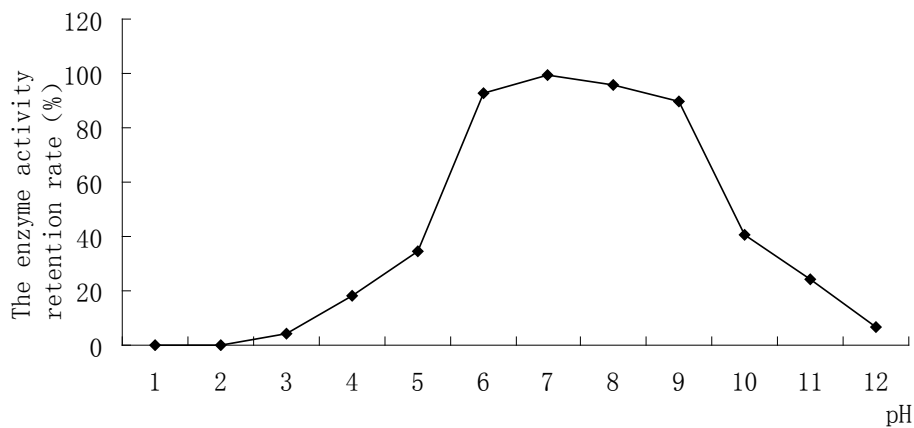


Fig.4 Effect of pH on the activity of nattokinase

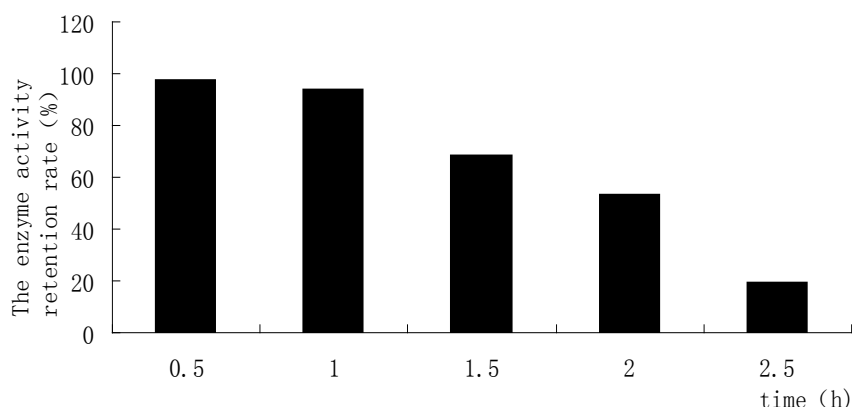


Fig.5 The stability of nattokinase under pH3

Conclusion

The optimal condition of isolation and purification of nattokinase by salting out method was studied. The $(\text{NH}_4)_2\text{SO}_4$ saturation of 30% was selected to remove impurity protein, $(\text{NH}_4)_2\text{SO}_4$ saturation of 60% was selected to precipitate nattokinase. When the temperature is 37°C , the activity of nattokinase keeps very well. While at pH3, the activity of nattokinase was very low. But pH is back to 7 in 1h, the activity of nattokinase can still be restored. Nattokinase can be developed into oral thrombolytic drug.

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