

## Study on Nattokinase-Extraction with two-phase aqueous system

Yingge Yang

Department of Bioengineering, Xuzhou Vocational college of Bioengineering, Xuzhou, 221006, China

email:ygyang\_1982@163.com

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**Abstract.** The extraction of nattokinase with two-phase aqueous system was studied. Distribution behavior of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and PEG6000/MgSO<sub>4</sub> two-phase aqueous systems were studied respectively. PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system is suitable for the extraction of nattokinase. Various factors affecting the extraction of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system were studied. The mass fraction of PEG400 was determined to be 24%, The mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was determined to be 27%, the addition of crude enzyme was determined to be 15%. Under these conditions, purification of nattokinase reached 2.11.

### Introduction

Nattokinase is a kind of alkaline protease. The study found that, nattokinase has the ability to dissolve thrombus effectively[1]. At present, nattokinase is extracted by the combination of salting out method and two to three step chromatography. Chromatography media is mainly agarose, dextran, cellulose, etc. Although the high pure nattokinase can be obtained, but the cost is high[2]. Two-phase aqueous extraction techniques refers to the two kinds of polymer or polymer and a salt water solution mixed together[3]. Because of the incompatibility between the polymer and the polymer and salt, two phase are formed. Two-phase aqueous extraction technology has the characteristics of mild conditions, easy method, continuous operation. Two-phase aqueous extraction technique has been successfully applied to the separation and purification of biological products[4]. Natto was used as materials, the extraction technology of nattokinase with two-phase aqueous system has been explored.

### Materials and methods

Reagents used in the study are shown as follow. Natto the laboratory fermentation, PEG400 CR Sinopharm Chemical Reagent Co.Ltd., PEG6000 CR Sinopharm Chemical Reagent Co.Ltd., (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> AR Xilong Chemical Co.Ltd., Mg SO<sub>4</sub> AR Sinopharm Chemical Reagent Co.Ltd., NaCl AR Shanghai Guangnuo Chemical Co.Ltd., Fibrinogen BR Sigma, Urokinase 10KU CALBIOCHEM

Apparatus used in the study are shown as follow. Vortex mixing device XW-80A Haimen equipment manufare Co.Ltd., Super clean worktable SW-CJ-2F Suzhou purification equipment limited company, Biochemical culture box MJ-300BS Shanghai Weicheng Instrument Co.Ltd., Spectrophotometer 756 Shanghai optical instrument factory.

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

Two-phase aqueous system was prepared. The amount of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was weighed, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> with 40% mass fraction was prepared. A certain amount of PEG400 was added. The solution was mixed with mixing device. Two-phase system were formed after resting. The mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and PEG400 were calculated. Two-phase aqueous systems were prepared.

Distribution behavior of nattokinase was studied. Nattokinase enzyme solution was added to

two-phase aqueous system. The solution was mixed thorough with mixing device. Two-phase system were formed after resting. Comparison R, distribution coefficient K and recovery of enzyme activity Yt were calculated respectively.

Comparison  $R = \frac{\text{up phase volum}}{\text{down phase volum}}$ , Distribution coefficient  $K = \frac{\text{up phase enzyme activity}}{\text{down phase enzyme activity}}$ , Recovery of enzyme activity  $Y_t = \frac{RK}{1+RK} \times 100\%$

The activity of nattokinase were determined by 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 tranparent circle diameter was determined, the area was calculated. Enzyme activity was calculated according to urokinase standard curve.

Protein concentration was determined by ultraviolet absorption method[5]. Protein concentration (mg/ml) = 1.45A<sub>280nm</sub> - 0.74A<sub>260nm</sub>

## Results

The phase diagram is often used to represent the formation conditions and the quantitative relationships of the two-phase aqueous system[6]. Substance which formed phase can limitlessly mixed with water. When their composition above the curve, system will be divided into two phases. When their composition below the curve, system can not be separated. The experimental results are as follows. PEG200/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and PEG200/MgSO<sub>4</sub> can not form two-phase. PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> can form two-phase. PEG400/MgSO<sub>4</sub> can not form two-phase. PEG6000/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and PEG6000/MgSO<sub>4</sub> can form two-phase. Figure 1 shows that the phase diagram formed by PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase system. Figure 2 shows that the phase diagram formed by PEG6000/MgSO<sub>4</sub> two-phase system. PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase formation required a larger fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. PEG6000/MgSO<sub>4</sub> two-phase formation required a smaller fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Maybe due to the larger the molecular weight of PEG, the hydrophobic property, thereby the phase power is increased.

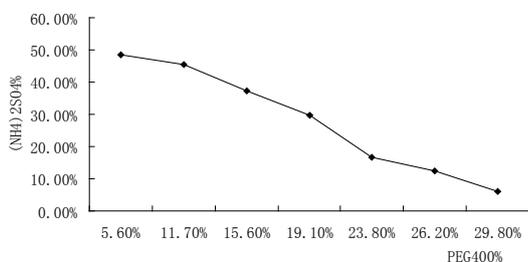


Figure 1 The phase diagram formed by PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase system.

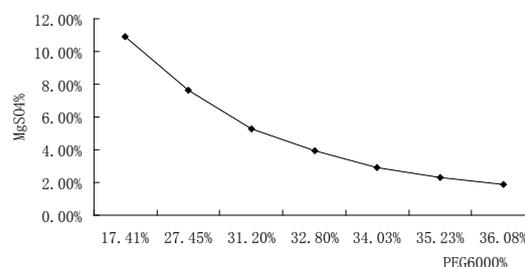


Figure 2 The phase diagram formed by PEG6000/MgSO<sub>4</sub> two-phase system

**Effect on distribution behavior of nattokinase with different two-phase aqueous system** was studied. The crude enzyme solution were added to PEG/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and PEG6000/MgSO<sub>4</sub> two-phase aqueous system respectively. The solution was mixed with mixing device. Up volum, down volum and its enzyme activity were determined respectively after resting. Table 1 shows that distribution behavior of nattokinase in PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system. Table 2 shows that distribution behavior of nattokinase in PEG6000/MgSO<sub>4</sub> two-phase aqueous system. All of the recovery rates of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system were around 50%. The highest recovery rate of nattokinase with PEG6000/MgSO<sub>4</sub> two-phase aqueous system was 37.89%. The recovery rate of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system was higher the recovery rate of nattokinase with PEG6000/MgSO<sub>4</sub> two-phase aqueous system. Research shows that, the hydrophobicity of PEG and phase viscosity increases as the molecular weight increases, and the phase boundary tension between two phases increases, which is not conducive to the protein into PEG phase[7]. If higher recovery rate of protein

is expected to obtain, the molecular weight of PEG should be reduced. Therefore, PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system was selected for the extraction of nattokinase.

Table 1 Distribution behavior of nattokinase in PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system

| PEG400% | (NH <sub>4</sub> ) <sub>2</sub> SO <sub>4</sub> % | R    | K    | Yt    |
|---------|---|------|------|-------|
| 25      | 22  | 1.18 | 1.14 | 57.36 |
| 17      | 24  | 0.74 | 1.59 | 54.06 |
| 16      | 24  | 0.5  | 2.66 | 57.08 |
| 13      | 29  | 0.35 | 2.79 | 49.40 |
| 20      | 25  | 0.81 | 1.82 | 59.58 |

Table 2 Distribution behavior of nattokinase in PEG6000/MgSO<sub>4</sub> two-phase aqueous system.

| PEG6000% | MgSO <sub>4</sub> % | R    | K    | Yt    |
|----------|---------------------|------|------|-------|
| 11       | 18                  | 0.61 | 1    | 37.89 |
| 8        | 28                  | 0.35 | 0.82 | 22.30 |
| 6        | 30                  | 0.20 | 1    | 16.67 |
| 4        | 33                  | 0.16 | 1.36 | 17.87 |
| 3        | 37                  | 0.12 | 0.68 | 7.54  |

Extraction of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system was studied. The mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was 25%, the addition of crude enzyme was 10%. Effect on extraction of nattokinase with the mass fraction of 20%、22%、24%、26%、28% PEG400 were studied respectively. The results are shown in table 3. With the increase of the mass fraction of PEG400, R and Yt of nattokinase increase, K of nattokinase increases first and then decreases. The higher mass fraction of PEG400 is conducive to the extraction of nattokinase. But R increased with the increase of the mass fraction of PEG400, up phase volum enlarged, which was inconducive to enrichment of nattokinase. So the mass fraction of PEG400 also should not be too big. As the mass fraction of PEG400 was 24% and 28%, the recovery rate of nattokinase almost equal. When the mass fraction of PEG400 was 28%, R was smaller. Comprehensive consideration, the mass fraction of PEG400 was determined to be 24%.

The mass fraction of PEG400 was 24%, the addition of crude enzyme solution was 10%. Effect on extraction of nattokinase with the mass fraction of 21%、23%、25%、27%、29% (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> were studied respectively. The results are shown in table 4. With the increase of the mass fraction of PEG400, R of nattokinase increase, K and Yt of nattokinase increases first and then decreases. When the mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was 27%, K and Yt of nattokinase is the highest. So the mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was determined to be 27%.

The mass fraction of PEG400 was 24%, the mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was 27%. Effect on extraction of nattokinase with addition of 5%、10%、15%、20%、25% crude enzyme were studied respectively. The results are shown in table 5. With the increase of the addition of crude enzyme, R and Yt of nattokinase increase, K of nattokinase decreases. When the addition of crude enzyme was 15%, Yt of nattokinase is the highest. So the addition of crude enzyme was determined to be 27%.

The mass fraction of PEG400 was 24%, the mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was 27%, the addition of crude enzyme was 15%. The content of protein and enzyme activity of nattokinase in up phase was determined. The results are shown in table 6. The recovery rate of nattokinase reached 86%, the purification of nattokinase reached 2.11.

Table 3 Effect on extraction of nattokinase with the mass fraction of PEG400

| PEG40<br>0% | (NH <sub>4</sub> ) <sub>2</sub><br>SO <sub>4</sub> % | R    | K    | Yt    |
|-------------|--|------|------|-------|
| 20          | 25   | 0.82 | 1.82 | 59.87 |
| 22          | 25   | 1.82 | 2.01 | 78.53 |
| 24          | 25   | 1.94 | 2.94 | 85.08 |
| 26          | 25   | 2.32 | 2.04 | 82.56 |
| 28          | 25   | 5.00 | 1.60 | 88.89 |

Table 4 Effect on extraction of nattokinase with the mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>

| PEG40<br>0% | (NH <sub>4</sub> ) <sub>2</sub><br>SO <sub>4</sub> % | R    | K    | Yt    |
|-------------|--|------|------|-------|
| 24          | 21   | 1.28 | 1.12 | 58.91 |
| 24          | 23   | 1.38 | 1.77 | 70.95 |
| 24          | 25   | 1.94 | 1.94 | 79.01 |
| 24          | 27   | 2.62 | 2.12 | 84.74 |
| 24          | 29   | 1.75 | 1.39 | 70.87 |

Table 5 Effect on extraction of nattokinase with addition of crude enzyme

| Addition of<br>crude enzyme (%) | R    | K    | Yt    |
|---------------------------------|------|------|-------|
| 5                               | 0.97 | 3.12 | 75.16 |
| 10                              | 2.62 | 2.12 | 84.74 |
| 15                              | 2.78 | 2.08 | 85.25 |
| 20                              | 4.42 | 1.98 | 89.74 |
| 25                              | 5.05 | 1.85 | 90.33 |

Table 6 Recovery of nattokinase enzyme activity and total protein two-phase aqueous system

| Test                         | Crude<br>enzyme<br>solution | up<br>phaseextraction |
|------------------------------|-----------------------------|-----------------------|
| NK recovery /%               | 100                         | 86                    |
| Protein<br>recovery/%        | 100                         | 41                    |
| Specific<br>activity/(IU/mg) | 3060                        | 6456                  |
| Purification fold            | 1                           | 2.11                  |

## Conclusion

Compared with PEG6000/MgSO<sub>4</sub> two-phase aqueous system, PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system was more suitable for extraction of nattokinase. Various factors affecting the extraction of nattokinase with PEG400/(NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> two-phase aqueous system were studied. The mass fraction of PEG400 was determined to be 24%, The mass fraction of (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> was determined to be 27%, crude enzyme was determined to be 15%. Under these conditions, purification of nattokinase reached 2.11. This study not only has opened up new avenues for separation and purification of nattokinase, but also broadens the scope of application in biological macromolecules with two-phase aqueous system.

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