Optimization of spectrophotometric method for determination Nattokinase activity in dietary supplements

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Abstract

Nattokinase is an enzyme with a strong fibrinolytic activity that can be used for preventing thrombolytic diseases. The fibrinolytic activity of the Nattokinase in dietary supplements is determined by spectrophotometric method. In this study, the conditions of the assay were examined and finally optimized as pH 8.5, reaction temperature: 40°C, and reaction time: 60 minutes. The proposed method was validated and successfully applied to determine the Nattokinase activity in 10 dietary supplements. The results showed that the proportion of measured activity compared to label product activity were within in the range 79.1 - 98.0%. The effect of herbal compounds and excipients on Nattokinase activity were also surveyed. From the evaluated results and application to commercial samples, this method will be a valuable contribution to quality control of dietary supplement products containing Nattokinase.

Keywwords: *Nattokinase, dietary supplements, fibrinolytic activity, spectrophotometric method.*

1. INTRODUCTION

Nattokinase is an enzyme extracted from a popular Japanese food called "Natto". Natto is a traditional food made of soybean fermented with *Bacillus subtilis*. Natto has been used as a folk remedy for diseases of the heart and blood vessels for hundreds of years. Common names for the Nattokinase include natto extract, subtilisin NAT and fermented soybeans. It belongs to the serin protease family, the sequence of its primary structure includes 275 amino acid residues, with the molecular weight of 27.778 Dalton. It has strong fibrinolytic properties. It remains stable between from pH from 6.0 to 12.0 but loses its fibrinolytic activity quickly if pH is less than 5.0 or temperature higher than 50°C [1].

Nattokinase is a fibrinolytic enzyme, meaning that it breaks down fibrin, an insoluble protein produced by the conversion of fibrinogen (a protein in the plasma of blood for clotting) by thrombin (a blood-clotting enzyme). It degrades fibrin clots both directly and indirectly. It degrades fibrin directly in clot lysis assays with activity comparable to plasmin and degrades fibrin indirectly by affecting plasminogen activator activity. It helps in normal blood circulation, blood flow, blood viscosity. It also supports the body's normal blood-clotting mechanism, supports the body's production of plasmin, which reduces fibrin and helps to maintain normal blood pressure level. It is also used for pain, fibromyalgia, chronic fatigue syndrome, endometriosis, uterine fibroids, muscle spasms, tissue oxygen deprivation, infertility, cancer [1-5].

The determination method for fibrinolytic activity is very important in the research of Nattokinase. Astrup & Mullerts (1952) develop the Fibrin plate method to measure the fibrinolytic method. This method includes a simulated thrombus artificially formed by fibrinogen

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and thrombin in a petri dish. The position of the area on the petri dish that has contact with fibrinolytic enzyme will generate a lytic circle, which stands for the fibrinolytic activity of the enzyme applied. Then, a standard enzyme is used to create a standard curve in order to compare and calculate the activity of the. The serological plate was developed from a fibrin plate assay for determining the activity of nattokinase. The serological plate method was based on the maximum absorbance of fibrin at 655 nm in visible light (OD_{655}), and the absorbance intensity is decreasing along with the fibrin lyses [6]. Clot Lysis Time (CLT) was modified from Astrup's method. Thrombus can be generated in vitro, and the time used for dissolving the preformed thrombus is recorded as CLT, which has a good linear relationship with the logarithm of enzyme concentration within the range of 10 - 70 µg [7].

Ultraviolet spectrophotometry method is a relatively new method for Nattokinase activity determination indirectly through the estimation of its hydrolysate. After the fibrinolytic reaction, the OD_{275} of soluble hydrolysate products of Nattokinase are measured and compared to the standard units [6]. This method is not affected by external factors and it has been demonstrated to be an accurate and sensitive method, so we chose spectrophotometric method for this study.

Nowadays, dietary supplements which improve brain function are becoming more and more popular and diverse. In these products, Nattokinase is often supplemented with herbal compounds that support brain functions such as *Ginkgo biloba*, *Polyscias fruticose*. These compounds as well as the excipients in the product may affect the fibrinolytic activity of the enzyme Nattokinase. Therefore, it is necessary to optimize the method for determination of the Nattokinase activity and study the factors affecting Nattokinase activity in dietary supplements products to provide safe, quality products to consumers.

2. MATERIALS AND METHODS

2.1. Reagents and chemicals

Nattokinase was provided by Febico, Taiwan; sodium borate decahydrate, sodium chloride, trichloroacetic acid, acetic acid, sodium acetate trihydrate, triton X-100, calcium sulfate dihydrate were purchased from Merck, Germany; fibrinogen and thrombin from bovine plasma were provided by Sigma Aldrich, Singapore; distilled water was obtained from Merck Millipore water purification system.

2.2. Equipment

Spectrophotometric analysis was performed using a double beam UV-VIS spectrophotometer UV 2401 (Shimadzu, Japan), fitted with a 10 × 10 mm cuvette holder, and scanned within a wavelength range of 198 - 1.000 nm. Analytical balance (Model ME54E, Mettler Toledo, Switzerland), pH meter (Model S220, Mettler Toledo, Switzerland), centrifuge (Model Z326K, Hermle, Germany), water bath (Model WNB22, Memmerts, Germany) were among the equipment employed for the study.

2.3. Methods

2.3.1. Fibrinolytic activity assay

Nattokinase was dissolved and diluted with diluent solution (2 mM of calcium sulfate dihydrate plus 10 mM of sodium chloride and 0.005% Triton X-100). For both sample and blank tubes, incubate 1.4 mL of 50 mM sodium borate buffer with 0.4 mL of 0.72% Fibrinogen solution in 5 min. Then, add 0.1 mL of 20 U/mL thrombin into tubes and mix. After 10 min, add 0.1 mL Nattokinase solution into the sample tube and incubate for exact time and temperature (to be

investigated). Add 2.0 mL of 0.2 M trichloroacetic acid to stop the reaction, add 0.1 mL Nattokinase solution into a blank tube and incubate for further 20 min. The tubes were centrifuged at 6000 rpm for 5 minutes and the absorbance of supernatants were measured at 275 nm against distilled water.

The fibrinolytic activity of the Nattokinase was calculated by this formula:

$$\text{Nattokinase activity (FU/g)} = \frac{A_T - A_B}{0.01} \times \frac{1}{T} \times \frac{1}{0.1} \times \frac{V}{m} \times D$$

Where A_T = absorbance of sample solution, A_B = absorbance of blank solution,

T = reaction times (minute), V = volume of Nattokinase extractant (mL),

m = weight of sample (g), D = dilution factor.

One unit (1 FU) of Nattokinase activity is defined as the amount of the enzyme which increases the absorbance of the filtrate at 275 nm by 0.01 per minute under the conditions specified in the procedure.

2.3.2. Optimization of pH

The enzyme activity was measured in the range of pH from 5.5 to 10.0 using the standard activity assay procedure at related pH. The optimal pH was investigated by measuring the fibrinolytic activity after incubating the enzyme solution at pH 5.5, 7.0, 8.5, 10.0 for 60 minutes at 40°C.

2.3.3. Optimization of reaction time

The enzyme activity was measured in the range of time 30 - 75 minutes using the standard activity assay procedure at related incubate time. The optimal incubate time was investigated by measuring the fibrinolytic activity after incubating the enzyme solution for 30, 45, 60, 75 min for 60 minutes in 50 mM sodium borate buffer (pH 8.5) at 40°C in 50 mM sodium borate buffer (pH 8.5).

2.3.4. Optimization of reaction temperature

The enzyme activity was measured in the range of 30, 40, 50, 60, 70°C using the standard activity assay procedure at related temperature. The optimal temperature was investigated by measuring the fibrinolytic activity after incubating the enzyme solution at 30, 40, 50, 60, 70°C for 60 minutes in 50 mM sodium borate buffer (pH 8.5).

2.3.5. Effect of herbal compounds on Nattokinase fibrinolytic activity

The examined herbal compounds include *Ginkgo biloba* extract, *Polyscias fruticosa* extract, *White willow bark* extract, *Rehmannia glutinosa* extract, *Paeoniae lactiflora* extract, *Angelica sinensis* extract, *Wolfiporia extensa* extract, *Eucommia ulmoides* extract, *Codonopsis pilosula* extract. Prepare the herbal extracts solution in 50 mM sodium borate buffer (pH 8.5) at a concentration of 10% (w/v) to investigate the effect on Nattokinase activity. Mix 1.0 mL of the enzyme solution with 1.0 mL of the herbal extracts solution, pre-incubated for 15 min at 40°C then the fibrinolytic activity was determined.

2.3.6. Effect of excipients on Nattokinase fibrinolytic activity

The examined excipients include talc, sodium benzoate, calcium carbonate, magnesium stearate, tapioca starch. Prepare the excipients solution in 50 mM sodium borate buffer (pH 8.5) at a concentration of 10% (w/v) to investigate the effect on Nattokinase activity. Mix 1.0 mL of the enzyme solutions with 1.0 mL of the excipient solution, pre-incubated for 15 min at 40°C then the fibrinolytic activity was determined.

2.3.7. Method application

The optimized and validated method was applied for determination of the fibrinolytic activity in commercial dietary supplements containing Nattokinase. Measurement of activity was taken in three replicates. The measured activity was compared with labeled activity to estimate the quality of products.

3. RESULTS AND DISCUSSION

3.1. Optimization of pH

In this study, the pH of sodium borate buffer solution from 5.5 to 10.0 were examined. The results are shown in Figure 1.

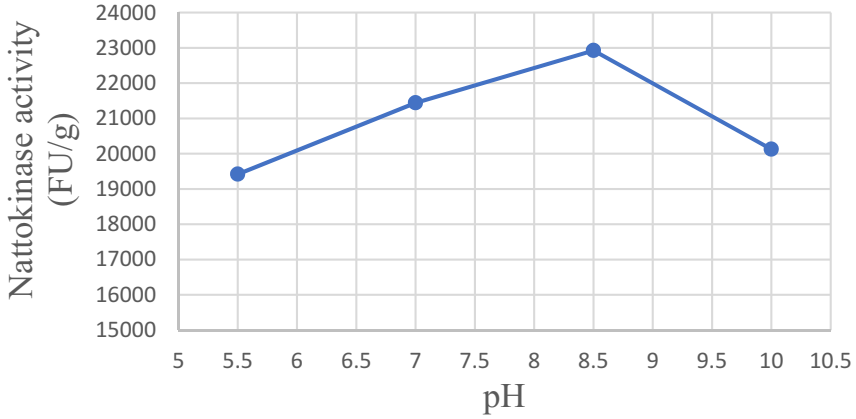

***Figure 1**. Nattokinase activity at different pH value (5.5 - 10.0)*

The Nattokinase activity increased when the pH increased from 5.5 to 8.5 but decreased when the pH increased to 10.0. At pH 8.5, Nattokinase activity was highest and statistically different to those at other pH values. According to this result, pH 8.5 was the optimal pH to determine Nattokinase activity.

3.2. Optimization of reaction time

In this study, the reaction time from 30 to 75 minutes were investigated. The results are shown in Figure 2.

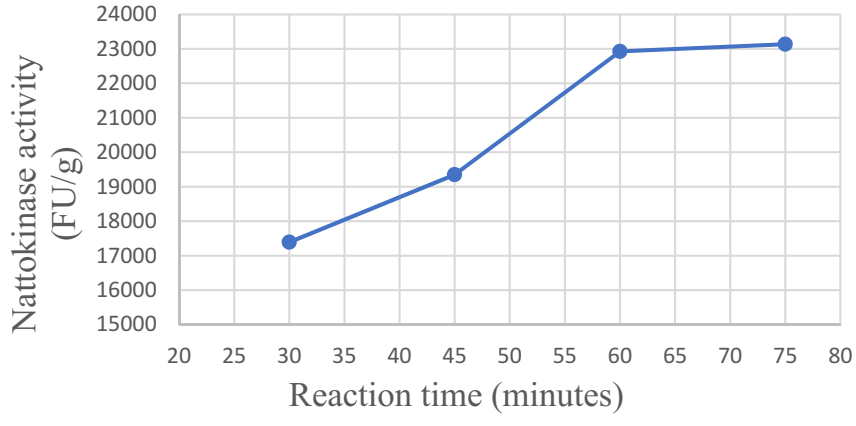

***Figure 2.** Nattokinase activity at different reaction time (30 - 75 minutes)*

The results showed that the fibrinolytic activity of Nattokinase increased when the incubate time increased from 30 to 75 minutes. However, the difference between the Nattokinase activity

at the reaction time 60 and 75 minutes were not statistically significant. According to these results, the optimal reaction time for the Nattokinase activity assay was 60 minutes to decrease the time of the assay.

3.3. Optimization of reaction temperature

In this study, the reaction temperature from 30°C to 70°C was examined. The results were shown in Figure 3.

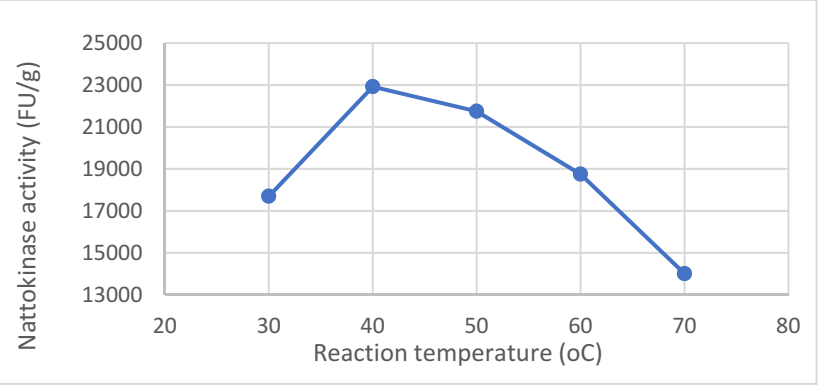

***Figure 3**. Nattokinase activity at different reaction temperature (30 – 70°C)*

The result showed that the fibrinolytic activity of the Nattokinase was highest at temperature 40°C and the activity decreased when the temperature increased from 40°C to 70°C. According to the survey results, the optimal temperature for the Nattokinase activity assay was 40°C.

3.4. Method validation

After examined, the conditions of the assay were selected as pH 8.5, reaction temperature: 40°C, and reaction time: 60 minutes. The method was validated by determining its specificity, precision, the limit of detection and quantification. Repeatability (RSD %) was 1.66% and reproducibility (RSD %) was 2.770%. The limit of detection (LOD) and the limit of quantification (LOQ) were 5.07 FU/g and 16.7 FU/g. This method was applied to study the effect of herbal compounds and excipients on the Nattokinase activity in dietary supplements.

3.5. Effect of herbal compounds on Nattokinase fibrinolytic activity

In this study, we selected 09 herbal compounds which improve brain function to study the affection of these compounds to the Nattokinase activity. The results are shown in Figure 4.

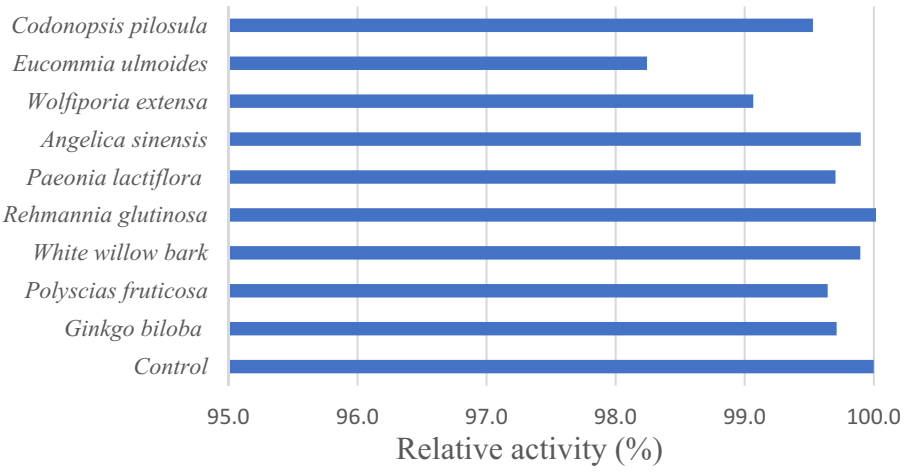

***Figure 4.** Effect of herbal compounds on Nattokinase activity*

According to this result, the fibrinolytic activity of Nattokinase is not affected by the presence of these herbal extracts at a concentration of 10% (w/v). The Nattokinase activity in mixture solutions with herbal compounds was retained at least 98.2% (compare to the Nattokinase activity of control solution that not mixed with herbal compounds). Thus, it is possible to combine these herbal extracts with the Nattokinase enzyme in dietary supplements without affecting the fibrinolytic activity of the Nattokinase.

3.6. Effect of excipients on Nattokinase fibrinolytic activity

In this study, we selected 05 excipients to examine the affection of excipients to the Nattokinase activity, include Talc, calcium carbonate, sodium benzoate, magnesium stearate and tapioca starch. The results are shown in Figure 5.

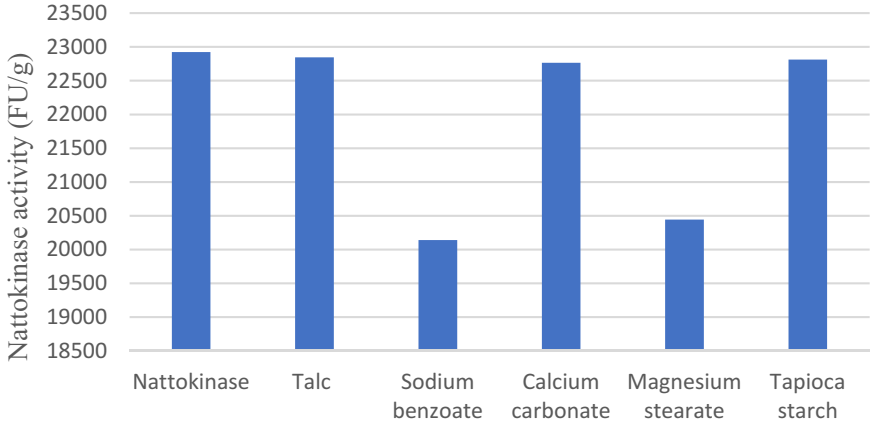

***Figure 5**. Effect of excipients on Nattokinase activity*

The fibrinolytic activity of the Nattokinase was not affected by the presence of talc, calcium carbonate, tapioca starch. However, the Nattokinase activity decreased by the presence of sodium benzoate and magnesium stearate in the mixture. According to this result, the manufacturer should replace sodium benzoate, magnesium stearate by the other excipients in dietary supplements containing the Nattokinase.

3.7. Analysis of commercial samples

The currently validated method was applied for the determination of the Nattokinase activity in 10 commercial products. The results of the analysis are presented in Table 1.

***Table 1.** Analysis of commercial dietary supplements containing Nattokinase*

Samples	*Dosage form*	*Measured activity (FU)*	*Labeled activity (FU)*	*Percentage (%)*
Sample 1	Hard capsule	1581 ± 3.79	2000	79.1
Sample 2	Hard capsule	823 ± 3.51	1000	82.3
Sample 3	Hard capsule	443 ± 2.79	500	88.6
Sample 4	Hard capsule	1755 ± 3.00	2000	87.8
Sample 5	Hard capsule	163 ± 3.02	200	81.5
Sample 6	Soft capsule	294 ± 4.04	300	98.0
Sample 7	Soft capsule	94.9 ± 2.62	100	94.9

Samples	*Dosage form*	*Measured activity (FU)*	*Labeled activity (FU)*	*Percentage (%)*
Sample 8	Soft capsule	147 ± 4.04	150	98.0
Sample 9	Soft capsule	488 ± 3.51	500	97.6
Sample 10	Soft capsule	93.7 ± 1.01	100	93.7

The measured activity was lower than the label product activity in all samples analyzed, the reason might be that Nattokinase is not stable and the loss of activity that have occured. The percentages of measured activity compared to labeled activity for hard capsules were within in the range 79.1 - 88.6% while the figure for soft capsules were 93.7 - 98.0%. The lowest proportion of measured activity compared to label product activity was 79.1% in sample 1. However, there were only 10 samples analyzed in this research so it is necessary to analyze a large number of samples to estimate the quality of dietary supplements products containing Nattokinase.

4. CONCLUSION

In this study, the conditions of the Nattokinase activity assay method about pH, reaction time and temperature for determination fibrinolytic activity of the Nattokinase were optimized. This method was validated and applied to determine the fibrinolytic activity of the Nattokinase in dietary supplements. The effect of herbal compounds and excipients on Nattokinase activity were also investigated. The results showed that the Nattokinase activity was not affected by examined herbal compounds. However, the fibrinolytic activity decreased by the presence of some excipients, such as magnesium stearate or sodium benzoate.

5. REFERENCES

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Tối ưu hoá phương pháp quang phổ hấp thụ phân tử xác định hoạt độ Nattokinase trong thực phẩm bảo vệ sức khoẻ

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Tóm tắt

Nattokinase là một enzyme có hoạt tính phân hủy fibrin mạnh và được bổ sung trong các chế phẩm thực phẩm bảo vệ sức khỏe có tác dụng dự phòng, hỗ trợ điều trị bệnh lý huyết khối. Hoạt tính tiêu fibrin của Nattokinase trong thực phẩm bảo vệ sức khỏe được xác định bằng phương pháp quang phổ hấp thụ phân tử. Trong nghiên cứu này, các điều kiện tối ưu của quy trình phân tích đã được khảo sát và xác định, phản ứng được tiến hành ở pH 8,5, nhiệt độ phản ứng là 40^{o}C và thời gian phản ứng là 60 phút. Phương pháp xác định hoạt độ Nattokinase đã được thẩm định đạt yêu cầu và áp dụng để phân tích trong10 mẫu trong thực phẩm bảo vệ sức khỏe. Kết quả phân tích cho thấy hoạt độ Nattokinase trong 10 mẫu thử nằm trong khoảng 79,1 - 98,0% so với giá trị công bố trên nhãn. Ngoài ra, ảnh hưởng của các loại dược liệu và tá dược có trong sản phẩm tới hoạt tính của Nattokinase cũng được nghiên cứu. Từ kết quả thẩm định và áp dụng phân tích trên mẫu sản phẩm thương mại, cho thấy phương pháp phân tích này có thể áp dụng để kiểm soát chất lượng của các sản phẩm thực phẩm bảo vệ sức khỏe trên thị trường có chứa Nattokinase.

Từ khóa: *Nattokinase, thực phẩm bảo vệ sức khỏe, hoạt độ fibrinolytic, quang phổ, hấp thụ phân tử.*