

# Rapid Detection of Milk Protein based on Proteolysis Catalyzed by Trypsinase

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## Abstract

Using pH-state method, when degree of casein hydrolysis was set as criterion, the optimal enzymatic reaction conditions were obtained as follows: pH 7.5 and temperature 55 °C. Based on the proteolysis catalyzed by trypsinase, the relationship between protein content (x) and initial velocity of enzymatic reaction (y) was discussed and the equation of  $y=0.1177x$  ( $R^2=0.9927$ ) was found as standard curve for the calculation of protein content. In addition, the protein contents in five kinds of commercial fresh milk were determined by enzyme hydrolysis method and GB method (biuret method). Protein content calculated by enzyme hydrolysis method could be confirmed by GB method and the analysis of t-test indicated that there was no notable difference between two methods. Furthermore, the determinate time of the rapid method was only 8 minutes (including samples' preparation) and was 1/20 times than that of biuret method.

**Keywords:** Protein, Trypsinase, The initial velocity, PH-state

Nutrient-rich milk is the ideal natural food, protein is one of the major nutritional components of milk, which also is an important quality indicator of a large number of dairy products (LIU Dong-hong, TANG Jia-ni, LV Yuan, YE Xing-qian, 2008). However, in recent years, "Big Head" and "melamine" event happened time to time. They also reflected the existence of significant gaps in dairy testing (TANG Jia-ni, LV Yuan, FU Yu, YE Xing-qian, LIU Dong-hong, 2009), so how to detect the protein content of dairy products quickly and accurately, has restricted the development of China's dairy industry.

The nitrogen content of milk divides into non-protein nitrogen and true protein (Depeters EJ, Ferguson JD, 1992). Protein content in milk is currently detected by the main methods for Kjeldahl method, Coomassie Brilliant Blue G-250 method, biuret, etc (QIN Li-hu, KOU Yun-juan, REN Jiang-hong, SUN Wu-bin, 2001) (TANG Ping, TIAN Jing, SHE Zhen-bao, WENG Qian-feng, XU Guo-wang, 2006) (ZHANG Ai-mei, LI Lin-wei, ZANG Yu-bo, 2006). But these methods both have complicated operation, consumption of reagents and more in need of special equipment or material. Therefore, we use enzymatic reaction with the relations between the substrate concentration and the initial velocity, and then establish a rapid detection method of true protein in milk. When we test samples, only need to measure the initial velocity of hydrolysis then can get the true protein content, in the meanwhile because this method is used to detect the true protein, so it also can prevent unscrupulous trader's adulterate melamine into milk.

## 1. Materials and methods

### 1.1 Materials and reagents

Carbon tetrachloride, potassium sodium tartrate, trichloroacetic acid, anhydrous sodium carbonate, disodium hydrogen phosphate, sodium dihydrogen phosphate, anhydrous copper sulfate, sodium hydroxide, barium hydroxide, zinc sulfate, Folin - phenol reagent, trypsin were purchased from China National Medicines Corporation Ltd. Casein were purchased from National Institutes for Food and Drug Control.

### 1.2 Experimental methods

#### 1.2.1 Folin-phenol method to measure trypsin activity

Determination of trypsin activity was reference to Wang Furong (GUO Yong, 2002) (WANG Fu-rong, PANG Yu-zhen, 1981) and others methods, the measured tyrosine (y) and absorbance (x) of the standard curve was  $y = 0.009x$  ( $R^2 = 0.9927$ ), K was 111.11 μg/OD. Activity Definition: In the above measurement conditions, we use the amount of enzyme which produces 1 μg tyrosine per minute needed as a unit. Therefore, the obtained activity of trypsin used for the 57.33u/mg.

### 1.2.2 Determination of protein degree of hydrolysis (DH%) by pH-state method

The degree of hydrolysis was determined by pH-state method (MA Ge-li, PENG Xin-bang, WEI Jun, MA Tian-hua, 2007), when hydrolysis begins, adjust the pH of the reaction system as pH value "a" (a for each of the initial reaction pH), then add 0.1mol / L NaOH solution into system to adjust pH, the pH values remain at the setting value "a", recording the volume of NaOH which used in reaction after 15mins. Optimized pH values, "a" set 7.0,7.5,8.0,8.5 and 9.0; optimized temperature, "a" was set to 7.5.

### 1.2.3 Determination of the initial velocity of hydrolysis of proteins by pH-state method

The concentration of casein solution were prepared 6.60,8.25,11.00,16.50,33.00 mg/ml, use pH-state method to detect the initial velocity of enzyme reaction.

Take 30ml casein solution, make the temperature constantly at 55°C for 3min, then take another 0.1mol / L NaOH standard solution to adjusting pH into 7.50, add 171.99 u trypsin, immediately recording time. When the pH value decreased 0.05, transferred back to pH 7.50 with standard NaOH solution, repeat the experiment for 5 times, recording the reaction time t (s) and consumption volume v ( $\mu$ l) each time, according to above records to make a figure, the slope is the initial velocity of hydrolysis V ( $\mu$ l / s). According to the casein concentration and its corresponding initial velocity, we can get their curve.

### 1.2.4 Determination of the protein content of milk samples by enzymatic hydrolysis

Take fresh milk samples A, B, C, D, E, the samples were diluted three times, and then detect the initial velocity of hydrolysis, took them into 1.2.3 curves obtained, respectively, then we can get the protein content of them.

### 1.2.5 Statistical analysis

The Data for each experiment were the average of three parallel, and limited the upper and lower error in figures. Using Enzyme initial velocity method to measure out the protein content of milk samples, then compare with the samples which was detected by biuret method. And used paired t-test method to judge whether the results of two methods were significantly different.

## 2. Results and Analysis

### 2.1 Optimize the condition of trypsin catalyzed hydrolysis of casein

#### 2.1.1 The effect of pH on the degree of hydrolysis of casein

According to researches on the conditions of trypsin hydrolysis from Li Peijun (LI Pei-jun, YUAN Yong-jun, HU Ting, WANG Li-hui, 2005), Li Guiqin (LI Gui-qin, LIU Jin, HAN Qing-bo, 2003), we take 30ml 33.00mg/ml casein solution and adjust pH to 7.0,7.5,8.0,8.5 and 9.0, enzyme volume of 57.33 u, reaction at 45 °C for 15 min, obtained pH values under different degree of hydrolysis, the results shown in Figure 1.

It can be seen from Figure 1, when pH between 7.0 and 8.0, the maximum degree of hydrolysis at pH7.5 when the degree of hydrolysis of proteins was 2.17%. Therefore, we selected the optimal hydrolysis pH 7.5.

#### 2.1.2 The effect of temperature on the degree of hydrolysis of casein

According to the research on trypsin hydrolysis of casein temperature at pH7.5 from Wang zheng (WANG Zheng, YANG Guo-yu, PANG Yu-shan, 2005), we take 30ml 33.00mg/ml casein solution, enzyme amount was 57.33 u, set temperature at 35 °C, 40 °C, 45 °C, 50 °C, 55 °C and 60 °C, reaction of 15 min, obtained the degree of hydrolysis of casein in different temperatures, the results shown in Figure 2.

It can be seen from Figure 2 that the minimum degree of hydrolysis of proteins at 35°C; and then with increasing temperature, the speed gradually accelerated enzymatic hydrolysis, the maximum degree of hydrolysis at 55°C when the degree of hydrolysis of 2.39%. Then with the temperature increasing, the degree of hydrolysis of casein decreased. So choose the best hydrolysis temperature was 55°C.

### 2.2 Analysis of the relation between the concentration of Initial velocity and casein hydrolysis curves

We got the corresponding initial velocity and relative standard deviation (RSD) under the different casein concentration, as shown in Table 1.

As can be seen from Table 1, with the (casein) concentration of substrate increasing, the initial velocity of enzymatic reaction was also increasing. According to the general concepts of enzyme kinetics, enzyme and substrate binding for single-substrate reaction, if the enzyme concentration and other reaction conditions constant, we can get rectangular hyperbola between the reaction rate (velocity, V) and substrate concentration [S]. This curve can be roughly divided into three stages: a. When [S] is low, V with [S] increased linearly; b. with [S] continues to increase, the magnitude of increase in declining order reaction was mixed; c. When [S] up to a

certain extent, we gain the maximum rate (maximum velocity,  $V_{max}$ ) and this time as a zero order reaction (JIA Hong-ti. 2005).

It is assumed measuring  $V$  meeting the Michaelis curve at the concentration of casein 0-33mg/ml, do  $1/V-1 / [S]$  of the double reciprocal graph, the curve should be a straight line. Of  $1 / V$  (y) and  $1 / [S]$  (x) plot, the curve equation was  $y = 8.166x + 0.0043$  ( $R^2 = 0.9858$ ), the line with assumptions, but fitness was not very good, may be casein was chosen for the concentration of too concentrated, leading to the error. Therefore, we get the  $V$  and  $[S]$  a direct linear relationship, shown in Figure 3, can be seen from the figure, the curve of the linear relationship, and the fitness greater than the double reciprocal curves, so I chose it as a standard curve to calculate the enzyme hydrolysis of the protein content.

### 2.3 Determination of the protein content of milk samples with enzymatic hydrolysis method

Select 5 different brands of fresh milk samples by enzymatic hydrolysis measured the initial velocity, bring them into the linear equation  $y = 0.1177x$  ( $R^2 = 0.9927$ ), take biuret method as the comparison, measured the protein content of 5 samples, together with the results listed in Table 2.

From Table 2, using enzymatic hydrolysis method that milk A, B, E protein content smaller than the biuret method, while the milk C, D larger than biuret measured. Therefore, using the t-test tests them, and judge whether there is a significant difference between them. The results showed: calculated  $|t| = 0.625$ , critical  $t = 2.132$ , calculated  $|t| <$  critical  $t$  value, both measured no significant difference in the results. Moreover, the time of determination of enzyme hydrolysis method only about 8min, is required 1 / 20 by biuret method.

### 3. Conclusion

In this experiment, we use the degree of hydrolysis as an index to optimize the hydrolysis conditions of trypsin, the optimal pH and temperature were 7.5 and 55 °C. And we detected the initial velocity under different casein concentration with the pH-state method. Moreover we get the standard curve  $y = 0.1177x$  ( $R^2 = 0.9927$ ). In the experiment, we select casein as substrate, because the total casein in milk protein content of more than 80%, and the use of a broad-spectrum enzyme trypsin, which not only hydrolyzed casein in milk and also hydrolyzed some other proteins, such as lactoglobulin and so on. Using this method and the biuret method to detect the protein content of five different brands of fresh milk respectively, the results show that two methods basically consistent; t-test analysis showed no significant difference between them; and the time of former detect process only need 8 min which only is 1/20 of the latter.

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Table 1. Initial velocity of enzymatic under various casein content

Casein Content (mg/ml)	0	6.60	8.25	11.00	16.50	33.00
V(μl/s)	0	0.7845	1.0751	1.2512	2.1354	3.7728
RSD(‰)	0	9.0	11.4	12.6	12.8	17.2

Table 2. Comparison of the results based on the two methods

Sample Number	Sample Name	Enzyme Hydrolysis Method		Biuret Method	
		Protein Content (g/100ml)	RSD (‰)	Protein Content (g/100ml)	RSD (‰)
1	Milk A	3.13	12.8	3.31	12.4
2	Milk B	3.24	18.5	3.35	6.3
3	Milk C	3.42	10.2	3.28	18.3
4	Milk D	3.36	7.4	3.27	11.9
5	Milk E	3.28	9.1	3.41	9.1

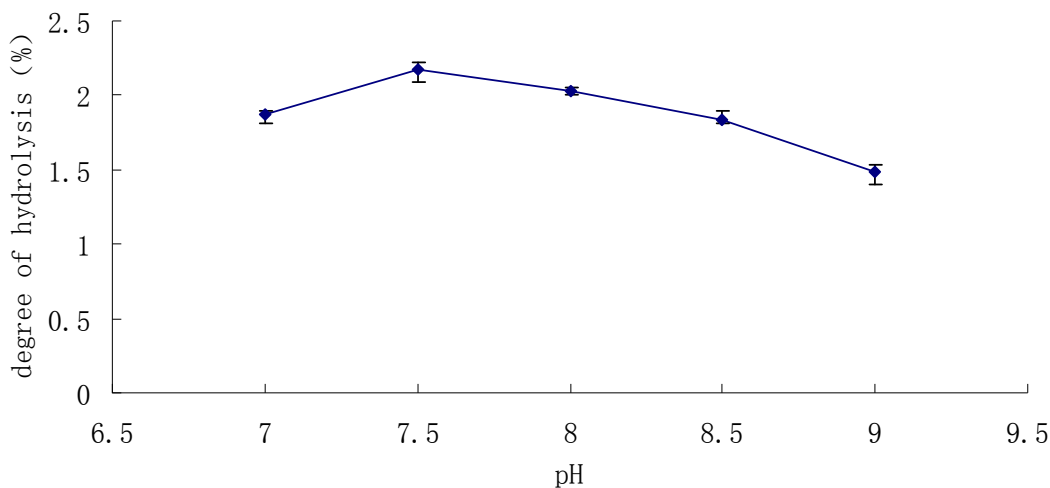


Figure 1. Influence of pH on the degree of casein hydrolysis

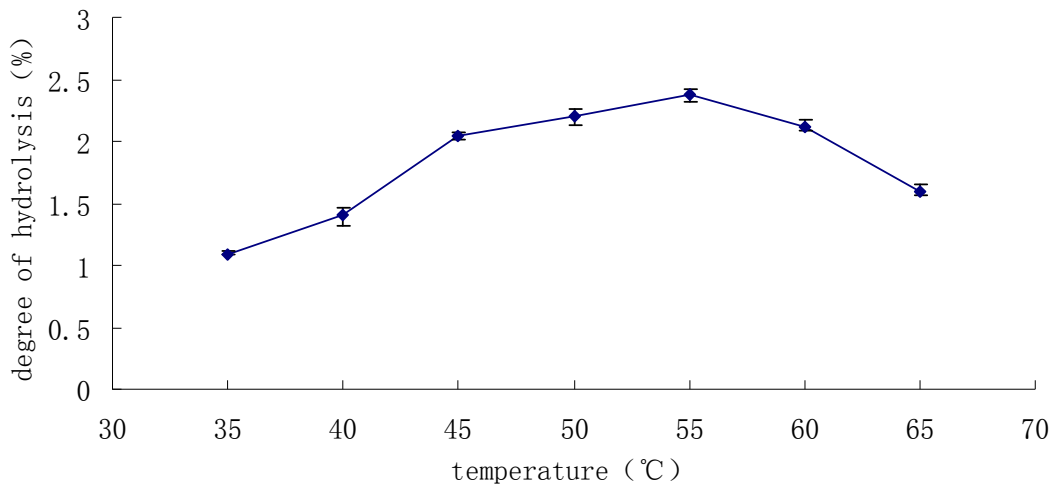


Figure 2. Influence of temperature on the degree of casein hydrolysis

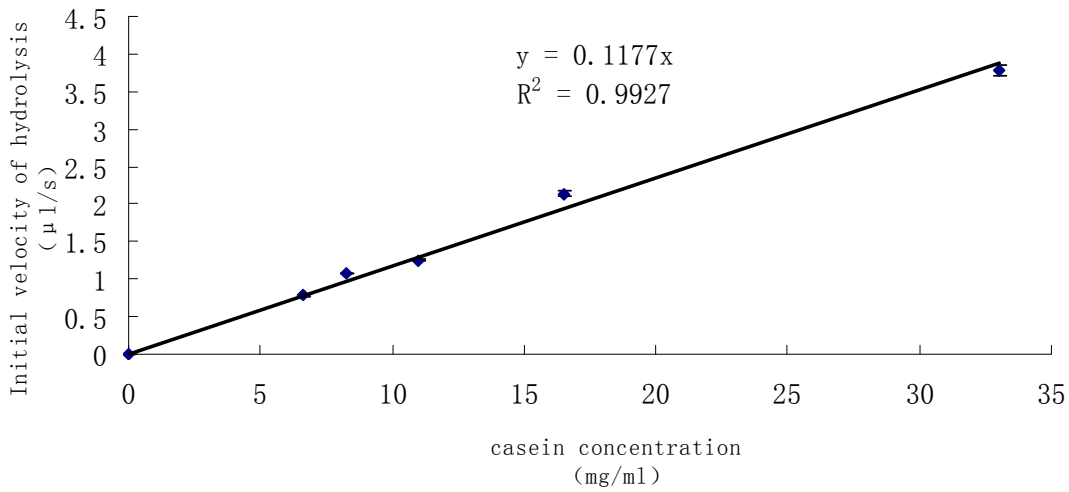


Figure 3. The relation between casein content and initial velocity of enzymatic