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# EFFECT OF TEMPERATURE, pH, ENZYME TO SUBSTRATE RATIO, SUBSTRATE CONCENTRATION AND TIME ON THE ANTIOXIDATIVE ACTIVITY OF HYDROLYSATES FROM GOAT MILK CASEIN BY ALCALASE

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**Abstract:** The effect of hydrolysis temperature (45, 50, 55, 60 and 65°C), pH (7.0, 7.5, 8.0, 8.5 and 9.0), enzyme to substrate (E/S) ratio (1.0, 1.5, 2.0, 2.5 and 3.0%), substrate concentration (2, 3, 4, 5 and 6%) and hydrolysis time (30 -240min) on antioxidant peptides hydrolysated from goat's milk casein by Alcalase was investigated using single factor experiment. In order to obtain high DPPH radical-scavenging activity, metal-chelating activity and superoxide radical scavenging activity, the optimal conditions were hydrolysis time of 150 min, temperature of 50°C, pH 8.0, E/S ratio of 2.0% and substrate concentration of 4.0%. The hydrolysis time, hydrolysis temperature, pH, E/S ratio and substrate concentration had a significant influence on degree of hydrolysis, metal-chelating activity, DPPH and superoxide radical scavenging activity on casein hydrolysate of goat milk by Alcalase, the results were beneficial for further provide theoretical basis for production of antioxidant peptides.

**Key words:** Alcalase, goat milk casein, enzymatic hydrolysis, antioxidative peptides

## INTRODUCTION

Reactive oxygen species in the organisms are produced by non-enzymatic

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and enzymatic reactions, and antioxidant enzymes and endogenous antioxidants in the body under synergy of has been removed (Murphy et al., 2011). But when the body is aging, illness or fatigue, the body's free radicals may be destroyed, and excess oxygen free radicals cause a series of oxidative damage to the body (Head, 2009; Kim et al., 2001). Excessive free radicals can cause irreversible damage to the body. It can cause damage to the body at molecular level, cell level and level of tissue and organs by attacking the life molecules and various kinds of cells (Dröge 2002).

Bioactive peptide refers to those peptides with special physiological function, these small peptides not only easy digestion and absorption, but also has a variety of human metabolism and physiological regulation function, such as the promotion of immunity, anti-bad thrombus peptide, antibacterial, lowering blood pressure, antioxidant, opioid antagonist activity, promoting in mineral absorption effect (Nagpal et al., 2011; Udenigwe, 2014). Antioxidant peptides as biological active peptide, have inhibition of lipid peroxidation effect, scavenging free radicals, maintain the balance of free radicals, and improve the body's aging and disease resistance functions. In the body, adequate antioxidant was intake and can not only reduce the content of free radicals, but also can improve the immunity and prevent lipid peroxidation (Lobo et al., 2010; Ramos et al., 2003).

Goat milk nutritional value is high, which the composition is similar to human milk and is called ‘the king of milk’ by the people (Park, 2007; Rafter, 2003). It has rich nutritional value and more than milk, which performance of protein, fat, vitamins and minerals (Haenlein 2004). In goat milk, casein expect for $\alpha_{s1}$-casein, the content of $\alpha_{s2}$ - casein, β- casein and κ- casein was higher than that of bovine milk (Alferez et al., 2006). $\alpha_{s1}$ - casein as the main allergens, which content in goat’s milk is far lower than milk, so drinking goat milk is not lead to allergic reactions (Hodgkinson et al., 2012).

In our previous work, the effect of five proteases on the antioxidative activity in goat's milk casein hydrolysate was studied and we found that Alcalase was the optimal proteinase (Shu et al., 2015). In the present research, the influence of temperature, time, temperature, pH, E/S ratio and substrate concentration on the antioxidative peptides activity and degree of hydrolysis in hydrolysate from goat milk casein by Alcalase was investigated by the single factor tests.

## MATERIALS AND METHODS

**Materials:** Goat milk was provided by Hongxing Dairy Co., Ltd. (Weinan, China). Alcalase, Tris-HCl, ferrozine and 1,1 – diphenyl – 2 - picrylhydrazyl (DPPH) were obtained from Xi’an Luosenbo Co., Ltd. (Xi’an, China), (St. Louis, MO, USA). The other chemicals were analytical pure unless otherwise

specified.

**Preparation of goat milk casein:** goat milk powder was added to the distilled water to obtain the reconstituted milk, and then centrifuged at 5000g for 15min. Then, adjust pH to 4.6 with 1M HCl (stirring intensity is 8000 rmp). Finally centrifuged at 6000×g of centrifugation for 15 min, and the precipitate of goat milk casein were freeze-dried.

**Preparation of antioxidative peptides:** goat milk casein was added to distilled water with 0.1M NaOH and stored in water bath. Temperature and pH values were adjusted to the best. In the hydrolysis process, hydrolysis pH was maintained at the prescribed value by continuously adding 0.1M NaOH. Hydrolysis was stopped by heating at 95℃ water bath for 10 min to made enzymes inactivation. The hydrolysate was centrifuged at 8000g for 15min after pH was regulated to 3.4. The supernatant was collected, regulated pH to 8.3, then the supernatant was used to determine the antioxidative activity by UV Spectrophotometer.

**Determination of degree hydrolysis (DH) for casein:** Determination of protein hydrolysis by pH-state (Adler-Nissen, 1986). The calculation formula of the hydrolysis is as follows:

$$DH\ (\%) = [(B \times M_b) / (\alpha \times M_p \times h_{tot})] \times 100\% \quad (1)$$

where (1), B is the consumption of NaOH (mL), $M_b$ is the concentration of NaOH, α is dissociation degree of α-amino (α=0.44), $M_p$ is the mass of protein, and $h_{tot}$ (8.2mmol/g) is the total number of peptide bonds in proteins.

**Determination of antioxidative activity in hydrolysate from goat milk casein**

**DPPH radical-scavenging activity:** The modified method of (Wu etal, 2003) was applied to determine DPPH radical-scavenging activity. That 2ml of hydrolysate sample was added to 2ml of 0.1 mM DPPH in 95% ethanol. Under the condition of no light and room temperature, the mixture was incubated with shaken vigorously for 30 min and the absorbance was measured at 517 nm. The calculation formula of the scavenging effect is as follows:

$$\text{DPPH radical-scavenging activity}\ (\%) = (A_0 - A_1 + A_2)/A_0 \times 100\% \quad (2)$$

In formula (2), $A_0$ is the absorbance of the control, $A_1$ is the absorbance of sample with DPPH solution, and $A_2$ is the absorbance value of the sample mixed to 95% ethanol not contained DPPH solution.

**Metal-chelating activity:** The method of (Decker and Welch, 1990) was applied to estimate the ability of chelating pro-oxidative $Fe^{2+}$ of casein. Each of these hydrolysis samples, including 0.2 ml of 5mM ferrozine, 0.1 ml of 2mM $FeCl_2$, 1 ml of hydroliysate sample and 3.7 ml of distilled water of the reaction, and added in sequence, then measured the absorbance at 562 nm

after 20 min. The calculation formula of the chelating activity is as follows:

$$\text{Chelating activity (\%)} = (A_2 - A_1)/A_2 \times 100\% \quad (3)$$

In the formula (3), $A_1$ is the absorbance value of sample and $A_2$ is the absorbance value of the control (the sample was replaced by distilled water).

**Superoxide radical scavenging activity:** The method of (Marklund and Marklund, 1974) was applied to measure the superoxide radical ($O_2^{\cdot -}$) scavenging activity. Mixed 5.6mL of 50mM Tris-HCl-(1mM) EDTA buffer (pH8.2) with 0.2 mL of hydroliysate sample. Instead of the sample solution, the distilled water was used as blank, then added 0.1mL of 5mM pyrogallol solution to the mixture. The reaction began with the addition of pyrogallol. The calculation formula of the capability for scavenging superoxide anion radicals is as follows:

$$\text{Scavenging activity of } O_2^{\cdot -}(\%) = [1 - (A_2/\text{min})/(A_1/\text{min})] \times 100\% \quad (4)$$

where $A_1$/min is the absorbance per minute of the control group comprising buffer and pyrogallol; $A_2$/min is the absorbance per minute of the samples.

## RESULTS AND DISCUSSIONS

### Effect of hydrolysis temperature on antioxidative activity in casein hydrolysates

The selected substrate concentration was 2%, E/S was 1%, pH= 8, under the condition of hydrolysis of 2 h; the result is shown in Figure 1. Within a certain range, the degree of hydrolysis increases with the increase of temperature. The DH reaches the maximum at 55℃, then the DH decreases when the temperature increases to a certain temperature.

According to Xiao (2006), the degree of hydrolysis of oxya chinensis protein by Alcalase had a maximum value at 55℃. This is because at temperature smaller than 55℃, the enzyme activity is lesser, and hydrolysis speed is low; when temperature reaches 55℃, peptide bond is easy to be bound, so hydrolysis speed increases; when the temperature is too high, the inactivation of enzymes decreases the hydrolysis speed.

The changes of antioxidant activity are not synchronized with the degree of hydrolysis. DPPH radical-scavenging activity and $O_2^{\cdot -}$radical scavenging activity reaches the maximum at 50℃, being 60.92% and 36.08%.

$Fe^{2+}$ chelating activity reaches the maximum (87.68%) at 55℃, 87.68%. But $Fe^{2+}$ chelating activity at 50℃ was nearly the same as that of $Fe^{2+}$ chelating activity at 55℃. In comparing the results comprehensively, 50℃ was chosen as the optimum temperature to hydrolyze casein. He (2008) also indicated that decapterus maruadsi protein hydrolysated with Alcalase has strong reducing power and hydroxyl radical scavenging capacity in 50 ℃, 0.640 and 56.58%, respectively.

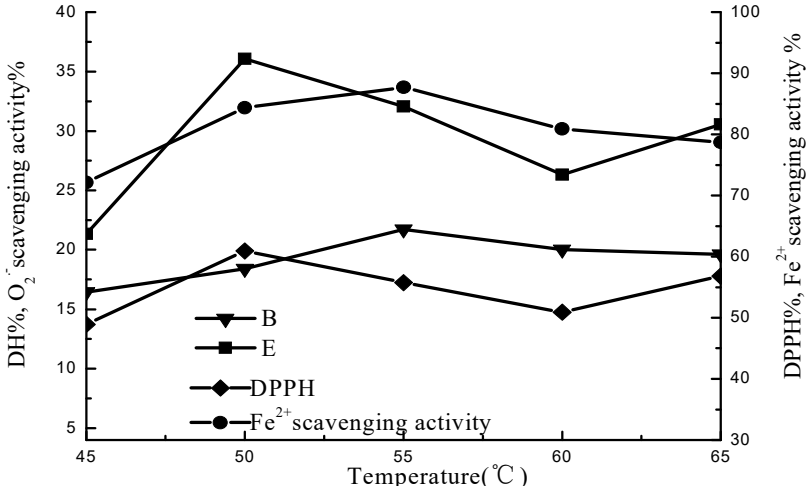


Figure 1. Effect of temperature on antioxidative peptides activity

**Effect of pH on on antioxidative activity in hydrolysates from goat milk casein by Alcalase**

When the selected substrate concentration is 2%, E/S is 1%, T = 50℃, under the condition of hydrolysis of 2 h, pH varies according to Figure 2.

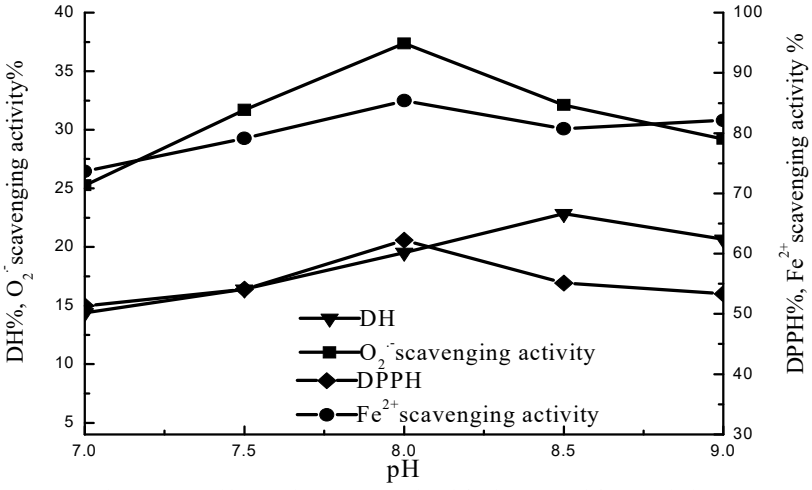


Figure 2. Effect of pH on antioxidative peptides activity

From the view of kinetics of enzyme reaction, the catalytic active site of an enzyme contains some charged amino acid side chain groups; these groups in combination with the conversion substrates and substrates for the product, which must be in a particular state of dissociation. The pH of the environment affects the conformation of enzyme protein and enzyme molecule and the substrate molecular dissociation state, so that is affecting

the stability of the enzyme, the combination of enzyme and substrate, and the conversion of enzyme catalysis substrates to the products, and then affect the catalytic effect of enzyme (Millán et al., 1999). Based on this, the influence of pH is important to be analysed.

The degree of hydrolysis was the maximum at pH of 8.5, 22.84%. Han and Ren (2003) found that corn protein hydrolysated with Alcalase had the maximum value in 8.5, 28.53%. Due to the different substrate, degree of hydrolysis was not the same. DPPH radical-scavenging activity, $O_2^{-}$radical scavenging activity and $Fe^{2+}$ chelating activity were the maximum at pH of 8.0, 62.22%, 85.36% and 37.38%. This may be because the generated peptides were degraded into inactive fragments or free amino acids, which leads to antioxidative peptides activity was reduced with increase of degree of hydrolysis. Therefore, the selection of the 8.0 was the optimum pH to carry out the next experiment. Zhang (2007) also reported that Alcalase of the hydrolysate had a strong antioxidant activity at pH of 8.0.

**Effect of substrate concentration on on antioxidative activity in hydrolysates from goat milk casein by Alcalase**

The selected pH was 8.0, E: S was 1%, T = 50℃, under the condition of hydrolysis of 2 h. The result was shown in Figure 3.

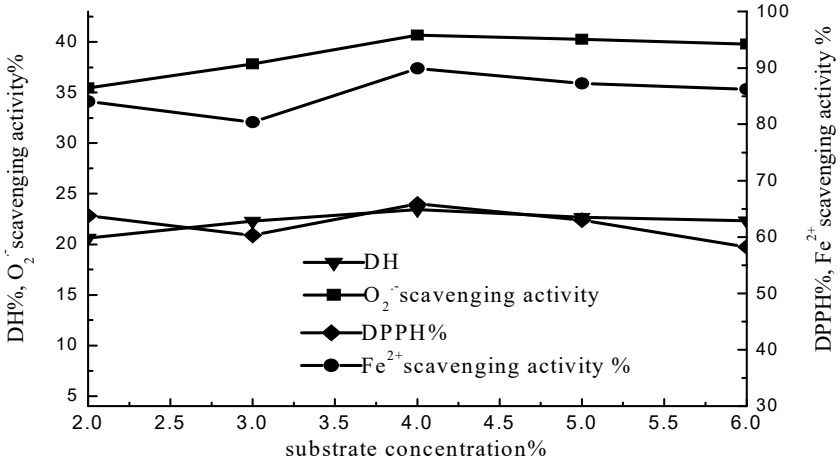


Figure 3. Effect of substrate concentration on antioxidative peptides activity

As shown in figure 3, the degree of hydrolysis increased with the increase of substrate concentration. The degree of hydrolysis reached the maximum at 4%, 23.44%. The degree of hydrolysis was slightly reduced When the substrate concentration above 4%. This is because the water play the role of reaction medium and transport in the enzymatic hydrolysis process, water

was conducive to molecular diffusion and movement in the reaction, so that the substrate and protease evenly distributed in order to better contact (Peng 2002). Water can make the product quickly dispersed, and prevent the high concentration of local products to inhibit the hydrolysis reaction, so that the degree of substrate concentration increased (Ferreira and Hultin, 1994).Antioxidative peptides activity change was roughly the trend of first increases and then decreases, reached the maximum at 4%. This shows that early hydrolysis, the increase of substrate concentration to promote the hydrolysis process, so the degree of hydrolysis and antioxidative peptides activity were increased. Therefore, 4% was chosen as the optimum substrate concentration to hydrolyze casein.

**Effect of E/S on on antioxidative activity in hydrolysates from goat milk casein by Alcalase**

The selected the pH was 8.0, substrate concentration was 4%, T = 50℃, under the condition of hydrolysis of 2 h. The result was shown in figure 4.

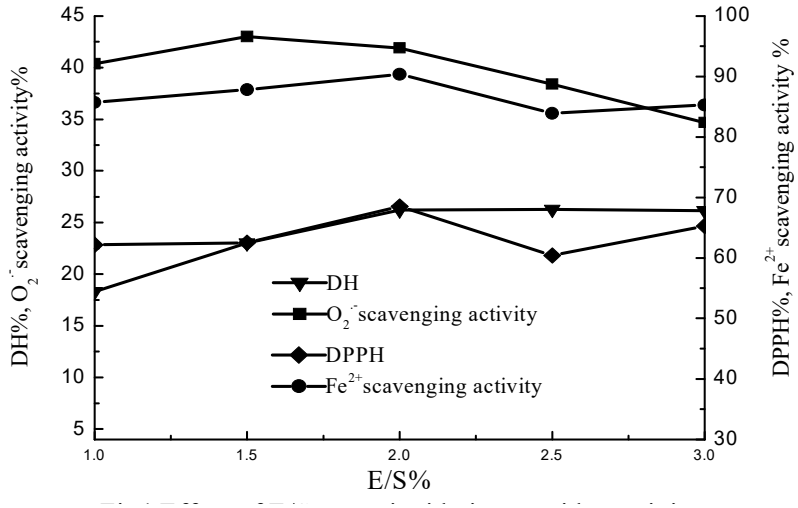


Figure 4. Effect of E/S on antioxidative peptides activity

The degree of hydrolysis reached the maximum at 2.5%. This was because when enzyme quantity was small, enzyme and substrate can completely combine with the substrate excessive, and degree of hydrolysis increased. When enzyme added amount was more than 2.5%, the substrate concentration was relatively small. Some enzymes can't combine with casein, leads to the degree of hydrolysis only a little change. The DPPH radical-scavenging activity and $Fe^{2+}$ chelating activity reached the maximum at 2%, 68.49% and 90.39%. $O_2^-$radical scavenging activity was reached the maximum at 1.5%, 43.01%. In comparing the results comprehensively, 2%

was chosen as the optimum E/S to hydrolyze casein. It showed that the antioxidant activity was increased with the increase of E/S in a certain range, after reaching a certain value, reduced with the increase of hydrolysis degree.

**Effect of hydrolysis time on antioxidative activity in casein hydrolysates**

The selected the pH was 8.0, substrate concentration was 4%, E/S was 2% and T = 50℃, under the condition of hydrolysis of 4 h, the result was shown in figure 5. DH of casein with the increase of hydrolysis time, the degree of hydrolysis was increased, and the degree of hydrolysis was no longer change after being hydrolyzed to a certain time. Because as the reaction progresses, the substrate concentration gradually decreases, the reaction site was saturated by enzyme molecules. And formed a strong competitive inhibition with product concentration gradually increased. The DPPH radical-scavenging activity and $O_2^{\cdot-}$ adical scavenging activity reached the maximum value at 150min, 71.75% and 46.13%. $Fe^{2+}$ chelating activity was the maximum at 90min, 90.18%. So, 150min was the optimum hydrolysis time.

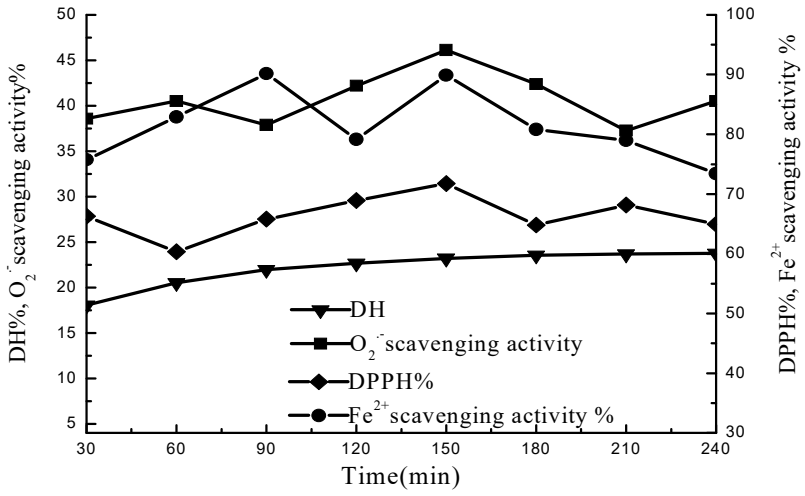


Figure 5. Effect of time on antioxidative peptides activity

## CONCLUSIONS

The temperature, pH, E/S ration, substrate concentration, hydrolysis time have a significant effect on the antioxidative activity of casein hydrolysates from goat milk by Alcalase. The optimal hydrolysis time, temperature, pH, E/S ration and substrate concentration were 150 min, 50℃, 8.0, 2.0% and 4.0% for the high degree of hydrolysis, metal-chelating activity, DPPH and superoxide radical scavenging activity.

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