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EFFECT OF FIVE PROTEASES INCLUDING ALCALASE, FLAVOURZYME, PAPAIN, PROTEINASE K AND TRYPSIN ON ANTIOXIDATIVE ACTIVITIES OF CASEIN HYDROLYSATE FROM GOAT MILK

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Abstract: Oxidation was related to the pathogenesis of human diseases. Adequate intake of antioxidant activity of food can reduce the levels of free radicals, prevent lipid peroxidation, and help the body against diseases. In the paper, casein from goat milk was hydrolyzed by five commercial proteases, namely, Alcalase, flavourzyme, papain, proteinase K and trypsin. The antioxidant activities of casein hydrolysates were assessed by evaluating hydrolysis degree, DPPH radical-scavenging activity, metal-chelating activity and superoxide radical scavenging activity. The results showed as follows: the DH of proteinase K, Alcalase, and trypsin were higher significantly than those of papain and flavourzyme. The Fe^{2+}-chelation activity and superoxide radical scavenging activity of casein hydrolysates from goat milk by Alcalase was higher than the others, the DPPH scavenging activities of casein hydrolysates by Alcalase and papain was higher than the others and the DPPH scavenging activities by Alcalase and papain had no significant diffierence ($p < 0.05$), so the optimal proteinase for hydrolysis casein from goat milk to produce antioxidant peptide was Alcalase.

Keywords: goat milk casein, Alcalase, enzymatic hydrolysis, antioxidative peptides

INTRODUCTION

In our body, appropriate concentrations of free radicals play an important role both in biological function and physiological effect (Poli et al., 2004). However, cells composition and some biological macromolecules damage was the result of excessive accumulation of free radicals attacking on lipids, proteins and nucleic acids, leading to cell death and the oxidation of tissue

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damaged when the body cells and removal mechanism of free radical were in imbalances(Wang et al., 2009; Butterfield et al., 2002). Studies confirm that oxidative stress caused by free radicals was associated with a variety of pathological conditions, such as cardiovascular disease, cancer, neurodegenerative diseases, diabetes, ischemia-reperfusion injury, aging and other diseases (Dalle-Donne et al., 2006; Dhalla et al., 2000; Jenner, 2003). The health of cells and tissues of humans under normal physiological conditions was maintained through a balance between antioxidation and oxidation (Lobo et al., 2010). The body's antioxidant enzymes neutralize these reactive species and avoid oxidation (Shinde et al., 2012). However, in some cases, the destruction of drugs antioxidant system caused by such as prolonged aerobic exercise, malnutrition and injuries, the body's defense system of antioxidant is not sufficient to removal of free radicals during metabolism process, which cause the excessive accumulation of free radicals in the body, lead to damage to the cells and tissues (Halliwell and Gutteridge, 1990).

Therefore, it is necessary to replenish the in vitro antioxidant to remove excess free radicals, alleviate damage caused by free radicals on the body (Halliwell and Gutteridge, 1990; Hettiarachchy et al., 1996; Ji, 1995) .The body's antioxidant obtained from in vitro according to its source can be divided into chemical synthetic antioxidants and natural antioxidant. Natural antioxidants due to its natural, small side effects or completely without any side effects (Jae et al., 2005).Therefore, in view of health reasons, people are increasingly tend to choose use of natural antioxidants. At present, people have already received a variety of biological active peptides, which through enzymolysis or fermentation from kinds of milk protein, soybean protein and corn protein, etc (Teng et al., 2005; Xu et al., 2007; Liu et al., 2006).Some bioactive peptides have realized industrialization as health foods and drugs, and achieved great economic benefits (Wang and Mejia, 2005). Therefore, the full use of the rich resources of proteins to developed good biological activity for human health and social development are of great significance.

Goat milk has rich nutrition, which was known as "the king of milk" in the world (Agnihotri and Prasad, 1993). Goat milk was recognized as the closest to human milk (Rafter, 2003). Compared with cow milk and human milk, the concentration of α_{s2}-casein, β-casein and κ- casein were found in goat milk were higher than in cow milk (Alferez et al., 2006; Kondylie et al., 2007), and α_{s1}-casein content is much lower than cow milk, which is as the main allergen, so goat milk does not cause allergic reactions (Jin, 1993). In this study, effect of five proteases including Alcalase, flavourzyme, papain, proteinase K and trypsin on antioxidative activities of casein hydrolysates from goat milk was investigated to provide reference for further optimization.

MATERIALS AND METHODS

Materials

Goat milk powder was brought from Hongxing Dairy Co., Ltd. (Weinan city, China). Alcalase, flavourzyme, papain and proteinase K bought from Sigma-Aldrich (St. Louis, MO, USA). Trypsin was provided by Luo Senbo Technology Co., Ltd. (Xi'an, China). 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-bis (4-phenylsulphonic acid)-1,2,4-triazine (ferrozine) and Tris-HCl were bought from Sigma-Aldrich. All other reagents used were of analytical grade.

Preparation of goat milk casein

Goat milk powder was mixed in distilled water at a ratio of 1:9 (w/v) , and subsequently centrifuged at 5000 × g for 15 min. Then, skim milk was heated at 45℃, and dropwised add 1M HCL with stirring intensity at 800 RPM until the pH is 4.6, make the casein precipitation down, after that centrifuged at 6400×g for 15 min, the supernatant was discarded and casein precipitation was freeze-dried.

Preparation of enzymatic hydrolysates

Freeze-dried casein obtained from goat milk was mixed in distilled water at a ratio of 2:100 (w/v), the proteins were hydrolyzed by five kinds of enzymes including Alcalase, flavourzyme, papain, proteinase K and Trypsin at an enzyme/substrate ratio of 1:100 (w/w). Temperature and pH of hydrolysis were adjusted to the optimal values for each enzyme: Alcalase, 55℃ and pH 8.0; flavourzyme, 50℃ and pH 6.0; papain, 50℃and pH 6.0; proteinase K, 60℃and pH 8.0; and trypsin, 37℃and pH 8.0. During the reaction, hydrolysis pH was maintained at the prescribed value by continuously adding 0.1M NaOH according to certain time interval and sampled 4ml. Hydrolysis were stoppde by heating at 95℃water bath for 15 min to ensure enzyme deactivation. Then pH was adjusted to 4.0, then the hydrolysates were centrifuged at 6500g for 15min, and the supernatant was collected. After that pH was adjusted to 8.0, and the hydrolysates were centrifuged at 6500g for 15min again, the supernatant was collected. Antioxidative peptides were determined using UV Spectrophotometer (shanghai metash instruments co.,ltd, Shanghai).

Measurement of protein hydrolysis

Protein hydrolysis was measured using the method of pH-state (Adler-Nissen, 1986). Casein was mixed in distilled water with help to dissolve in 0.1M NaOH. Temperature and pH were adjusted to the optimum values for each proteinases. The casein was hydrolyzed by the five proteinases mentioned above. During process of casein hydrolysis, hydrolysis pH was maintained at the prescribed value by continuously adding 0.1M NaOH, and the consumption of alkali liquor was recorded every 30min for 6h. The degree of

hydrolysis was quantified according to the following equation:

$$DH\% = [\ (B \times Mb)/(\alpha \times Mp \times h_{tot}\ )\ ] \times 100\%$$

where B represents the volume of NaOH (mL), Mb represents the concentration of 0.1M NaOH. α represents the degree of dissociation of α-amino(α=0.44). Mp represents the quality of protein. And h_{tot} represents the protein of the total number of peptide bonds (h_{tot}=8.2mmol/g).

Measurement of antioxidative activity

DPPH radical-scavenging activity

DPPH radical-scavenging activity was determined according to the method of (Wu *etal,* 2003) with slight modifications. Briefly, 2ml of hydrolysates sample was mixed with 2ml of 0.1 mM DPPH in 95% ethanol. The mixture was shaken thoroughly and incubated for 30 min at room temperature in the dark. The absorbance was determined wavelength of 517 nm. The scavenging effect was quantified according to the following equation:

DPPH radical-scavenging activity (%)=$[1-(A_1-A_2)/A_0]\times 100$

Where A_1represents the absorbance of the sample with DPPH solution, A_0 represents the absorbance of the control of the DPPH- ethanol solution and A_2 represents the absorbance of the sample added to 95% ethanol without DPPH solution.

Metal-chelating activity

The ability of chelating pro-oxidative Fe^{2+} of casein hydrolysates was determined using the method of (Decker and Welch, 1990). For each hydrolysates sample, the reaction mixture included 1 ml of hydrolysates sample, 3.7 ml of distilled water, 0.1 ml of 2mM $FeCl_2$, and 0.2 ml of 5mM ferrozine, added in sequence. The absorbance was measured at wavelength of 562 nm after 20 min. The chelating activity was quantified according to the following equation:

$$\text{Chelating activity (\%)} = [1 - (A_1/A_2)] \times 100$$

Where A_1 represents the absorbance of sample and A_2 represents the absorbance of the control (distilled water replaced the sample).

Superoxide radical scavenging activity

Superoxide radical (O_2^-) scavenging activity assay was measured according to the method of (Marklund and Marklund, 1974). 0.2 mL of hydrolysates sample was mixed with 5.6mL of 50mM Tris-HCl-EDTA buffer (1mM, pH8.2). The mixture was shaken thoroughly. Reaction mixture was incubated at 25℃ for 10 min. After incubation, 0.1mL of 5mM pyrogallol solution was added. Reaction was initiated by the addition of pyrogallol and using 0.2ml of distilled water instead of the sample as the control. Absorbance was determined at 325 nm wavelength and recorded every 30 s for 5 min. The

capability for scavenging superoxide anion radicals was quantified according to the following equation:

$$Scavenging\ activity\ of\ O_2^-(\%)=[(A_1/min-A_2/min)/(A_1/min)]\times 100$$

where A_1/min represents the absorbance per minute of the control solution containing pyrogallol and buffer, and A_2/min represents the absorbance per minute of the sample.

RESULTS AND DISCUSSIONS

Effects of five proteinases on degree of hydrolysis of casein from goat milk

The casein from goat milk was hydrolyzed for 5 h under the optimum reaction conditions by five proteinases including Alcalase, flavourzyme, papain, proteinase K and Trypsin, respectively. The degree of hydrolysis (DH) was calculated according to the pH - stat method and the results were as shown in Figure 1.

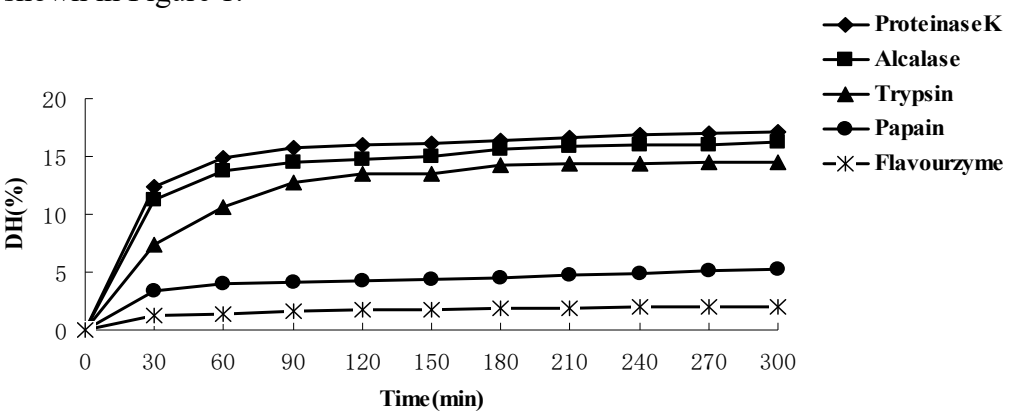

Fig1 hydrolysis curves for casein with 5 different proteases

With the increase of hydrolysis time, DH of casein from goat milk first increased quickly and then tended to remain unchanged to a certain time. Among then, the degree of hydrolysis of proteinase K was the maximum and lavourzyme was the minimum, the DH of proteinase K, Alcalase, and trypsin (all more than 10%) was higher significantly than those of papain and flavourzyme (both less than 5%). The order of hydrolysis rate is as follows: proteinase K＞Alcalase＞trypsin＞papain＞flavourzyme.

Effects of five proteinases on DPPH radical-scavenging activity of casein hydrolysates from goat milk

DPPH is a stable free radical which was synthesized and had an unpaired

valence electron. It can be characterized by a deep purple color, with absorbance in the range of 517 nm when dissolved in ethanol. If DPPH radicals encounter a proton-donating substrate, the radicals would be scavenged (Sánchez-Moreno, 2002). The figure 2 showed that the DPPH clearance rate of five proteinases was not like the degree of hydrolysis has a unified variation with the hydrolysis time increasing. The DPPH scavenging activities of Alcalase and flavourzyme reached the maximum value at 150 min. Proteinase K and trypsin reached the maximum value at 180min. Papain reached the maximum value at 90min. Among then, the DPPH scavenging activities of papain was maximum.

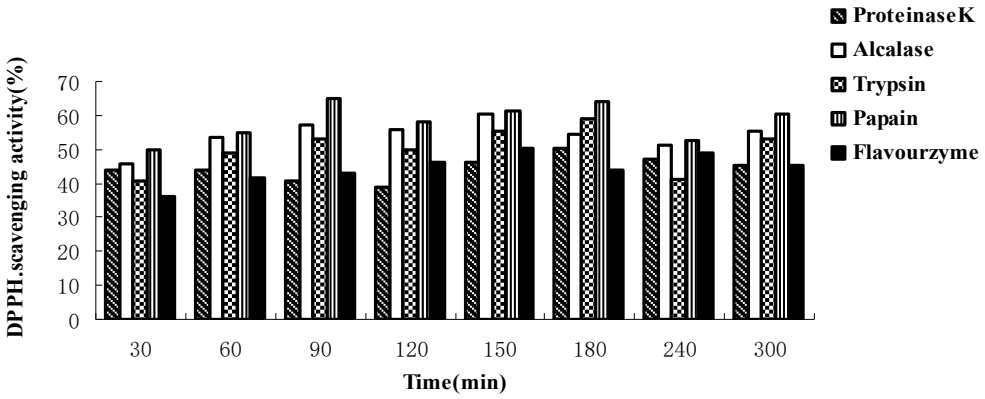

Fig2 DPPH. scavenging activity of casein at different enzymatic hydrolysates

Effects of five proteinases on O_2^{-}·scavenging activity of casein hydrolysates from goat milk

Superoxide radical was a biologically unstable and venenous species. It cannot directly initiate lipid oxidation, and was normally formed first as potential precursor of other kinds of free radicals and oxidizing agents, superoxide radical and its derivatives leads to cell or tissue injury (Richmond *et al*., 1981). So O_2^{-}·scavenging activity as an important indicator of antioxidant. As shown in figure 3, Papain and Alcalase were higher on superoxide radical scavenging activity. Proteinase K reached the maximum value at 120 min, 31.18%. Papain, Alcalase and trypsin reached the maximum value at 180min, 35.96%, 37.51% and 30.06%, respectively. Flavourzyme reached the maximum value at 150min, 28.53%. The casein hydrolysates by Alcalase exhibited a stronger scavenging activity than the others.

Effects of five proteinases on Fe^{2+}-chelation activity of casein hydrolysates from goat milk

Transition metal ions was very strong free radical agent, which can catalytic

various of reactive oxygen species,such as hydroxyl radical and superoxide anion (Saiga, *etal*, 2003).The presence of metal ions in order to capture free radicals was quickly consumed, so the chelation of metal ions contributes to antioxidation. As shown in figure 4, metal ions chelation was increased with the hydrolysis time increasing.

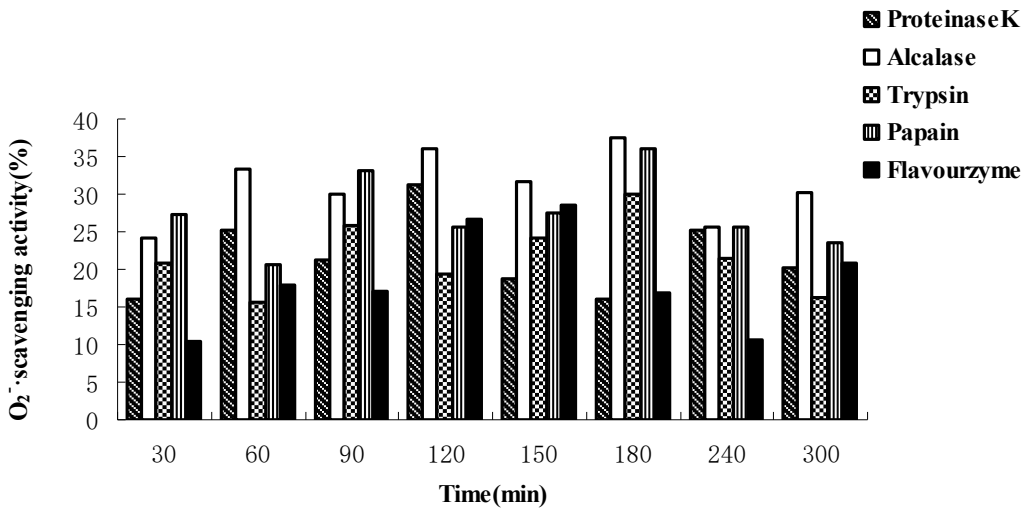

Fig3 O_2^-· scavenging activity of casein at different enymatic hydrolysates

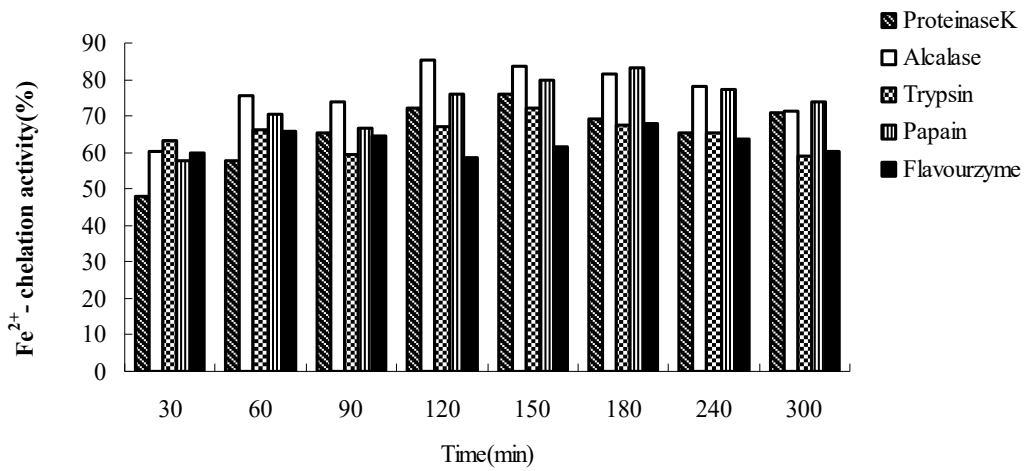

Fig4 Fe^{2+}-chelation activity of casein at different enzymatic hydrolysates

The casein hydrolysates by Alcalace reached the maximum value at 120 min, 85.31%.The casein hydrolysates by proteinase K and trypsin reached the maximum value at 150min, 75.80% and 72.16%，respectively. Papain and flavourzyme reached the maximum value at 180min, 83.29 and 68.02%. Continue to increase the hydrolysis time, metal chelating ability gradually decline. The Fe^{2+}-chelation ability of casein hydrolysates by Alcalace was

higher than other enzymes

CONCLUSIONS

The five proteinase including Alcalase, flavourzyme, papain, proteinase K and trypsin had significant effect on the DH of casein from goat milk, the DH of proteinase K, Alcalase, and trypsin was higher significantly than those of papain and flavourzyme. The Fe^{2+}-chelation activity and superoxide radical scavenging activity of casein hydrolysates from goat milk by Alcalase at 120min were higher than those of the other four proteinases, the DPPH scavenging activities of casein hydrolysates from goat milk by Alcalase and papain was higher than those of the other three proteinases and the DPPH scavenging activities by Alcalase and papain had no significant diffierence ($p < 0.05$), so the optimal proteinase for hydrolysis casein from goat milk to produce antioxidant peptide was Alcalase.

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