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10.1515/aucft-2016-0006

COMPARISON OF ACE INHIBITORY ACTIVITY IN SKIMMED GOAT AND COW MILK HYDROLYZED BY ALCALASE, FLAVOURZYME, NEUTRAL PROTEASE AND PROTEINASE K

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Abstract: Angiotensin I converting enzyme (ACE) inhibitory peptides derived from milk proteins have obvious effect of lowering blood pressure, safe and non-toxic side effects. This study compared four commercial proteases, namely alcalase, flavourzyme, neutral protease and proteinase K for their ACE inhibitory activity in skimmed goat and cow milk and identified the best one with higher ACE inhibitory activity. The degree of hydrolysis (DH) of alcalase and proteinase K were much higher than flavourzyme, neutral protease for both skimmed goat and cow milk. Alcalase was the best enzyme to produce ACE inhibitory peptides from goat milk, with the ACE inhibitory activity 95.31%, while proteinase K was the optimal protease for hydrolyzing cow milk, with 81.28% ACE inhibitory activity. Furthermore, no correlation was obtained between the ACE inhibitory activity and DH for both goat and cow milk.

Keywords: goat and cow milk, enzymatic hydrolysis, ACE inhibitory peptides

INTRODUCTION

Hypertension is a common disease which is related to the incidence of coronary heart disease, and the treatment of it could reduce the cardiovascular and related diseases (Collins et al., 1990). Angiotensin converting enzyme is a dipeptide hydrolase that catalyses formation of angiotensin-II and deactivate

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soothing peptide which results in increase of blood pressure. Hence, substances such as natural ACE inhibitory peptides can give a decrease of blood pressure by inhibiting ACE activity. In addition, ACE inhibitory peptides are also not easy to release from the ACE binding region. Many peptides had been extracted from different food proteins (Ariyoshi 1993; Dziuba et al., 1999), including algae proteins (I-Chuan et al., 2009), canola meal (Wu et al., 2009) and the tuna back bone (Lee et al., 2010). These biologically active peptides have the advantages of being natural and healthier in comparison to traditional antihypertensive drugs.

Recently, a variety of ways for producing ACE inhibitory peptides had been obtained including chemical synthesis, recombinant DNA technology, extract from natural foods, enzymatic hydrolysis and fermentation by lactic acid bacteria (Aluko, 2015). Among them, ACE inhibitory peptides from food proteins by enzymatic hydrolysis method has the characteristics of low cost, high safety of products, no side effects, easy to be absorbed and only acts on patients with hypertension (Bougatef et al., 2008; Han et al., 2008).

Many evidences have approved that milk protein not only have energetic and nutritional functions, but also can act as physiological regulators (Schanbacher et al., 1998; Tome & Debabbi, 1998). Most studies are concentrated on casein or whey protein to produce ACE inhibitory peptides; the ACE inhibitory peptides can be released within sequence of the parent protein and are hydrolyzed by enzymes or by food processing (Korhonen et al., 2006). Thus, screening for proper proteases to hydrolyze the protein is a key factor to obtain high activity ACE inhibitory peptides.

Because they are different cleavage sites of proteases for peptide bond and different amino acids composition between goat and cow milk, the proteases may hydrolyze milk from different amino acid sites and produce peptides with different composition and activity. Choose of proper protease is important for further development of active functional foods and provide theoretical basis for its industrial production. This research has as goal to test four proteases in order to choose the proper enzyme for its potential to hydrolysis goat or cow milk with high ACE inhibitory activity, and to make a comparison whether there are differences for ACE inhibitory activity between goat and cow milk.

MATERIALS AND METHODS

Reagents: Skimmed goat and cow milk were purchased from Fonterra & Anchor, New Zealand. Hippuryl-histidyl-leucine (HHL) and ACE all obtained from Sigma Chemical Co. Ltd. Alcalase, flavourzyme, neutral

protease and proteinase K were all bought from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of enzymatic hydrolysates: Skimmed goat and cow milk were mixed with distilled water at a ratio of 5:100 (w/v), respectively, and the proteins were hydrolyzed by four proteases: alcalase, flavourzyme, neutral protease and proteinase K, at an enzyme/substrate ratio of 5:100 (w/w). The reaction was performed with the four proteases under conditions of their optimum temperature and pH, which are: alcalase, 55℃and pH 8.5; flavourzyme, 50℃ and pH 6.0; neutral protease, 65℃and pH 6.5; proteinase K, 65℃and pH 8.0, respectively. Because pH of the solution decreases continuously with the extension of the reaction time, 0.10 mol/L NaOH was added into the solution to maintain the optimum pH of each protease; the consumption of NaOH was recorded. After the enzymatic reaction, the samples were heated in water bath at 90 for 15 min to make sure enzymatic hydrolysis reaction was stopped. The skimmed goat and cow milk were adjusted to pH 3.4-3.6 with 1mol/L HCl, the hydrolysates were centrifuged at 8000g for 15 min and then the supernatant was collected. After that, the obtained acidic supernatants were adjusted to pH 8.3, and the supernatant was collected after centrifugation once again. The enzymatic hydrolysis experiments were performed around 5-6 h until the hydrolysis reaction finished (when the pH of samples was no longer changed). The pH of skimmed goat and cow milk were measured by a pH-meter.

Assay for degree of hydrolysis and ACE inhibitory activity

Protein hydrolysis was determined using the method of pH-state (Adler-Nissen, 1986) with some modifications. The study of present work use four proteases which directly hydrolyze skimmed milk which containing whey and casein protein. Thus the parameter of equation (1) was determined according to ratio of two kinds of proteins and the ratio of whey and casein protein for skimmed goat and cow milk were 25:75, 15:85, respectively. The degree of hydrolysis (DH) was measured according to the following equation:

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

where B represents the volume of NaOH (mL); M_b represents the concentration of 0.1 mol/L NaOH; α represents the degree of dissociation of α-amino (α=0.442); M_p represents the quality of protein; h_{tot} is the protein of the total number of peptide bonds (h_{tot} for goat and cow milk were 8.35 mmol/g and 8.29 mmol/g, respectively).

ACE inhibitory activity was determined based on the method of the spectrophotometric assay (Cushman & Cheung, 1971; Guo et al., 2009). ACE inhibitory activity of skimmed milk was measured at 228 nm and determined according to the following equation:

$$\text{ACE inhibitory activity (\%)} = (b\text{-}a)/(b\text{-}c) \times 100\% \qquad (2)$$

where *a* represents absorbance with samples, for HHL and ACE reacted at simultaneously (Sample group); *b* represents absorbance with the sample added after HHL and ACE having reacted (Control group); *c* represents absorbance with ACE inactivation before the reaction (Blank group).

RESULTS AND DISCUSSIONS

Effects of four proteases on degree of hydrolysis for goat and cow milk

The skimmed goat and cow milk were hydrolyzed for 5-6 h under their optimum temperatures and pH by four proteases including alcalase, flavourzyme, neutral protease and proteinase K, respectively. The DH of skimmed goat and cow milk are described in Figure1a and Figure 1b, respectively.

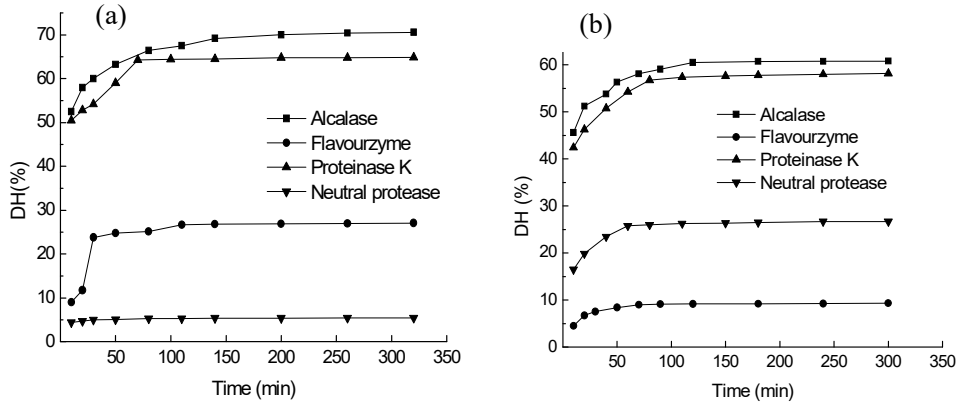

Figure 1. Degree of hydrolysis for skimmed milk with four proteases: (a) for goat milk and (b) for cow milk

As is shown in Figure 1, the DH for the skimmed goat and cow milk are first increasing quickly and then remain nearly unchanged with the increase of hydrolysis time. This result may be obtained because the enzyme no longer acts on skimmed milk and the hydrolysis reaction achieves its maximum value. The DH of alcalase and proteinase K are much higher than those of flavourzyme, neutral protease for both goat and cow milk. Besides, alcalase is the best protease to hydrolyze skimmed milk with the highest degree of hydrolysis among the four proteases. The degree of hydrolysis for neutral protease is no more than 6% in goat milk, and the degree of hydrolysis for flavourzyme is no more than 10% in cow milk. The order of hydrolysis rate for goat milk is as follows: alcalase＞proteinase K＞flavourzyme＞neutral

protease. And the cow milk is as follows: alcalase＞proteinase K＞neutral protease＞flavourzyme.

The DH of goat milk is higher than that of cow milk except under the action of neutral protease as shown in Figure1a and Figure1b. At the initial stage of protease hydrolysis (within first hour), hydrolysis is very fast because the number of peptide bond is high, and they are sensible and break fast. With the extension of hydrolysis time, hydrolysis speed gradually decreased because peptide bond reduces and the enzymatic action of substrate proteins reaches saturation state. Thus, long hydrolysis time cannot improve the ACE inhibitory activity and it is not conducing to the production of ACE inhibitory peptides.

Effects of four proteases on ACE inhibitory activity for skimmed goat and cow milk

The results of ACE inhibitory activity for skimmed goat and cow milk under the same conditions which was hydrolyzed by different proteases at the optimum temperature and pH are shown in Figure 2. It could be seen that the change trends of ACE inhibitory activity is unstable and chaotic for both skimmed goat and cow milk. Alcalase is the best enzyme to produce ACE inhibitory peptides for goat milk with the ACE inhibitory activity is 95.31%. It is consistent with Anne's (Anne, 2001) study showing that the alkaline protease is the optimum enzyme to produce ACE inhibitory peptides for its fast hydrolysis speed and high vitality. Li et al (Li et al., 2005) tested five proteases (alcalase, papain, pepsin, neutrase and trypsin) and found that alcalase was the suitable protease for its higher ACE inhibitory activity. Similarly, the ACE inhibitory activity obtained by alkaline protease hydrolysis gelatin was the highest (Kim et al., 2001). Alcalase belongs to one of the alkaline protease and it is an ideal enzyme to meet requirements of industrial production, while for skimmed cow milk, proteinase K is the optimal protease with the ACE inhibitory activity 81.28%; this result may be due to the proportion of casein and whey protein, which is different in goat and cow milk. Although the alcalase is the optimal protease for cow milk with biggest DH, ACE inhibitory activity is the key factor to measure protease's ability of producing ACE inhibitory peptides. Besides, there is no significant difference in DH of skimmed cow milk between alcalase and proteinase K. Thus, proteinase K is the optimal protease for cow milk to produce ACE inhibitory peptides.

The highest ACE inhibitory activity for flavourzyme, proteinase K and neutral protease in skimmed goat milk were 51.56%, 81.25% and 65.63%, respectively. And the highest ACE inhibitory activity for flavourzyme, alcalase and neutral protease in skimmed cow milk were 60.34%, 81.08% and

44.58%, respectively. Proteinase K and alcalase are both good proteases to hydrolyze skimmed goat and cow milk in order to obtain high activity ACE inhibitory peptides. Alcalase was used to hydrolyze casein of skimmed goat milk and ACE inhibitory activity reached at 89.09% (Chen et al., 2013), which was lower than the present study (95.31%). These could be explained that the alcalase also acts on the whey proteins and produces ACE inhibitory peptides. ACE inhibitory activity is no more than 22% except beginning 20 min for neutral protease in skimmed cow milk.

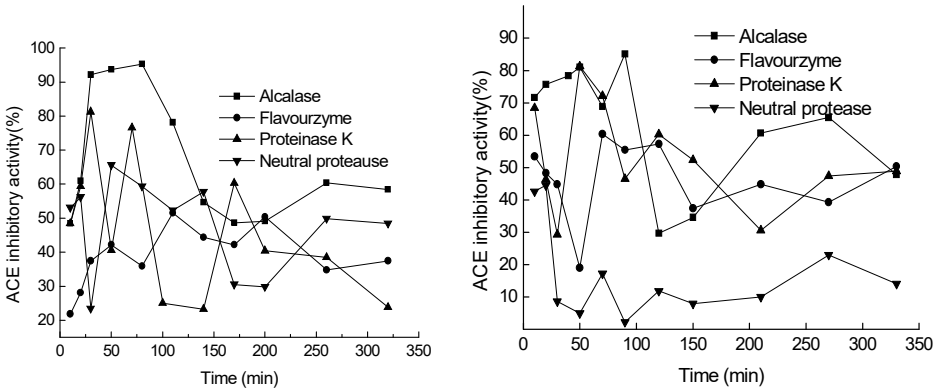

Figure 2. ACE inhibitory activity for skimmed milk with four proteases: (a) for goat milk and (b) for cow milk

The relationship between the degree of hydrolysis and ACE inhibitory activity

There is no correlation between the degree of hydrolysis and ACE inhibitory activity for both skimmed goat and cow milk as shown in Figure 1 and Figure 2. It can be seen from the ACE inhibitory peptides or analogues obtained previously that ACE is a enzyme with a relatively wide class of specificity. This is due to the mechanism of enzyme action on the substrate is very complex. ACE inhibitory peptides will generate by enzymatic hydrolysis in a certain degree, while further hydrolysis is likely to inhibit the sequence of ACE degradation of the stomach. Hence, the ACE inhibitory activity is reduced. Of course, there is another chance that the sequence with a higher ACE inhibitory activity is likely to expose. The formation of high active ACE inhibitory peptides should be the results of the two reactions reach the best degree. Mullally's (Mullally et al., 1997) used PTN 3.0S protease to produce ACE inhibitory peptides by hydrolyzing whey protein and found that there is no clear relationship between ACE inhibitory activity and DH, and activity of ACE inhibition peptides almost unchanged with the degree of hydrolysis increased. Lv's (Lv et al., 2003) study had drawn the same conclusion, the ACE inhibitory activity had no correlation with the DH.

CONCLUSIONS

The four proteases tested in this study, namely alcalase, flavourzyme, neutral protease and proteinase K had significant effects on ACE inhibitory activity and the DH for skimmed goat and cow milk. The DH of alcalase and proteinase K were much higher than those of flavourzyme, neutral protease for both goat and cow milk. Long hydrolysis time cannot improve the inhibitory activity and it is not conducing to the production of ACE inhibitory peptides. The optimal protease for skimmed goat and cow milk to produce ACE inhibitory peptides with high activity is alcalase and proteinase K, respectively. There is no clear relationship between the DH and ACE inhibitory activity for both skimmed goat and cow milk.

ACKNOWLEDGEMENTS
The work was partly supported by the Science and Technology Overall Planning for innovation Engineering project of Shaanxi Province (No. 2016KTZDNY02-08), the Science and Technology Bureau of Weiyang District (No.201507), Shaanxi Innovation and Transformation Project of Agricultural Science and Technology (No.NYKJ-2015-004).

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