As. J. Food Ag-Ind. **2009**, 2(04), 167-174

OPEN ACCESS

Asian Journal of Food and Agro-Industry

ISSN 1906-3040

Available online at www.ajofai.info

Research Article

Production of fermented milk high in activity of angiotensin converting enzyme inhibition by extending fermentation time and protease addition

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This paper was originally presented at Food Innovation Asia 2009, Bangkok, Thailand.
Received 19 June 2009, Revised 3 December 2009, Accepted 8 December 2009.

Abstract

Milk was fermented for 24 h with or without maintaining pH. For the sample that maintained pH, milk was fermented with and without protease**.** Starter cultures proliferated until completing fermentation (15 log CFU/ml) in the samples made without protease. In the protease added sample, starter culture reached 12 log CFU/ml (6 h) and declined afterward. Free amino groups and % ACE inhibition in the protease added sample were 389 mg/ml and 92% (6 h), respectively. Those samples made without protease had only 32 mg/ml and 88% after 24 h fermentation. The added protease is effective in increasing % ACE inhibition.

Keywords: dairy, angiotensin converting enzyme (ACE), fermented milk, **f**ermentation time, protease, proteolysis, Thailand.

Introduction

Milk fermentation has been shown to be a successful strategy to produce antihypertensive peptides or angiotensin-converting enzyme (ACE) inhibitory peptides. These ACE-inhibitory peptides inhibit ACE (EC 3.4.15.1), which regulates an increase of blood pressure [1]. The first fermented milk with documented antihypertensive activity to help lower blood pressure was marketed by the Japanese Calpis company [2].

Generally, the activity of ACE-inhibition found in dairy products was low compared with that of synthetic drugs, for example, Captopril [3]. Tsai *et al* [4], reported that extending fermentation time can increase the activity of ACE inhibition. The use of proteolytic enzymes (trypsin, pepsin and thermolysin) could be another effective strategy [5, 6]. In addition, the combination of both lactic acid bacteria and proteolytic enzymes in milk fermentation has been shown to give higher ACE-inhibitory activity than the use of either lactic acid bacteria or proteolytic enzymes alone [4, 6]. However, in these studies, the pH of fermented milk was not controlled. This may cause unsuitable pH conditions for the optimum growth and proteolytic activity of lactic acid bacteria and proteolytic enzyme used.

The purpose of this study was to investigate the combination of extending fermentation time and the use of protease on the activity of ACE inhibition in fermented milk made with controlled pH.

Materials and Methods

Bacterial strains

Streptococcus thermophilus TISTR 458 and *Lactobacillus delbrueckii* subsp. *bulgaricus* TISTR 892 strains were obtained from Thailand Institute of Scientific and Technological Research (TISTR), Bangkok, Thailand.

Production of fermented milk

Three fermenting conditions were used in this study. These included the fermentation that maintained pH at 5.0 made with and without adding protease (from *Aspergillus oryzae*, Sigma activity labeled 500 LAPU/g) at ratio 100:0.3 (v/v) of milk to protease. In addition, milk was fermented without maintaining pH and adding protease. Two litres of milk were fermented in 5 L fermenter (Biostat B. B, Brown) by using 2% (v/v) each starter culture at 42°C for 24 h. The agitation was set at 150 rpm. Sodium bicarbonate (1 N) was used in maintaining pH. The protease was filtered through 0.45 μm prior to addition. The samples were taken every 3 h. First sample was mixed 2 min for homogeneity.

Growth of starter cultures

The colony counts of *L. delbrueckii* subsp. *bulgaricus* was enumerated using the pour plate technique in deMan Rogosa and Sharpe (MRS) broth (Himedia) and incubated anaerobically at 42°C for 48 h. *S. thermophilus* was enumerated in M17 broth (Oxoid), incubated aerobically at 37°C for 48 h.

Determination of free amino groups

Samples (2.5 ml) were mixed with 5 ml of 0.75% trichloroacetic acid. The mixture was centrifuged at 6000 rpm for 10 min. The supernatant was used to determine the proteolysis of proteins during fermentation using *o*-phthaldialdelyde (OPA) method of Zahar *et al* [7].

Determination of ACE-inhibitory activity

Ten ml of sample was centrifuged at 6000 rpm for 20 min. The pH of supernatant was adjusted to 8.3 using 10 M NaOH, prior to measurement of the ACE-inhibitory activity according to the method of Cushman and Cheung [8] with some modifications of Donkor *et al* [9].

Results and Discussion

Growth of starter cultures

Cell counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* as well as pH profiles of fermented milk made without adding protease and maintaining pH are shown in Figure 1a. There was 8 log CFU/ml of both starter cultures at the beginning of fermentation. After 12 h fermentation, the cell counts of *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* increased to 11 and 12 log CFU/ml, respectively and the pH decreased to 4.6. At the end of fermentation, *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* grew to 15 and 16 log CFU/ml, respectively, and the pH further declined to 4.0.

Generally, *L. delbrueckii* subsp. *bulgaricus* can tolerate low pH condition better than *S. thermophilus* [10]. However, this conclusion is drawn from experiments commonly done in minimal media, as well as using only a single bacterial strain. Interestingly, in our study, *S. thermophilus* grew continuously even at pH below 4.6 (Fig. 1a). This may be due to the combined effects of using milk as fermentation medium and the use of mixed starter cultures. Derzelle *et al* [11], found that *S. thermophilus* adapted to milk used as fermentation medium by producing several enzymes involved in amino acid biosynthesis. In addition, *L. delbrueckii* subsp. *bulgaricus* enhances growth of *S. thermophilus* by creating small peptides as well as amino acids [12].

Similar growth pattern was observed in fermented milk made with maintaining pH (pH 5.0) without adding protease (Fig. 1b). However, the difference in cell counts between both starter cultures was less than that observed in fermented milk made without adding protease and maintaining pH. Fermentation at pH 5.0 may be a suitable condition for proliferation of both microorganisms.

The use of protease affected growth of both starter cultures in fermented milk made with maintaining pH (Fig. 1c). Both starters grew quickly to the maximum cell counts, 13 log CFU/ml at 6 h, then it slowly decreased to 10 log CFU/ml at 24 h.

To examine whether the result was due to the added protease, another fermentation trial was conducted by using heat inactivated protease (data not shown). It was found that the growth pattern of both starters was similar to that of fermented milk made without protease addition (Fig. 1b). This confirms that the added protease have an influence on growth of starter cultures. Gomes, *et al* [13], found that the addition of milk hydrolyzates to milk enhanced growth of *Bifidobacteria*. It might be possible that the added protease releases peptides from milk protein promoting rapid growth of both starters during the first 6 h. Nevertheless, the result after 6 h fermentation was unexpected. As best as is known, there has been no report on the effect of protease from *Aspergillus* ssp. on growth of bacteria. It might be possible that the added enzymes digest important proteins on the cell surface resulting in the inactivation of starters.

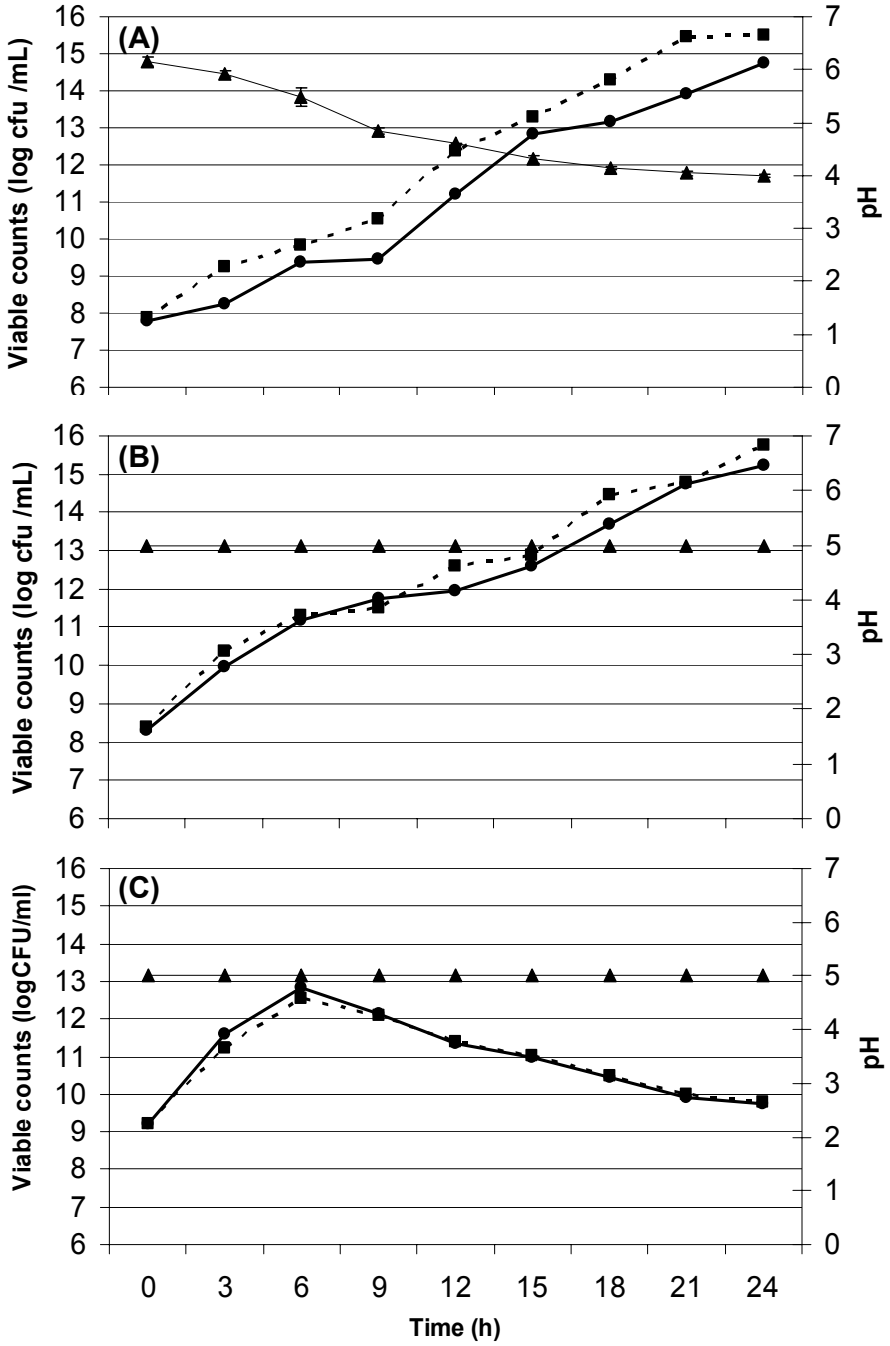

Figure 1. Cell counts and pH (▲) of fermented milk using *S. thermophilus* (●)and *L. delbrueckii* subsp. *bulgaricus* (■) as starter cultures. The fermentation without maintaining pH (A). The fermentation maintaining pH at 5.0 without adding protease (B) and the fermentation maintained pH at 5.0 with adding protease (C).

Free amino acid groups

The quantity of free amino acid groups representing degrees of proteolysis is shown in Figure 2. For samples made without protease addition (both maintaining and non-maintaining pH), the free amino groups increased slightly from 16 at the beginning to 32 mg/ml at the end of fermentation. This means that the maintenance of pH at 5.0 did not increase proteolytic activity

by starter cultures. Similarly, the growth pattern of starter cultures could be partly responsible for this result. *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus* have been reported to possess cell-surface proteinases [12]. However, the free amino groups found were very low compared with those of the sample made with protease addition.

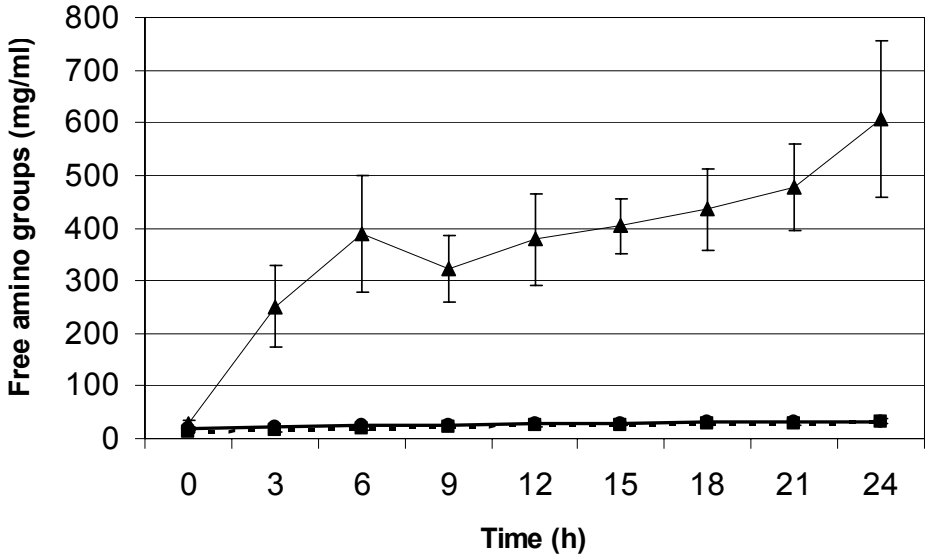

Figure 2. Free amino groups of the fermentation without maintaining pH (●) The fermentation maintaining pH at 5.0 without adding protease (■) and the fermentation maintaining pH at 5.0 with adding protease (▲).

When the protease was used, there was a rapid increase in free amino groups from 29 to 389 mg/ml during 6 h fermentation. Thereafter, free amino groups continued to increase at a decelerating rate. At the end of fermentation, there was 606 mg/ml of free amino groups, which is 19 times higher than that of samples made without protease addition. This may suggest that the majority of proteolytic activity is due to the added protease. Similar trends were observed by Chen, *et al* [14], and Tsai, *et al* [6]. These researchers investigated effects of protease facilitated lactic fermentation on proteolysis and ACE inhibitory peptides formation. Although another proteolysis determining technique was used, Chen, *et al* [14], reported a 39 times increase of free amino acid content (30 h fermentation) in protease facilitated lactic acid fermented milk compared with normal lactic acid fermentation. The differences between the result of this research and those findings might be partly due to the different protease used as well as the way their experiment was conducted.

ACE inhibitory activity

At 0 h fermentation, fermented milk made by non-maintaining and maintaining pH without protease addition showed 23.8 and 32.7% ACE inhibitory activity, respectively (Fig. 3). On the other hand, the sample made by adding protease showed 58.2% ACE inhibitory activity. The differences could be due to the proteolytic activity of the added protease because the measurement was conducted 2 minutes after the addition of protease.

During fermentation, the percentage of ACE inhibition in those samples made without protease addition increased continuously reaching the maximum at 24 h fermentation. These values were 87.6% and 87.8% for the samples made by non-maintaining and maintaining pH,

respectively. This suggests that the maintenance of pH at 5.0 did not affect the activity of ACE inhibition. The sample made by adding protease showed maximum % ACE inhibitory activity, 92%, at just 6 h fermentation. This value was the highest ACE inhibitory activity observed in this study. However, its ACE inhibitory activity was minimally higher than that of the samples made without protease addition at 24 h fermentation. These results contradicted those found by Chen, *et al* [14], even though their fermentation time used (30 h) was long, as was the case in this experiment (24 h). They found that the protease-facilitated lactic acid fermented milk (protease from *Aspergillus oryzae*) showed lower IC50 (higher ACE inhibition) than the sample made by using only lactic acid bacteria (*L. casei*, *L. acidophilus*, *S. thermophilus*,. *L. delbrueckii* subsp. *bulgaricus* and *Bifidobacterium).* Although the results of this research are reported as % ACE inhibitory activity, similar trends should be observed when the result is expressed as IC50 value. Different percentage of starter cultures used (0.1% vs 2% each starter culture in the current case) as well as activity of protease may explain the contradiction. Tsai, *et al* [6], also fermented milk by using lactic starter cultures (*S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*) and/or flavorzyme (protease from *Aspergillus oryzae*) for 5 h. They found that the lowest IC50 (the highest ACE inhibitory activity) was the sample made using starter cultures and protease.

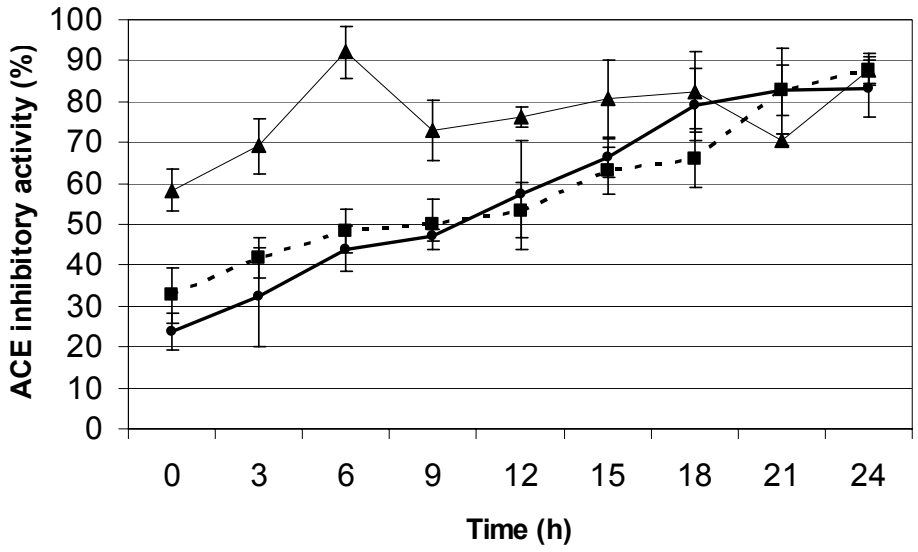

Figure 3. ACE inhibitory activity of the fermentation without maintaining pH (●). The fermentation maintaining pH at 5.0 without adding protease (■) and fermentation maintaining pH at 5.0 with adding protease (▲).

The fluctuation in ACE inhibition after 6 h fermentation in the sample made with protease addition could be due to the action of the added protease leading to a decrease of starter cultures (Fig. 1c) and an increase of proteolysis (Fig. 2). These results may suggest that the added protease not only digest proteins to active ACE inhibitory peptides, but also digest those active peptides to inactive ones. Similarly, Mao, *et al* [15], reported a decrease in ACE inhibitory activity after 240 min of alcalase treated casein hydrolyzate (360 min of total treatment). However, they reported that a good correlation between degree of enzyme hydrolysis and % ACE inhibitory activity was observed during 0 to 240 min. In this study, an increase in proteolysis activity correlated with increased percentage of ACE inhibitory activity, particularly during 0 to 6 h fermentation.

Conclusion

The addition of protease during milk fermentation is an effective method for the production of fermented milk high in ACE inhibitory activity. The use of protease could reduce fermentation time to only 6 h compared to 24 h when the starter culture is used alone. Extending fermentation time to further than 6 h in the sample made with protease addition may result in a reduction in ACE inhibitory activity.

Acknowledgements

A part of this study was supported by the Ministry of Education, Commission of Higher Education, subproject; Graduated Study and Research in Agricultural Biotechnology, Thailand.

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