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In Vitro Angiotensin-Converting Enzyme (ACE) Inhibition Activity Determination in Fermented Goat Milks

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Abstract

Seven fermented goat milks containing angiotensin-converting enzyme (ACE) inhibitory peptides were produced by using selected *Lactobacillus delbrueckii* subsp. *bulgaricus, L. lactis* subsp. *cremoris, L. lactis* subsp. Lactis, *Lactobacillus acidophilus, Bifidobacterium* and *Streptococcous thermophilus.* A rapid *In vitro* test for ACE inhibition activity determination in whey by competitive inhibition of angiotensin-converting enzyme was used. First, a solution of whey was mixed in a l to 10 ratio with a serum containing high ACE activity. Enzymatic activity (inhibition) was determined by spectrophotometric kinetic method using 0.8 mmol/L FAPGG (N-[3-(Furyl)-Acryloyl]-L-Phenylalanyl-Glycyl Glycyne) as the substrate. The inhibition activity was determined from a standard curve of inhibitor concentration versus percent of ACE inhibition. The most inhibition activity was detected for the milk fermented by a mixture of *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcous thermophilus.*

Key words: ACE, Competitive Inhibition, Fermented milk, Goat milk, Whey.

Introduction

Milk proteins are precursors of many different biologically active peptides. These peptides are inactive within the sequence of the precursor proteins but can be released by enzymatic proteolysis during intestinal digestion or during food processing. Milk protein-derived bioactive peptides may function as exogenous regulatory substances with hormone-like activity on the different intestinal and peripheral target sites of the mammalian organism. Opiate, antithrombotic, antihypertensive, immunomodulating, antibacterial, antigastric, human immunodeficiency virus type 1 proteinase inhibitory, and mineral carrying are some properties that have been attributed to several of the bioactive sequences identified[1-4]. Angiotensin-I-converting enzyme (ACE; kininase II; EC 3.4.15.1) is a multifunctional ectoenzyme

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located in different tissues which plays a key physiological role in the regulation of local levels of several endogenous bioactive peptides[5, 6]. ACE has been classically associated with the renin-angiotensin system which regulates peripheral blood pressure, where it catalyzes both the production of the vasoconstrictor angiotensin-II and the inactivation of the vasodilator bradykinin. ACE inhibition results mainly in an antihypertensive effect but may also influence different regulatory systems involved in modulating blood pressure, immune defense, and nervous system activity[7]. Naturally occurring peptides in snake venom were the first reported competitive inhibitors of ACE[8,9]. Thereafter, many other ACE inhibitors were discovered from enzymatic hydrolysates or the related synthetic peptides of bovine and human caseins (CNs), as well as plant and other food proteins[10]. Although chemical and physical treatments may have some influence, proteolysis by naturally occurring enzymes in milk, exogenous enzymes, and enzymes from microbial starters such as lactic acid bacteria can potentially generate bioactive sequences from milk protein precursors during dairy processing. Lactic acid bacteria are known to produce inhibitors of the enzyme in various amounts during fermentation[11,12,13]. The inhibitors are formed by the bacterial proteinases when the lactic acid bacteria hydrolyze milk proteins, mainly casein, into peptides, which can be used as nitrogen sources necessary for growth. All of the ACE inhibitors known to date that are formed in milk during fermentation are peptides that act as competitive inhibitors, such as Ile-Pro-Pro and Val-Pro-Pro[14]. In some cases it has been demonstrated that fermented milk rich in inhibitory substances can lower systolic blood pressure in spontaneously hypertensive rats (SHR) after oral administration[13-15]. Since the inhibitors formed are peptides, which are formed by degradation of caseins, proteolysis plays an important role. In theory, both specificity and overall proteolytic activity may have a significant role in the formation. The fact that the proteolytic systems of lactic acid bacteria are quite unspecific, *i.e.,* that they cleave, for instance, caseins at a high number of places[16], combined with the fact that ACE can be inhibited by many different peptidic structures (for instance, Val-Pro-Pro [14], Ile-Tyr, and Lys-Val-Leu-Pro-Val-Pro [18], all of which inhibit ACE in the micromolar range), led to the investigation of a possible correlation between crude proteolysis (measured as free amino groups) and ACE inhibition. It was shown that, in general, the higher the number of free amino groups formed during fermentation, the higher the chance that the same fermented substance will be able to inhibit ACE significantly, even though this rule is not always strictly obeyed. Among the strains showing a relatively high amount of free amino group formation, two strains capable of producing milk with high activity *in vitro* against ACE were identified and were shown to be able to decrease the in *vivo* activity of circulatory ACE in rats, measured as a decreased ability to convert angiotensin I to angiotensin II after oral administration of the fermented milk.

In this study, we used selected *Lactobacillus delbrueckii* subsp. *bulgaricus, L. lactis* subsp. *cremoris, L. lactis* subsp. Lactis, *Lactobacillus acidophilus, Bifidobacterium* and *Streptococcous thermophilus* to produce fermented milk containing ACE-inhibitory peptides. The ACE-inhibitory peptides were determined in whey by competitive inhibition of angiotensin-

converting enzyme using spectrophotometric kinetic method.

Materials and Methods

Microorganisms and culture fermentation conditions :

Raw goat milk was obtained from a local dairy. Bacterial strains, which we used, are listed in Table 1. All strains were obtained from the Chr. Hansen Culture Collection (Chr. Hansen A/S, Horsholm, Denmark). Bacterial strains were propagated in heat-treated 9.5% reconstituted skim milk, and fermentations were carried out at 37°C overnight, using a 1% inoculum. Skim milk cultures were used to inoculate (1%, vol/vol) 50 ml of fresh skim milk. Incubation was carried out under sterile conditions several times at the temperatures indicated in Table 1. Three batches of skim milk were inoculated with each strain. Fermented milk was produced with milk under sterile conditions in order to exclude enzyme interference by contaminant microorganisms. The extent of fermentation in the milk was monitored by pH.

Sample preparation :

The fermented milk was centrifuged at 4°C in an Eppendorf 5804R centrifuge (Eppendorf GmbH, Hamburg, Germany) at 4,500 rpm. The whey supernatant was adjusted to pH 8.3 with 10 N NaOH and was given 14,000 rpm (RCF 20,000 g) in a bench centrifuge (157MP; Ole Dich, Hvidovre, Denmark) for 10 min. The supernatant was used in the measurement of peptide content and measurement of *in vitro* ACE inhibition.

Measurement of peptide content

The whey peptide content was quantified using a modified version of the method by Church *et al.*[21] and Frister *et al.*[22]. *o*-Phthaldialdehyde (OPA) solution was made by combining the following reagents an diluting to final volume of 50 mL with water: 25 mL of 100 mM sodium tetraborate; 2,5 mL of 20% (wt/wt) sodium dodecyl sulfate; 40 mg of OPA (dissolved in 1 mL of methanol) and 100 mg N,N-dimethyl-2-mercaptoethylammonium chloride. This reagent was prepared daily. To assay proteolysis with milk proteins as substrates, small aliquot (usually 10 to 50 µL containing 5 to 100 µg protein) was added directly to 1.0 mL OPA reagent in a 1.5 mL quartz cuvette; the solution was mixed briefly by inversion and incubated for 3 min at ambient temperature, and the absorbance at 340 nm was measured in Specol 1200 spectrophotometer. Peptide content was quantified using amino acids, casein, and casein peptone as standards, yielded linear standard curves in the range of 0 to 100 µMole ($r^2 = 0.999$).

Measurement of *in vitro* ACE inhibition.

Enzymatic activity (inhibition) was determined by Holmquist's kinetic method[19], modified by Ronca-Testoni[20], with 0.8 mmol/L FAPGG as the substrate in 50 mmol/L Tris buffer at pH 8.2 at 37 °C. The enzymatic activity was determined by monitoring the decrease in absorbance at 340 nm as a result of hydrolysis of the substrate. (The substrate is a tripeptide blocked at the N-terminal by a furanacryloyl group which acts as a chromophoric monitor of substrate hydrolysis). A sample (50 µL) was added to the above mixture and the

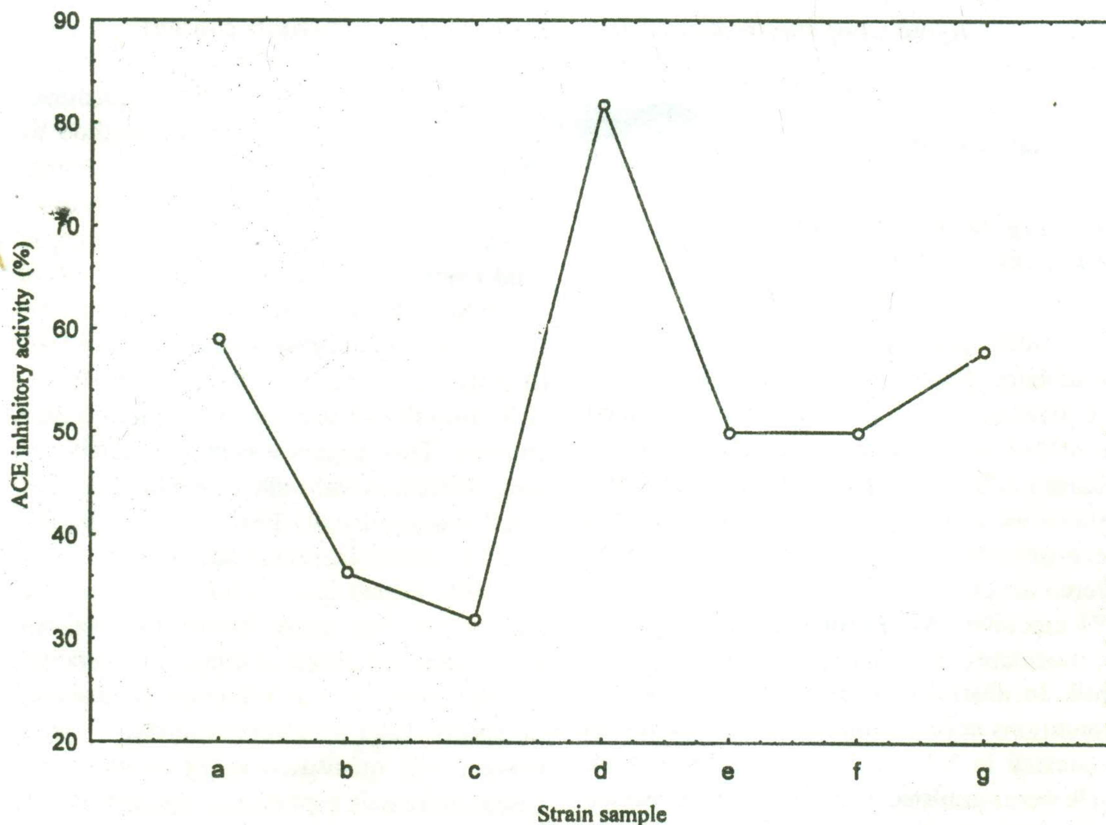

Fig. 1. ACE inhibitory activities of fermented goat milks. Sample a to g, various type bacterial strains given in Table 1.

cuvet was placed in the constant temperature cuvet compartment and allowed to equilibrate for 5 minutes. The initial absorbance value of the sample was recorded and the final absorbance was recorded exactly 25 minutes later. The percent of inhibition was calculated as follows: $(B - A) / (B - C) \times 100$, where A is optical density in the presence of both ACE and the whey peptide fraction or synthesized peptide, B is optical density without the whey peptide, and C is optical density without ACE. The inhibition values reported are the means of four determinations.

Results and Discussion

Seven different bacterial strains (Table 1) were screened for the production of ACE inhibitory activity during fermentation (Table 2). In addition, in this study we measured the amount of total peptides produced as a result of the fermentation. Since the fermentation was carried out from the same sample of goat milk the results obtained represent the degree

Table 1. Bacterial strains and condition for fermented goat milks

Symbol of culture	Strains composition	Temperature (°C)	Time (h)	pH
a	*Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus* (CH1)*	38	5	4.6
b	*Lactobacillus acidophilus, Bifidobacterium* and *Streptococcus* thermophilus (ABT 5)*	38	6	4.6
c	*Lactobacillus lactis subsp. cremoris* and *Lactobacillus lactis subsp. Lactis* (R 704)*	30	6	4.8
d	*Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus.* (YC 381) *	38	6	4.6
e	*Lactobacillus delbrueckii subsp. Bulgaricus* and Streptococcus *thermophilus.*	42	4	4.8
f	*Rhizomucor miehei*	38	6	4.6
g	*Aspergillus niger*	42	4	4.8

* product name of Chr. Hansen Culture Collection

Table 2. The inhibitory activity of angiotensin-converting enzyme (ACE) and peptide contents in fermented goat milks

Symbol of culture*	ACE inhibitory activity (%)	Peptide content (μM)
a	59.0	23.37
b	36.3	9.87
c	31.8	9.65
d	81.8	23.30
e	50.0	23.97
f	50.0	22.76
g	57.8	8.91

* See Table 1

of hydrolysis of the proteins from the goat milk as well. According to Table 2, the lowest degree of hydrolysis is caused by the fungus *Aspergillus niger,* while the highest amount of peptites is produced by the bacterial strain composition *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus.* Our study has shown that there is no strong correlation between the concentration of total peptides and ACE inhibition activity ($r = 0.732$). This confirms the theory that each bacterial strain produces different fragments of peptides due to specific hydrolysis of the primary sequence of aminoacids. The results obtained for the inhibitory activity of angiotensin converting enzyme are shown in Table 2 and Fig. 1. Fermentation with strain composition d (*Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus*) yielded *in vitro* the highest ACE inhibitory activity, while strain composition c (*Lactobacillus lactis subsp. cremoris* and *Lactobacillus lactis subsp. Lactis*) exhibits the lowest activity. It is worth noting that the microbal coagulans *Rhizomucor miehei* and *Aspergillus niger* demonstrate significant ACE inhibition activity of 50.0% and 57.8%, respectively. If we compare the results for ACE inhibition activity and the content of total peptides of the strain compositions a and d it is apparent that they have the same amount of total peptides (23.3 μM) but substantially different ACE inhibition activity (36.3% and 81.8%, respectively). It is interesting to note that the cultures a and d (product name of Chr. Hansen Culture Collection CH 1 and YC 381, respectively) are practically the same microorganizms but are, most likely, in different relative amounts.

Conclusion

Fermentation of goat milk with different bacterial strains yields whey with a wide range of ACE inhibition activity (30% to 80%), which depends to a large degree on the type of culture used. The largest ACE inhibition activity of 82% is demonstrated by the strain composition *Lactobacillus delbrueckii subsp. Bulgaricus* and *Streptococcus thermophilus.* Our study has shown that there is no strong correlation between the concentration of total peptides and ACE inhibition activity ($r = 0.732$).

References

1. Meisel, H., Biochemical properties of bioactive peptides derived from milk proteins: potential nutraceuticals for food and pharmaceutical applications. *Livest. Prod. Sci. 50,* 125-138 (1997).

2. Meisel, H., Overview on milk protein-derived peptides. *Int. Dairy J., 8,* 363-373 (1998).

3. Meisel, H. and W. Bockelmann, Bioactive peptides encrypted in milks proteins: proteolytic activation and thropho-functional properties. *Antonie van Leeuwenhoek 76,* 207-215 (1999).

4. Smacchi, E. and M. Gobbetti, Bioactive peptides in dairy products: synthesis and interaction with proteolytic enzymes. *Food Microbiol. 17,* 129-141 (2000).

5. Bruneval, P., N. Hinglais and F. Alhenc-Gelas, Angiotensin I converting enzyme in human intestine and kidney. Ultrastructural immunohistochemical localization. *Histochemistry 86,* 73-80 (1986).

6. Ondetti, M. A. and D. W. Cushman, Enzymes of the renin-angiotensin system and their inhibitors., *Annu. Rev. Biochem., 51,* 283-308 (1982).

7. Meisel, H., Casokinins as inhibitors of angiotensin-converting-enzyme, p. 153-159. *In* G. Sawatzki and B. Renner (ed.), New perspectives in infant nutrition. Thieme, Stuttgart, Germany (1993).

8. Ferreira, S.H., D.C. Bartlet and L.J. Greene, Isolation of bradykinin potentiating peptides from *Bothrops jararaca* venom. *Biochemistry 9,* 2583-2592 (1970).

9. Ondetti, M. A., N. J. Williams, E. F. Sabo, J. Pluvec, E. R. Weaver and O. Kocy, Angiotensin converting enzyme inhibitors from the venom of *Bothrops jararaca,* isolation, elucidation of structure and synthesis., *Biochemistry, 10,* 4033-4039 (1971).

10. Smacchi, E. and M. Gobbetti, Peptides from several Italian cheeses inhibitory to proteolytic enzymes of lactic acid bacteria, *Pseudomonas fluorescens* ATCC 948 and to the angiotensin I-converting enzyme. *Enzyme Microb. Technol., 22,* 687-694 (1998).

11. Gobbetti, M., P. Ferranti, E. Smacchi, F. Goffredi and F. Addeo, Production of angiotensin-I-converting-enzyme-inhibitory peptides in fermented milks started by *Lactobacillus delbrueckii* subsp. bulgaricus SSl and *Lactococcus lactis* subsp. *cremoris* FT4. *Appl. Environ. Microbiol. 66,* 3898-3904 (2000).

12. Meisel, H., A. Goepfert, and S. Gunther, ACE-inhibitory activities in milk products. *Milchwissenschaft 52,* 307-311(1997).

13. Yamamoto, N., A. Akino and T. Takano, Antihypertensive effects of different kinds of fermented milk in spontaneously hypertensive rats. *Biosci. Biotechnol. Biochem. 58,* 776-778 (1994).

14 Nakamura, Y., N. Yamamoto, K. Sakai, A. Okubo, S. Yamazaki and T. Takano, Purification and characterization of angiotensin I-converting enzyme inhibitors from sour milk., *J. Dairy Sci. 78,* 777-783 (1995).

15. Yamamoto, Y., N. Nakamura, K. Sakai, and T. Takano, Effect of sour milk and peptides isolated from it that are inhibitors of angiotensin-I-converting enzyme. *J. Dairy Sci. 78,* 1253-1257 (1995).

16. Kunji, E. R., I. Mierau, A. Hagting, B. Poolman, and W. N. Konings, The proteolytic systems of lactic acid bacteria. *Antonie Leeuwenhoek, 70,* 187-221 (1996).

17. Yokoyama, K., H. Chiba and M. Yoshikawa, Peptide inhibitors for angiotensin I-converting enzyme from thermolysin digest of dried bonito., *Biosci. Biotechnol. Biochem. 56,* 1541-1545 (1992).

18. Maeno, M., N. Yamamoto and T. Takano, Identification of an antihypertensive peptide from casein hydrolysate produced by a proteinase from Lactobacillus helveticus

CP790. *J. Dairy Sci. 79,* 1316-1321 (1996).

19. Holmquist, B., Bunning, P., Riordan, R.F., A continuous spectrophotometric assay for angiotensin converting enzyme, *Anal. Biochem., 95,* 540-548 (1979).

20. Ronca-Tastoni S., Direct spectophotometric assay for angiotesin -converting enzyme in serurn, *Clin Chem., 29,* 1093-1096 (1983).

21. Church, F.C., H.E. Swaisgood, D. H. Porter, and G.L. Catign, Spectrophotometric assay using o-phthaldialdehyde for determination of proteolysis in milk and isolated milk proteins. *J. Dairy Sci., 66,* 1219-1227 (1983).

22. Frister, H., Maisel, H., Schllimme, E., OPA method modified by use of N,N-dimethyl-2-mercaptoethylammonium chloride as thiol component, *Fresenius Z. Anal. Chem. 330,* 631-633 (1988).