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Optimization of Whey-Based Medium for Growth and ACE-Inhibitory Activity of *Lactobacillus brevis*

Jae-Eun Ahn, Seung-Yong Park[1] and Byong H. Lee*

Department of Food Science and Agricultural Chemistry, McGill University, Montreal, QC, Canada H9X 3V9

[1]Dept. of Food Service Industry, Cheonan Yonam College, Korea

ABSTRACT

A whey-based medium was formulated with *Lactobacillus brevis* to investigate whether any functional peptides could derive from whey protein. The optimal concentrations of the ingredients of the medium for the growth of *Lactobacillus* were determined as 2% whey protein concentrate and 1% glucose and 0.5% yeast extracts. The growth of *Lb. brevis* was improved with the supplementation of yeast extracts than glucose. The viable cells counts of *Lb. brevis* reached to 2.0×10^8CFU/mL in the whey-based medium. The whey protein hydrolysates recovered from the supernatant after centrifugation at 10,000 × g for 10min induced strong inhibitory activity against ACE. When the whey protein hydrolysate were partially purified by a membrane tubing below 8,000Da, the partially purified fraction remained 64.7 ± 3.6% of the ACE inhibition activity of the whey protein hydrolysates and IC_{50} was 38.8 ± 2.2mg/mL. The whey-based medium was proved to be effective in producing ACE inhibitory peptides by lactic bacteria fermented whey protein.

(**Key words** : whey-based medium, yeast extracts, *Lactobacillus brevis*, ACE inhibitory activity, whey protein hydrolysates)

INTRODUCTION

Angiotensin- I -converting enzyme (ACE) is a dipeptide-liberating exopeptidase, which plays an important role in peripheral blood pressure regulation. It raises blood pressure by converting the inactive decapeptide angiotensin I to the potent vasoconstrictor octapeptide angiotensin II, as well as inactivating the vasodilating nonapeptide, bradykinin. Inhibition of ACE may exert an antihypertensive effect as a consequence of the decrease of angiotensin II as well as increase of bradykinin.

Peptides that inhibit ACE have been found in many different food proteins (Ariyoshi, 1993; Okamoto *et al.*, 1995; Yamamoto, 1997), most extensively in milk proteins (Pihlanto-Leppälä, 1998; Takano, 1998, 2000; Yamamoto and Takano, 1999; Nurminen, 2000). Several casein-derived ACE inhibitors have been reported (Maruyama and Suzuki, 1982; Maruyama *et al.*, 1985, 1987a,b; Karaki *et al.*, 1990; Nakamura *et al.*, 1995a; Maeno *et al.*, 1996; Sugai, 1998). However, only limited studies have been carried out on whey protein-derived ACE inhibitors. Albutensin A, a peptide derived from serum albumin, was shown to inhibit ACE (Chiba and Yoshikawa, 1991). Synthetic di- and tetra-peptides corresponding to α-lactalbumin(α-la) and β-lactoglobulin(β-lg) sequences were shown to inhibit ACE (Mullally *et al.*, 1996, 1997a,b).

Whey proteins represent about 20% of total milk proteins and are well known for their high nutritional value and versatile functional properties in food products. These proteins can also provide elemental nitrogen for the growth of lactic acid bacteria (LAB), which possess proteinase that catalyzes the hydrolysis of native or denatured protein molecules, and peptidases that catalyze the degradation of the smaller peptides produced by proteinases action (Law and Kolstad, 1983; Christensen *et al.*, 1999; Siezen, 1999). Thus, the proteolytic system of lactic acid bacteria can convert whey proteins into smaller peptides, which may exhibit ACE inhibitory activity.

In this study, optimizing culture medium for the growth of lactic acid bacteria (LAB) during whey fermentation was investigated in order to produce whey hydrolysates that contain potential ACE inhibitory peptides.

*Corresponding author : Byong H. Lee, 21, 111 Lakeshore Rd., Ste-Anne-de-Bellevue, QC H9X 3V9, Canada. Food R&D Centre, Agriculture and Agri-Food Canada, 3600 Casavant Blvd West., St-Hyacinthe, QC, Canada J2S 8ES. E-mail: byong.lee@mcgill.ca

MATERIALS AND METHODS

1. Chemicals

Hydrochloric acid (HCl), sodium borate ($Na_2B_4O_7 \cdot 10H_2O$), Tween 80 ($C_{64}H_{124}O_{26}$), and glycerol ($HOCH_2CH(OH)\ CH_2OH$) were purchased from Fisher Scientific Company (Fair Lawn, NJ, USA), while glucose ($C_6H_{12}O_6$), sodium chloride (NaCl), ethyl acetate ($C_4H_8O_2$), Hip-His-Leu, and ACE from rabbit lung were obtained from Sigma Chemicals Co.(St. Louis, MO, USA). Whey powder, yeast extract, and MRS were obtained from Pascobel Inc.(Longueuil, QC, Canada), Becton Dickinson Co. (Cockeysville, MD, USA), and Institut Rosell Inc.(Montreal, QC, Canada), respectively.

2. Microorganisms and Cultures

Stock culture of *Lb. brevis* was maintained at −80℃ in MRS broth, containing 50% (v/v) glycerol. As required, this cultures was thawed and reactivated by two transfers in MRS broth.

10 μL of *Lb. brevis* from the frozenstocks was inoculated to 10 mL of MRS broth, containing 1% glucose and 0.1% Tween 80, and grown at 30 or 37℃. A portion (50 μL) of this culture was transferred to 50 mL MRS broth in a 125 mL Erlenmeyer flask and incubated at 30 or 37℃ for 15~18h on a rotatory shaker (Forma Scientific Inc., Marietta, OH, USA) at 150rpm. Prior to inoculations, MRS broth was autoclaved at 121℃ for 15 min.

3. Whey-based Medium

Table 1. Optimal composition of fermentation media designed for the growth of *Lactobacillus brevis*

Media	Whey powder(%)	Yeast extract(%)	Glucose(%)
1a*	1	0.5	0
1b*	2	0.5	0
1c*	5	0.5	0
1d*	10	0.5	0
2a**	2	0	0
2b**	2	0.5	0
2c**	2	1	0
3a***	2	0.5	0
3b***	2	0.5	1
3c***	2	0.5	2

* Media prepared to determine the effect of whey powder concentration.
** Media prepared to determine the effect of yeast extract concentration.
*** Media prepared to determine the effect of glucose concentration.

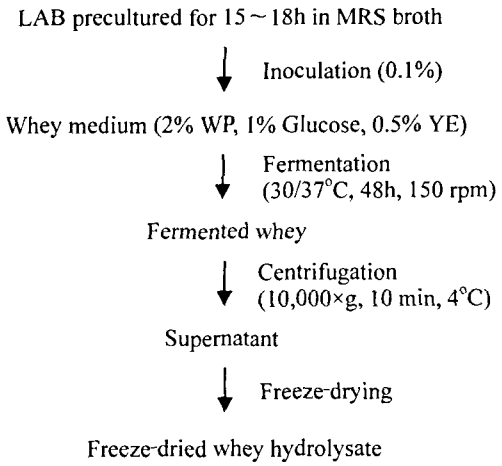

Fig. 1. Procedure for preparation of whey protein hydrolysate.

Whey-based medium, containing different concentrations of whey powder (12% protein), glucose, and yeast extract (Table 1) were prepared for fermentation with *Lactobacillus brevis*. Each medium (200 mL) in 500 mL Erlenmeyer flask and autoclaved at 121℃ for 15 min was inoculated with a seed culture at a rate of 0.1% (v/v) and similarly incubated at 30 or 37℃ for 24h. After serial dilutions of each sample in 0.85% saline solution, the viable cell count was achieved at 6h intervals by pour-plating on MRS agar.

4. Fermentation of Whey with *Lb. brevis*

For the fermentation of whey protein with *L. brevis*, 0.1% (v/v) of stock culture was inoculated in 500 mL Erlenmeyer flask containing 200 mL of sterile whey- based medium (pH 6.0). The whey-based medium was composed of 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract. The fermentation was carried out at 30 or 37℃, according to their optimal incubation temperature, for 48h with mild agitation (150 rpm). At 12h intervals, viable cell count and pH of each whey-based medium were monitored.

Whey protein hydrolysates were obtained from the growth medium and prepared as represented in Fig. 1. After the fermentation, each sample was centrifuged (10,000 × *g*, 10 min) to remove cell mass and insoluble denatured proteins and other components. The supernatant was collected, frozen, and freeze-dried using Flexi-Dry MP-Microprocessor controlled bench-top lyophilizer (FTS Systems, Inc., Stone Ridge, NY), equipped with a condenser and a vacuum pump. The freeze-dried hydrolysates were stored at −4℃ for further analysis.

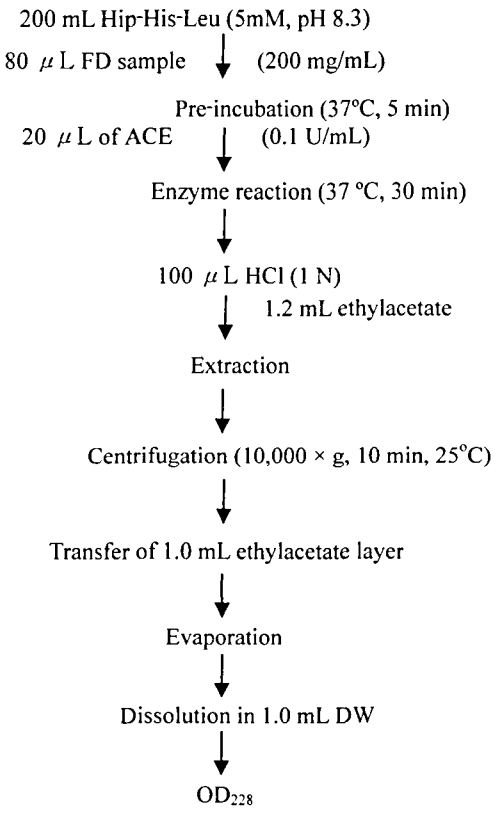

Fig. 2. Procedure for *in vitro* ACE inhibitory assay.

5. Partial Purification of Whey Protein Hydrolysates

The crude whey hydrolysates (freeze-dried), obtained from whey fermentation with *Lb. brevis* were dissolved in distilled water (200 mg/mL) and were partially purified by dialysis using a Spectrapor membrane tubing (6,000~8,000 Da cut-off, Spectrum Medical Industries Inc., LA, CA) and the portion, which molecules are smaller than 8,000 Da, was assayed for ACE inhibitory activity.

6. *In Vitro* Assay for ACE Inhibitory Activity

The inhibitory activity against ACE was measured *in vitro* by the method of Cushman and Cheung(1971), modified by Nakamura *et al.*(1995a). The procedure for the assay is represented in Fig. 2. After the Hip-His-Leu was dissolved in 0.1M sodium borate buffer (pH 8.3) containing 0.3M NaCl, 200 μL of 5 mM Hip-His-Leu solution was mixed with 80 μL of the freeze-dried whey hydrolysate, dissolved in distilled water at a concentration of 200 mg/mL (adjusted to pH 8.3), and then preincubated at 37℃ for 5 min. The reaction was initiated by the addition of 20 μL of ACE from rabbit lung dissolved in distilled water (0.1U/mL), and the mixture was incubated for 30 min at 37℃. The reaction was stopped by addition of 100 μL of 1M HCl. The hippuric acid liberated by ACE was extracted from the acidified solution into 1.2 mL of ethyl acetate by vortex mixing. After a brief centrifugation, a portion (1.0 mL) of ethyl acetate layer was transferred to a clean tube. The ethyl acetate layer was evaporated and the hippuric acid was dissolved in 1.0 mL distilled water and the amount formed was measured by a spectrophotometer (Ultraspec II, LKB) at 228 nm. For the determination of 50% inhibitory concentrations (IC_{50}), a series of dilutions (2.5, 5.0, 10.0, 50.0, 100.0, 200.0 L), which contained whey protein fractions (mg/mL), were prepared. The result was expressed as means ±SD in each analysis using SAS (2000).

RESULTS AND DISCUSSION

1. Optimization of Whey-based Medium

The major components of whey powder are lactose (74% w/w) and protein (12% w/w), which can provide carbon and nitrogen sources for the growth of lactic acid bacteria. In order to determine the optimum concentration of whey powder needed for the growth of *Lb. brevis*, four different culture media containing 1, 2, 5, and 10% (w/v) whey powder were fermented with *Lb. brevis*. Fig. 3 shows the kinetics of microbial growth at different concentrations of whey powder. The growth profiles obtained from all fermentations were very similar except for the medium containing 1% whey powder, which showed a brief stationary phase, followed by death phase. In

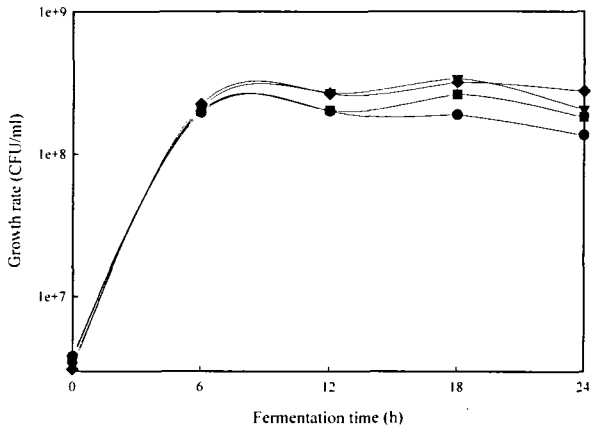

Fig. 3. Growth of *Lb. brevis* in whey-based medium at different whey powder concentration; ● 1% WP, 0.5% YE; ■ 2% WP, 0.5% YE; ▼ 5% WP, 0.5% YE; ◆ 10% WP, 0.5% YE.

other media, the stationary phase was much longer, extending to 18h of fermentation. Only a slight increase of biomass was observed with an increase in whey powder concentration up to 5%. Above this level no significant increase was observed in growth. The culture medium containing 2% whey powder appeared to be optimal in this study as only a slight increase in biomass was achieved by increasing whey powder by more than two folds.

Growth of *Lb. brevis* was very low in the absence of yeast extract, but the yield increased more than 10 fold when yeast extract was added at a concentration of 0.5% (Fig. 4). On the other hand, only a slight difference on the growth rate was observed in 1% yeast extract. This observation is in agreement with those of Arasaratnam *et al.*(1996), who reported that growth of *Lb. delbrueckii* in whey fermentation increased significantly with yeast extract supplementation up to 10g/L, but was not increased above this level. Arasaratnam *et al.*(1996) suggested that this could be resulted from carbon source limitation.

It is well known that lactic acid bacteria have complex nutritional requirements for growth (Cox *et al.*, 1977). Because cheese whey is relatively low in essential amino acids in free form needed for the growth of these bacteria, most whey fermentation requires supplementation, especially with yeast extract, to achieve good growth and productivity (Aeschlimann and von Stockar, 1990; Arasarathnam *et al.*, 1996; Amrane and Prigent, 1997). In addition to nitrogen in the form of amino acids and peptides, yeast extract also supplies growth factors, such as vitamin B groups and several organic acids including pyruvic and glyceric acid (Taso and

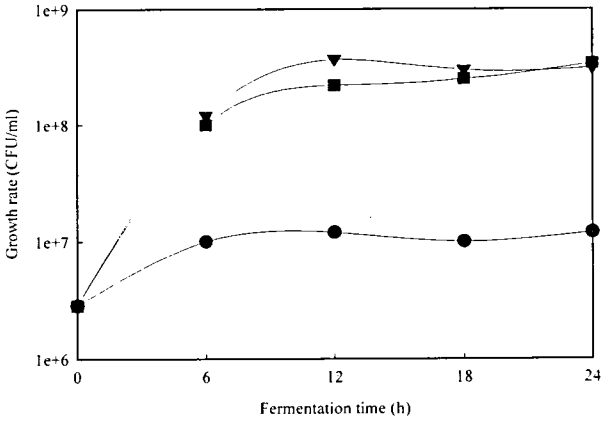

Fig. 4. Growth of *Lb. brevis* in whey-based medium at different yeast extracts concentration; ● 0% YE, 2% WP; ■ 0.5% YE, 2% WP; ▼ 1% YE, 2% WP.

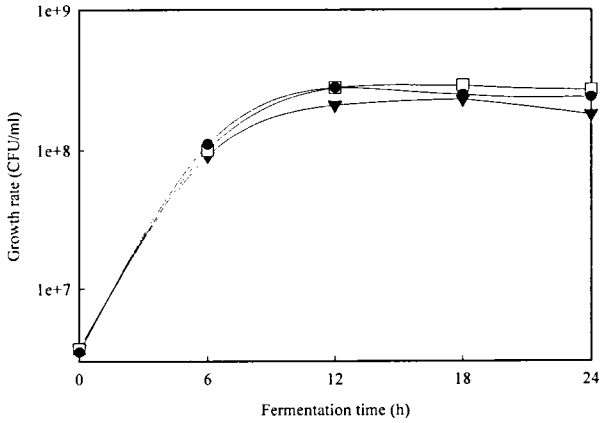

Fig. 5. Growth of *Lb. brevis* in whey-based medium at different glucose concentration; ▼ 0% glucose, 2% WP, 0.5% YE; ❑ 1% glucose, 2% WP, 0.5% YE; ● 2% glucose, 2% WP, 0.5% YE.

Hanson, 1975; Cox *et al.*, 1977).

Whey-based growth medium composed of 2% (w/v) whey powder alone can supply carbon source for the growth of *L. brevis,* as it contains approximately 15g lactose per liter. However, lactose is not a fermentable or preferred carbon source for many microorganisms (Yang and Silva, 1995). The effect of glucose supplementation on the growth rate of *Lb. brevis* was observed by preparing three whey media, containing equal amount of whey powder (2% w/v) and yeast extract (0.5% w/v), but different concentrations of glucose (0, 1, and 2% w/v). In Fig. 4, the growth of *Lb. brevis* slightly increased with glucose supplementation. However, the growth rates at 1% and 2% supplementations were almost identical. This could be due to inhibitory effect exerted by higher sugar concentration as suggested by Goncalves *et al.*(1991).

2. Growth of *Lb. brevis* in Whey-based Medium

Whey-based medium was fermented with *L. brevis* for the purpose of producing potentially active peptides that inhibit ACE activity from the *Lactobacillus* species. This was based on the fact that LAB possess a number of proteinases and peptidases, which hydrolyze milk proteins to small peptides and free amino acids required for cell growth during fermentation (Law and Kolstad, 1983; Christensen *et al.*, 1999; Siezen, 1999). Whey- based medium subjected to fermentation was composed of 2% (w/v) whey powder, 1% (w/v) glucose, and 0.5% (w/v) yeast extract.

Fig. 6 shows the kinetics of microbial growth and pH chan-

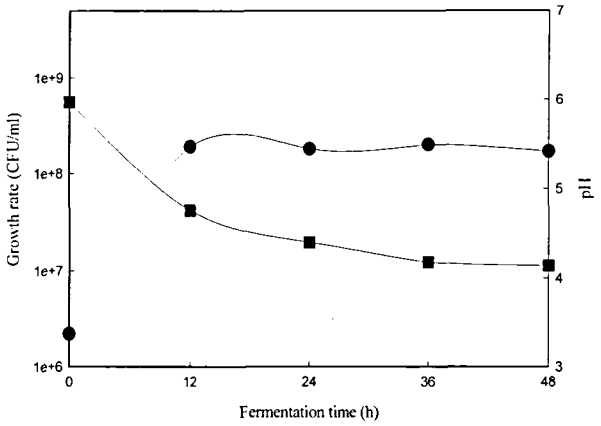

Fig. 6. Growth of *Lb. brevis* in whey-based-growth medium; ● viable cell count; ■ pH.

ge during fermentation with *Lb. brevis*. The maximal growth (about 2.0 × 10^8CFU/mL) was reached at 15h.

However, the growth was continued for another 36h to give ample time to hydrolyze whey proteins that are globular molecules with a high degree of secondary and tertiary structure, thus making them less susceptible to proteolysis in contrast to caseins, having an open and largely random structure (Thomas and Pritchard, 1987). During fermentation, the pH decreased from about pH 6.0 to pH 4.1 due to lactic acid production. However, the pH decreased steadily even after the maximal microbial growth was reached. In fact, *Lb. brevis* maintained a very slow death phase. It was assumed that cell proliferation and lysis were undergoing simultaneously, suggesting that proteolytic activity also continued.

3. ACE Inhibitory Activity of Whey Protein Hydrolysates

Table 2 shows the ACE inhibitory activity of the crude whey protein hydrolysates produced by *Lb. brevis*.and partially purified. The crude whey protein hydrolysates of *Lb. brevis* induced the high activity against ACE and when the hydrolysates were partially purified by dialysis using a Spectrapor membrane tubing (Spectrum Med. Industr. Inc., CA), The ACE inhibitory activity was remained in the molecules less than 8,000 Da with high recovery rate and determined the IC_{50} was 38.8 ±2.2 mg/mL. Nakamura *et al.* (1995a,b) demonstrated that *Lb. helveticus*, which generated potent ACE inhibitory peptides from fermented sour milk. On the other hand, Pihlanto-Leppälä *et al.*(1998) failed to find ACE inhibitory activity from fermentation of cheese whey with various lactic acid bacteria that are commonly used for the fermentation of dairy products. A number of factors may have attributed to such contradictory results, which include the use of different genus or species of LAB, the use of lower whey protein concentration as well as the fortification of yeast extract in addition to glucose supplementation, and the application of longer fermentation time of 48h, in contrast to 6 and 22h in their studies. All these factors may have induced strong proteolytic activity by LAB to produce higher amount of functional bio-peptides with ACE inhibitory activity. Such assumption is supported by a study by Pihlanto-Leppälä *et al.*(1998), who observed the increase in proteolytic activity as well as ACE inhibitory activity of fermented cheese whey that were further treated with pepsin and trypsin.

Table 2. ACE inhibitory activity of crude whey hydrolysates and partially purified fraction after whey fermentation with *Lb. brevis*

Purification Steps	Sample Conc. (mg/mL)	ACE Inhibitory Activity (%)*	IC_{50}[1] (mg/mL)*
Crude	200	100	100.0
Partially purified	50	64.7 ± 3.6	38.8 ± 2.2

[1] Concentration of freeze-dried whey hydrolysates required to inhibit 50% of ACE activity.

* The values represent means of two different experiments with standard deviation less than 6%.

In conclusion, whey protein was found to be effective substrate in the growth of *Lactobacillus brevis* and in liberating of functional bio-peptides like ACE inhibitory peptides.

국문요약

유청을 기초로 하는 배지를 제조하여 *Lactobacillus brevis*를 배양하면서 유청 단백질로부터 기능성 펩타이드 생성을 알아보고자 하였다. *Lb. brevis*의 적정생장에 필요한 배지성분의 농도는 2% 유청 분말, 1%의 포도당 및 0.5%의 효모 추출물이었다. *Lb. brevis*의 생장은 효모 추출물의 보충이 포도당의 보충보다 더 효과적이었다. 이 유청 배지에서 *Lb. brevis*의 생장은 2.0 × 10^8CFU/mL에 달하였다. 생장 후의 유청 배지를 10,000 × g에서 10분간 원심분리하여 그 여액으로부터 얻은 유청단백분해물은 ACE 효소 억제 효과가 나타났다. 분자량 8,000Da 이하로 부분 정제한 분획의 ACE에 대한 억제효과는 유청단백분해물의 64.7 ±3.6%, IC_{50}은 38.8 ± 2.2 mg/mL로 나타났다. 따라서 유청을 기초로 한 배지는 젖산균으로부터 유청 단백질을 발효하여 ACE 억제 효과를 주는 펩타이드 생산에 적합한 배지임을 알 수 있었다.

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