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Isolation and Characterization of Three Novel Peptides from Casein Hydrolysates That Stimulate the Growth of Mixed Cultures of *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*

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ABSTRACT: In this study, sodium caseinate hydrolysates produced by papain with strong growth-stimulating activity for *Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb) were obtained. A series of separation methods including ultrafiltration, macroporous adsorption resin chromatography, gel filtration chromatography, and reverse-phase high-performance liquid chromatography (RP-HPLC) were applied to isolate and purify the peptide(s), which were mainly responsible for the activity. Finally, three novel growth-stimulating peptides, H-2-A, F2-c, and F2-b, corresponding to amino acid residues 29−35 and 103−108 of bovine α_{S2}-casein and 181−186 of bovine α_{S1}-casein, respectively, were obtained. With supplementation of H-2-A, F2-b, or F2-c at a protein concentration of 0.3%, the biomass yield of these two lactic acid bacteria (LAB) was enhanced by 193.3, 166.7, or 151.7%, respectively. In addition, there were significant ($p < 0.05$) increases in viable counts of St and lactic acid production of LAB in the presence of the purified peptides.

KEYWORDS: peptides, purification, growth stimulating, lactic acid bacteria, casein, enzymatic hydrolysates

INTRODUCTION

Lactic acid bacteria (LAB) play an important role in food fermentation processes during which carbohydrates are fermented to lactic acid as the primary metabolic end-product.[1,2] Worldwide, LAB constitute a majority in volume and value of the commercial starter cultures, with the largest amount being applied in the production of dairy products, such as cheese and yogurt. Yogurt is usually manufactured from cow's milk, with or without the addition of some derivatives of milk, and possesses a gel structure that is the result of coagulation of the milk proteins by a 1:1 ratio of *Streptococcus thermophilus* (St) and *Lactobacillus delbrueckii* subsp. *bulgaricus* (Lb).[1,2] LAB have a beneficial effect on human health, which mainly depends on the number of viable microbial cells that reach the human gut.[3] An important issue to support health claims is that yogurt and fermented milk contain an abundant and viable microflora of starter cultures at the time of consumption. A definition along these lines is incorporated in the food laws of many countries, with the minimum values ranging between 10^6 and 10^8 CFU/mL.[4] However, commercial products often contain less LAB than the minimum number required.[5]

The growth of LAB depends on adequate supplies of suitable sources of nitrogen and carbon. However, there is only limited available nitrogen source (free amino acids and small peptides) in milk.[6] LAB are weakly proteolytic.[7] Hence, more amino acids or small peptides are required for the growth of LAB.[8] The most common way to produce peptides is through enzymatic hydrolysis of the whole protein. A large number of studies have demonstrated that the hydrolysis of milk proteins by proteolytic enzymes can produce biologically active peptides including antihypertensive, antibacterial, opioid, and immunomodulatory peptides.[9,10] Casein contains all of the amino acids necessary for LAB growth and also has many oligopeptides that contain growth-stimulating amino acids.[7] It has been shown that the addition of casein hydrolysates can reduce the fermentation time for yogurt and improve the viability of LAB in yogurt.[5,11] Poch and Bezkorovainy also found that tryptic hydrolysates of κ-casein were the most potent growth enhancer for the genus *Bifidobacterium*.[12] Although there have been many reports on growth-promoting factors for LAB in recent years,[13−15] very little is known about those growth-stimulating factors from peptidic origin. Therefore, the objective of this study was to isolate and identify peptide(s) with growth-stimulating activity for the mixed cultures of St and Lb originated by enzymatic hydrolysis of sodium caseinate with papain.

MATERIALS AND METHODS

Materials. Sodium caseinate (89.50%, protein content) was purchased from Chr. Hansen (Guangzhou, China). The food grade papain (EC 3.4.22.2) with an enzyme activity of 5.1×10^5 U/g was obtained from Novozymes Biotechnology (Tianjin, China). The skim milk powder was provided by Fonterra (Auckland, New Zealand). All other chemicals and solvents used in this study were of analytical grade and obtained from Sigma-Aldrich (St. Louis, MO).

Hydrolysis. Sodium caseinate was reconstituted in distilled water to obtain a solution containing 10% (w/v) of protein. The solution was digested with papain (1%, w/w of substrate) at 55 °C for 1, 3, 6, 8, 12,

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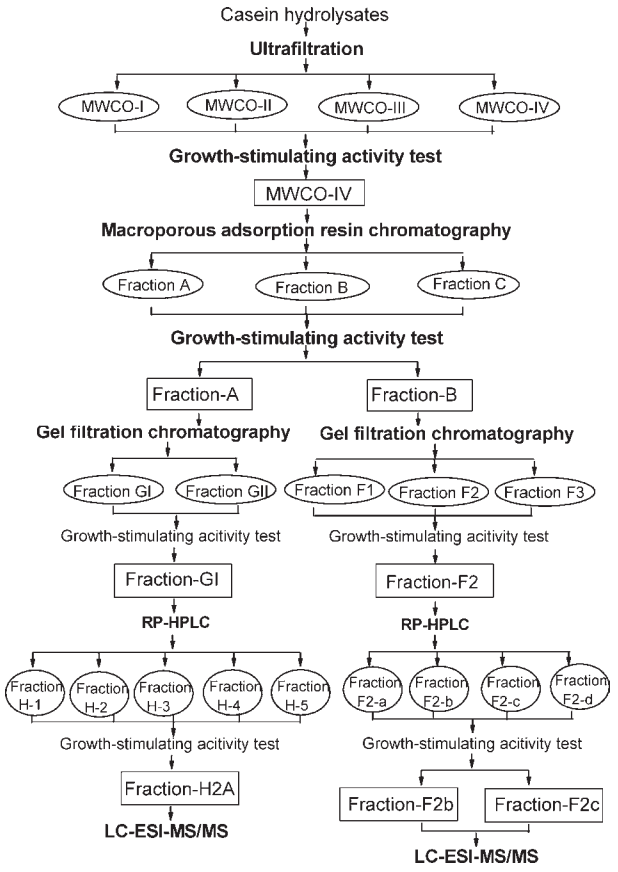

Figure 1. Schematic diagram for the isolation and purification of growth-stimulating peptides from casein hydrolysates.

and 24 h, respectively. The hydrolysis was done at pH 7.0 (pH-3E pH-meter, Rex, Shanghai, China) in a water bath shaker (New Brunswick Scientifics C24, Jintan, China). The reactions were terminated by immersing the reaction vessel in water at 95 °C for 10 min with stirring to ensure the inactivation of the enzyme. The resultant slurry was cooled in an ice bath and then centrifuged at 6000*g* for 20 min at 4 °C (Centrifuge, Sigma Aldrich, Munich, German) to eliminate the sediment. The obtained six casein hydrolysates (CH) samples, that is, CH-1, CH-3, CH-6, CH-8, CH-12, and CH-24, were kept at −18 °C prior to use.

Degree of Hydrolysis (DH). The DH, defined as the percentage of peptide bonds cleaved, was based on the number of free amino groups determined using the TNBS method described by Spellman et al.[16] DH values were calculated using the formula

$$\mathrm{DH}\ (\%) = \left(\frac{\mathrm{AN}_2 - \mathrm{AN}_1}{\mathrm{Npb}}\right) \times 100$$

where AN_1 is the amino nitrogen content of the protein substrate before hydrolysis (mg/g protein), AN_2 the amino nitrogen content of the protein substrate after hydrolysis (mg/g protein), and Npb the nitrogen content of the peptide bonds in the protein substrate (mg/g protein). A value of 114.8 was used for casein protein.[17]

Determination of Molecular Weight Distributions. The molecular weight distribution of CH was determined by gel permeation chromatography (GPC) on a Superdex peptide 10/300 GL column (Amersham Biosciences, Piscataway, NJ) with a UV detector set at 214 nm. The mobile phase (isocratic elution) was 0.02 M sodium phosphate buffer containing 0.25 M NaCl (pH 7.2), at a flow rate of 0.5 mL/min. A protein standard mixture (Amersham Biosciences) was used to calibrate the column. UNICORN 5.0 software (Amersham Biosciences) was used to analyze the chromatographic data.

Microorganisms and Culture Conditions. YC-380 (St and Lb in 1:1 ratio), a freeze-dried commercial starter culture, was purchased from Chr. Hansen China Co. and used throughout this work. The stock culture was fermented in the seed cultivation broth (14% skim milk culture, w/w) for 5 h until it coagulated. Then the fermentation broth (MRS broth) was prepared by inoculating 20 mL of culture medium with 1 mL of seed culture. The anaerobic fermentations were performed in 50 mL glass bottles at 37 °C for 24 h (Anaerobic incubator, Shanghai Fuma Test Equipment Co., Shanghai, China). The MRS broth medium had components as described by Degeest and De Vuyst.[18]

Microbial Growth. To evaluate the influence of the casein hydrolysates and casein hydrolysate fractions on the growth of the LAB, anaerobic fermentations were carried out in triplicate. Bacterial growth was measured by recording the value of optical density at 622 nm (OD_{622nm}) using an Unico 2100 spectrophotometer (Unico, Shanghai, China), and the changes in pH were measured with a pH-3E pH-meter. The hydrolysates or hydrolysate fractions were added at a protein concentration of 0.5% (w/w). The control contained 0.5% water instead of the casein hydrolysates or casein hydrolysate fractions. The protein content of water-soluble extracts was determined according to the Kjeldahl method. The protein concentration of the fractions collected from ultrafiltration or chromatography was estimated using the bicinchoninic acid assay (Pierce, Rockford, IL) using bovine serum albumin as the standard. The purified peptide(s) were quantified using amino acid analysis according to the method of Dong et al.[19]

Isolation and Purification of Peptides. The schematic diagram for the purification of casein hydrolysates is shown in Figure 1.

Ultrafiltration. The most active casein hydrolysates were fractionated through ultrafiltration membranes using a bioreactor (Vivaflow 200, Vivascience, Sartorius, Goettingen, Germany) with a range of molecular weight cutoff (MWCO) of 10, 5, and 3 kDa (PESU, Sartorius), respectively. MWCO-I, MWCO-II, MWCO-III, and MWCO-IV represented the fractions with molecular weight distribution of >10, 5−10, 3−5, and <3 kDa, respectively. All fractions recovered were lyophilized for growth-stimulating activity and protein concentration tests.

Macroporous Adsorption Resin Chromatography. The fraction MWCO-IV from ultrafiltration exhibiting the strongest growth-stimulating activity for the bacteria was redissolved in distilled water and loaded onto a macroporous adsorption resin NKA-11 column (2.6 × 60 cm; Tianjin Bohong Resin Technology, Tianjin, China) equilibrated with distilled water. Then, the column was eluted with a linear gradient of ethanol (5−80%) at a flow rate of 1.5 mL/min. The collection of the desire peak was pooled, concentrated using a rotary evaporator, freeze-dried, and tested for growth-stimulating activity and protein concentration.

Gel Filtration Chromatography. The fractions (A and B) showing powerful growth-stimulating activity after macroporous adsorption resin chromatography were redissolved in distilled water and separated with a Sephadex G-25 gel filtration chromatography column (1.6 × 90 cm; Amersham Biosciences), which was eluted with distilled water at a flow rate of 2.0 mL/min. Elution curves were obtained by measuring the absorbance at 220 nm. The fractions with the desired peaks were pooled and lyophilized for growth-stimulating activity and protein concentration tests.

RP-HPLC. The fractions with the highest growth-stimulating activity after gel filtration chromatography separation were further purified using RP-HPLC on an XBridge preparative C_{18} column (10 × 150 mm, 5 μm; Waters, Milford, MA). After injection of the fraction GI samples, the HPLC system (flow rate of 3.0 mL/min) was eluted with a linear gradient of acetonitrile (5−45%) containing 0.1% trifluoroacetic acid (TFA) and distilled water containing 0.1% TFA (95−55%). The elution peaks were detected at 220 nm, concentrated using a rotary evaporator, and lyophilized. Then, the peak with the highest growth-stimulating

activity was subjected to further purification by HPLC system (Waters) using a linear gradient of acetonitrile (10−20%) containing 0.1%TFA and distilled water containing 0.1% TFA at a flow rate of 2.0 mL/min. Fraction F2 samples were eluted at a flow rate of 2.0 mL/min with a linear gradient of acetonitrile (15−50%) containing 0.1% TFA and distilled water containing 0.1% TFA over 40 min. The effluent was monitored at 220 nm, and fractions were collected on the basis of the profile of the eluted peptides and evaporated before growth-promoting activity tests.

Peptide Identification. The final purified active peptides were analyzed for amino acid sequence and molecular mass determination using an online LC-MS/MS system. The peptide obtained from HPLC separation was dissolved in distilled water and analyzed by HPLC (Agilent, Santa Clara, CA) connected online to an HCTplus ion trap instrument (Bruker Daltonic, Bremen, Germany). A volume of 5 μL of purified fraction was loaded onto a 150 mm × 2.0 mm Luna 5 μm C18 column (Phenomenex, Torrance, CA). After injection of the fraction H-2-A samples, the elution was performed with a linear gradient of acetonitrile (0−50%) containing 0.1% TFA and distilled water containing 0.1% TFA, at a flow rate of 0.2 mL/min. For the analysis of fraction F2-b and fraction F2-c, the elution was performed with a linear gradient of acetonitrile (10−55%) containing 0.1% TFA and distilled water containing 0.1% TFA. A part of the flow at 100 μL/min was directed into the mass spectrometer (Bruker Daltonics, Bremen, Germany), which was operated in the positive electrospray ionization (ESI^+) mode, via the electrospray interface. The drying and ESI nebulizing gas used high-purity nitrogen. The following tuning parameters were used: nebulizing gas pressure, 20 psi; drying gas flow rate, 12 L/min; dry gas temperature, 300 °C. Spectra were recorded over the mass/charge (m/z) range of 100−1500 Da. About five spectra were averaged for both the MS and multiple MS (MS/MS) analysis modes. Peptide sequencing was performed by processing the MS/MS spectra using BioTools (version 3.0; Bruker Daltonics).

Effect of the Purified Peptides and Free Amino Acids on the Viable Counts and Lactic Acid Yield of Bacteria. To determine the influence of the purified peptides on the growth of the mixed cultures of St and Lb, the peptides were supplemented into MRS broth at a protein concentration of 0.1, 0.3, or 0.5% (w/w), respectively. A mixture of free amino acids, which was the same in amino acid composition of the purified peptide, was supplemented into MRS broth at the same concentration of the peptide selected. All of the fermentations were carried out as above.

Bacterial Enumeration. One milliliter of MRS broth supplemented with the purified peptide after 24 h of incubation was diluted with 9 mL of 0.1% peptone water and mixed uniformly. Subsequent serial dilutions were prepared and viable counts enumerated using a pour-tubed technique. The population of St was enumerated on ST agar by incubating the tubes aerobically at 37 °C for 48 h. Acidified MRS agar at pH 5.4 with anaerobic incubation at 37 °C for 72 h was used for the enumeration of Lb.[20] The bacterial counts were expressed as log CFU/mL.

Lactic Acid Concentration. The quantification of lactic acid concentrations was done using a HPLC system (Waters) equipped with a dual λ absorbance detector at 210 nm. One milliliter of MRS broth supplemented with the purified peptide after 24 h of incubation was filtered using a membrane filter (0.45 μm pore size, Sartorius, German) before it was subjected to HPLC. The quantification was performed on an XBridge C18 (4.6 mm × 250 mm, 5 μm) column (Waters), with a run time of 20 min. The mobile phases were used with 10 mM NaH_2PO_4 (pH 2.0) as mobile phase at a flow rate of 0.5 mL/min.

Statistical Analysis. Analysis of variance and significant difference were conducted to identify differences among means by one-way ANOVA using SPSS software (version 13.0 for Windows, SPSS Inc., Chicago, IL). The Student−Newman−Keuls test was used for comparison of mean values among treatments and to identify significant differences ($p < 0.05$) among treatments.

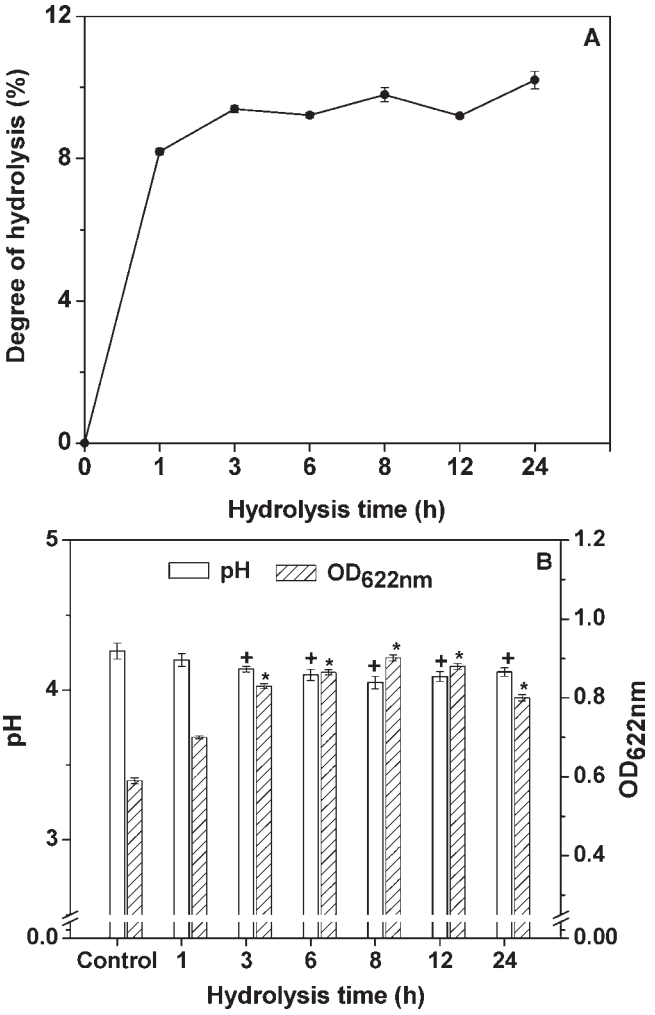

Figure 2. (A) Progress of hydrolysis of casein treated with papain. (B) The pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of six CH samples hydrolyzed for different times. Data are expressed as the mean value ± SD of three independent experiments. (∗) $p < 0.05$ versus the control at OD_{622nm}; (+) $p < 0.05$ versus the control group at pH.

RESULTS AND DISCUSSION

Evolution of Growth-Stimulating Activity during Casein Hydrolysis. The progress of the hydrolysis of sodium caseinate with papain was monitored by taking samples at different intervals. The DH of the hydrolysates was analyzed using the TNBS method, and the growth-stimulating activities of hydrolysates with different DH values were determined. As shown in Figure 2A, the hydrolysis of casein with papain proceeded rapidly during the first 1 h and then the slowed to achieve a final DH of 10.2% after 24 h. This indicated that most peptides were released during the first hour of hydrolysis. Although the biological activity that St and Lb perform in the process of fermentation is very complex,[21] we mainly focused on the effect of stimulators on the growth performance of the LAB in this study. The addition of the casein protein hydrolysates treated with papain for 8 h (CH8) to MRS broth resulted in the lowest pH and the

Table 1. Molecular Weight Distribution of the Casein Hydrolysates (CH) Prepared with Papain during Different Times (1, 3, 6, 8, 12, and 24 h)

	peak area[a] (%)					
mol wt (kDa)	CH1	CH3	CH6	CH8	CH12	CH24
>10	30.4 ± 1.8	28.3 ± 2.2	22.7 ± 1.4	20.6 ± 1.1	20.4 ± 1.3	15.4 ± 3.6
10−5	37.9 ± 3.1	33.3 ± 1.3	27.7 ± 2.0	25.1 ± 0.5	21.1 ± 2.8	33.7 ± 1.7
5−3	25.5 ± 2.4	29.7 ± 1.2	38.1 ± 2.6	40.9 ± 0.3	45.5 ± 3.1	44.7 ± 1.3
3−1	4.2 ± 0.9	5.1 ± 1.0	5.9 ± 0.7	6.6 ± 0.3	6.0 ± 1.9	5.2 ± 0.6
<1	2.0 ± 0.3	4.6 ± 0.9	5.6 ± 1.4	6.8 ± 0.6	6.0 ± 2.2	4.0 ± 1.1

[a] The values represent the mean of three independent experiments ± SD.

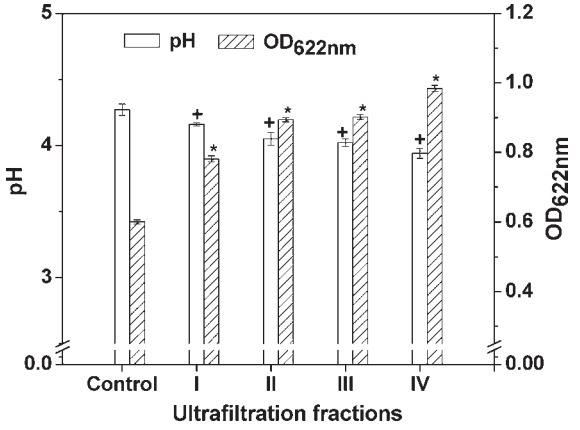

Figure 3. pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of four fractions obtained after ultrafiltration: MWCO-I, M > 10 kDa; MWCO-II, 5 kDa < M < 10 kDa; MWCO-III, 3 kDa < M < 5 kDa; MWCO-IV, M < 3 kDa. Data are expressed as the mean value ± SD of three independent experiments. (∗) $p < 0.05$ versus the control at OD_{622nm}; (+) $p < 0.05$ versus the control group at pH.

highest OD_{622nm} compared with the other hydrolysates (Figure 2B); that is, the hydrolysates with the maximum growth stimulating activity for the bacteria were CH8. From 8 to 24 h, as the hydrolysis time increased, the growth-stimulating activity of the hydrolysates had a tendency to decrease. Obviously, further treatment could result in the breakdown of growth-stimulating peptides into free amino acids or cause the aggregation of those peptides into polypeptides,[22] which could reduce the growth-stimulating activity of the hydrolysates. Contreras et al. also found that whey protein hydrolysates generated by thermolysin after 8 h at 80 °C showed higher antioxidant activity than the hydrolysates after 16 and 24 h.[23] It has also been reported that the ACE inhibitory activity of whey protein hydrolysates decreases with longer hydrolysis times.[24]

GPC was used to study the molecular weight distribution of the hydrolysates. Table 1 shows that the fractions with molecular weight >3 kDa were the main constituent for each of the six hydrolysates. Considering only the peak areas of the <3 kDa fractions, the order based on those areas was CH8 (13.4%) > CH12 (12.0%) > CH6 (11.5%) > CH3 (9.7%) > CH24 (9.2%) > CH1 (6.2), which was consistent with the order of their growth-stimulating activities. Therefore, the length of the peptides is an important factor affecting the growth of LAB.[25] Appropriate peptides can be absorbed by LAB more rapidly and more extensively than free amino acids.[26] Hence, a higher DH of the casein hydrolysates does not guarantee a higher growth-stimulating activity for yogurt LAB, which was in agreement with the report of Azuma et al.[27]

Ultrafiltration of Protein Hydrolysates Obtained with Papain. To isolate the growth-stimulating peptide(s), CH8 was separated by ultrafiltration into four fractions, MWCO-I (>10 kDa), MWCO-II (5−10 kDa), MWCO-III (3−5 kDa), and MWCO-IV (<3 kDa). The effect of four ultrafiltered fractions on the growth of the LAB was examined, obtaining the results presented in Figure 3. The MRS broth samples supplemented with all of the ultrafiltration fractions exhibited significantly ($p < 0.05$) lower pH values and higher OD_{622nm} values than the control. Furthermore, MWCO-IV showed the lowest pH and the highest OD_{622nm}, thus promoting a higher growth of LAB. Bouhallab et al.[15] also found that the growth-promoting activity for *Lactococcus lactis* of the peptides with molecular weight <3 kDa was much stronger than that of the peptides with molecular weight of approximately 6 kDa. The MWCO-IV fraction accounted for 13.31% of protein of the total hydrolysates and was thus selected for further purification.

Macroporous Adsorption Resin Chromatography of Fractions Obtained from Ultrafiltration. MWCO-IV was fractioned into three portions by macroporous adsorption resin chromatography (Figure 4). Because the column used was packed with macroporous adsorption resin, peptides with different hydrophilic or hydrophobic properties can be separated by gradient elution with different concentrations of ethanol. As shown in Figure 4, the growth-stimulating activity became lower when the fractions were eluted by higher concentrations of ethanol, suggesting that relatively hydrophilic compounds were responsible for the higher growth-stimulating activity for yogurt LAB. Etoh et al.[28] also reported that peptides with highly hydrophilic residues display higher growth activity for *Bifidobacterium*.

Gel Filtration Chromatography of Fractions Pooled from Macroporous Adsorption Resin Chromatography. Fraction A with the strongest growth-stimulating activity was separated by gel filtration chromatography, and two peaks (G-I and G-II) were collected (Figure 5). Fraction B with powerful growth-stimulating activity was separated into the three fractions F1, F2, and F3 (Figure 5). Fraction G-I displayed much more powerful growth-stimulating activity than G-II did, and fraction F2 displayed much stronger growth-stimulating activity than F1 or F3 did (Figure 5). Compared with the control, the pH value of MRS broth sample supplemented with G-I or F2 significantly decreased ($p < 0.01$), whereas the OD_{622nm} value increased significantly ($p < 0.01$).

RP-HPLC purification of Fractions Pooled from Gel Filtration Chromatography. The active fractions G-I and F2 were further separated by RP-HPLC. Five fractions (H1, H2, H3, H4, and H5) and four fractions (F2-a, F2-b, F2-c, and F2-d) were

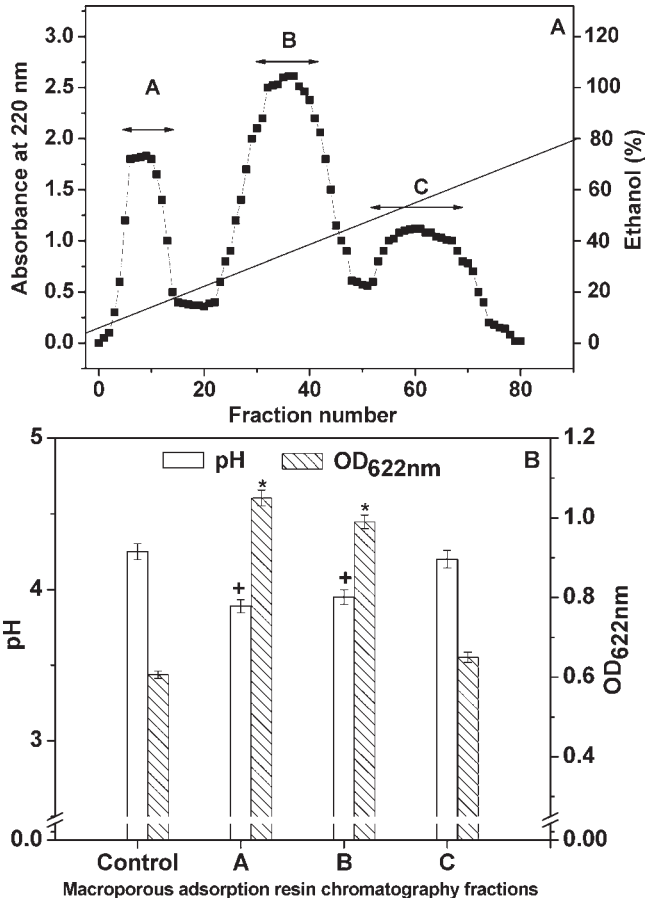

Figure 4. (A) Macroporous adsorption resin chromatography of the fraction MWCO-IV obtained from ultrafiltration. (B) pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of three fractions (A−C) obtained after macroporous adsorption resin chromatography. Data are expressed as the mean value ± SD of three independent experiments. (∗) $p < 0.05$ versus the control at OD_{622nm}; (+) $p < 0.05$ versus the control group at pH.

obtained, respectively (Figure 6A,B). Fraction H2 had a significantly higher growth-stimulating activity in comparison with the other four fractions (Figure 6C). For further purification, fraction H2 was rechromatographed by the second RP-HPLC. Further chromatographic separation of H2-A using analytical RP-HPLC showed only one symmetrical peak, suggesting reasonable homogeneity (data not shown). Compared with F2-a or F2-b, the pH value of MRS broth sample supplemented with F2-b or F2-c significantly decreased ($p < 0.01$), whereas the OD_{622nm} value increased significantly ($p < 0.01$) (Figure 6D). In addition, further chromatographic separation of F2-b or F2-c using analytical RP-HPLC showed only one, symmetrical peak, respectively (data not shown). Thus, the three purified peptides were designated H2-A, F2-b, and F2-c, respectively.

Identification of Growth-Stimulating Peptide. To identify the growth-stimulating peptides responsible for the activity observed, H2-A, F2-b, and F2-c were sequenced by LC-MS/MS. The molecular mass of peptide H2-A was determined to be 800.3 Da (Figure 7A). MS/MS analysis (Figure 7B) showed that the major ion products generated upon collision-induced dissociation of peptide H2-A agreed well with the *b*-type ions and *y*-type ions expected from theoretical MS/MS fragmentation of a peptide

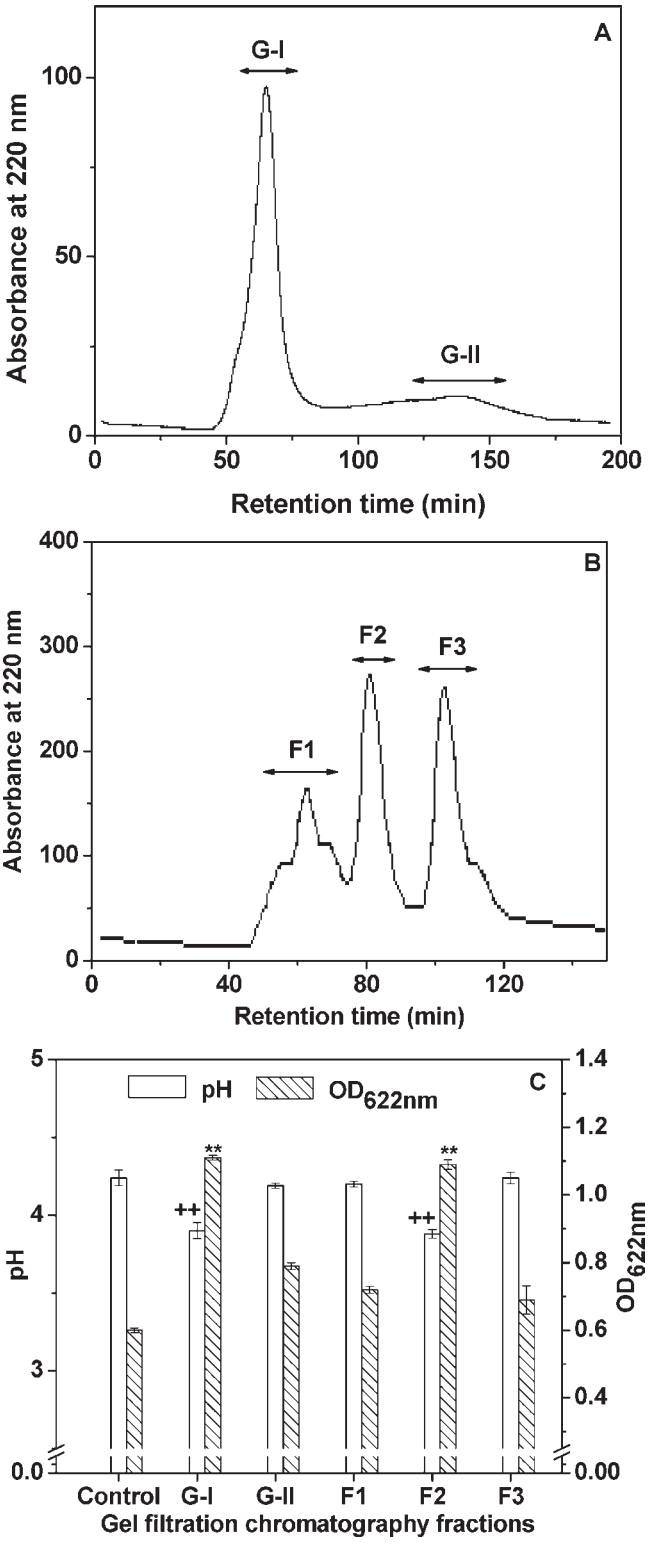

Figure 5. (A) Gel filtration chromatography of the fraction A obtained after Macroporous adsorption resin chromatography. (B) Gel filtration chromatography of fraction B obtained after macroporous adsorption resin chromatography. (C) pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of five fractions (G-I, G-II, F1, F2, and F3) obtained after gel filtration chromatography. Data are expressed as the mean value ± SD of three independent experiments. (∗∗) $p < 0.01$ versus the control at OD_{622nm}; (++) $p < 0.01$ versus the control group at pH.

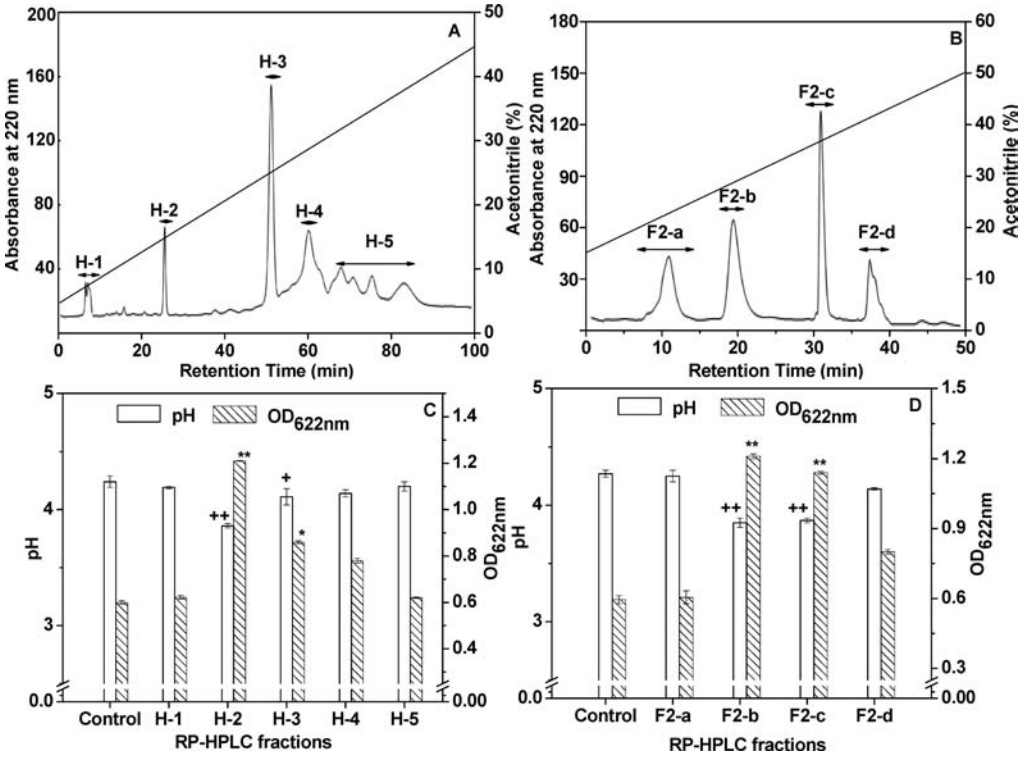

Figure 6. (A) RP-HPLC separation of the fraction G-I obtained after gel filtration chromatography. (B) RP-HPLC separation of fraction F2 obtained after gel filtration chromatography. (C) pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of five fractions (H1, H2, H3, H4, and H5) obtained after RP-HPLC separation. Data are expressed as the mean value ± SD of three independent experiments. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the control at OD_{622nm}; (+) $p < 0.05$ and (++) $p < 0.01$ versus the control group at pH. (D) pH and OD_{622nm} values of MRS broth after 24 h of incubation of yogurt starter culture with supplementation of four fractions (F2-a, F2-b, F2-c, and F2-d) obtained after RP-HPLC separation. Data are expressed as the mean value ± SD of three independent experiments. (∗) $p < 0.05$ and (∗∗) $p < 0.01$ versus the control at OD_{622nm}; (+) $p < 0.05$ and (++) $p < 0.01$ versus the control group at pH.

corresponding to residues 29−35 of bovine α_{S2}-casein, with the sequence Asn-Pro-Ser-Lys-Glu-Asn-Leu. The two purified peptides, F2-b and F2-c, had molecular masses of 665.1 and 650.4 Da, respectively (Figure 7C,D). MS/MS analysis of F2-b (Figure 7E) indicated that the major product ions generated upon collision-induced dissociation of F2-b agreed well with the *b*-type ions and *y*-type ions expected from theoretical MS/MS fragmentation of a peptide corresponding to residues 181−186 of bovine α_{S1}-casein, with a sequence of Asp-Ile-Pro-Asn-Pro-Ile. Also, peptide F2-c corresponded to residues 103−108 of bovine α_{S2}-casein, with the sequence Pro-Ile-Val-Leu-Asn-Pro (Figure 7F).

The heptapeptide H2-A and the hexapeptides F2-b and F2-c are three novel peptides with growth-stimulating activity for LAB, not previously reported. Casein-derived peptides consisted of seven or six amino acid residues including Arg-Pro-Lys-His-Pro-Ile-Lys, Ala-Val-Pro-Tyr-Pro-Gln-Arg, Met-Ala-Ile-Pro-Pro-Lys- Lys, Arg-Tyr-Leu-Gly-Tyr-Leu-Glu, Arg-Pro-Lys-His-Pro-Ile, Arg-Tyr-Leu-Gly-Tyr-Leu, Tyr-Leu-Gly-Tyr-Leu-Glu, Leu-Ala-Tyr-Phe-Tyr-Pro, Thr-Thr-Met-Pro-Leu-Trp, and Phe-Ala-Leu-Pro-Gla-Tyr and have been shown to display antihypertensive, ACE-inhibitory, antioxidant, opioid agonist, and immunomodulatory activities.[29−36]

All growth-stimulating peptides isolated from the papain digest of bovine casein in this work contained fewer than eight amino acid residues, which was in accordance with the general finding that peptides with four to eight residues exhibited stronger growth-stimulating activity for LAB than their parent native proteins or larger polypeptides did.[37,38] It is reported that St primarily requires Glu, His, and Met, as well as Leu, Cys, Val, Ile, Trp, Arg, and Tyr, for growth.[39,40] Poch and Bezkorovainy[12] proved that sulfhydryl groups contained in peptides are essential for growth-stimulating activity of peptides for *Bifidobacteria*. Peptide H2-A contained the two mentioned amino acid residues (Glu and Leu). Peptides F2-b and F2-c both had an Ile residue, and the residues of Leu and Val existed in peptide F2-c. The growth-stimulating activity order of these three peptides was H2-A > F2-b > F2-c (Figure 6C,D). Etoh et al. previously reported that the decapeptide Ala-Thr-Pro-Glu-Lys-Glu-Glu-Pro-Thr-Ala, which was purified from natural rubber serum powder, displayed growth-promoting activity for *Bifidobacterium*.[28] The peptide did not contain any sulfhydryl groups. The results indicated that sulfhydryl groups would not be indispensable for growth-promoting activity for LAB. Generally, these findings on structure−function relationship suggest that the growth-promoting activities of peptides for LAB depend on not only the length of peptides but also certain amino acid sequences.

Viable Counts and Lactic Acid Yield of the Bacteria. The effect of the purified peptides (H2-A, F2-b, and F2-c) at different protein concentrations on the growth of St and Lb is presented in Table 2. In all cases, the addition of the peptides at a protein concentration of 0.3% resulted in the highest OD_{622nm} and lowest pH in comparison with other protein concentrations. Compared with the control, the biomass yield of the bacteria was respectively enhanced by 193.3, 166.7, or 151.7% with the addition of H2-A, F2-b, or F2-c at a protein concentration of 0.3%. Gomes et al. previously found that

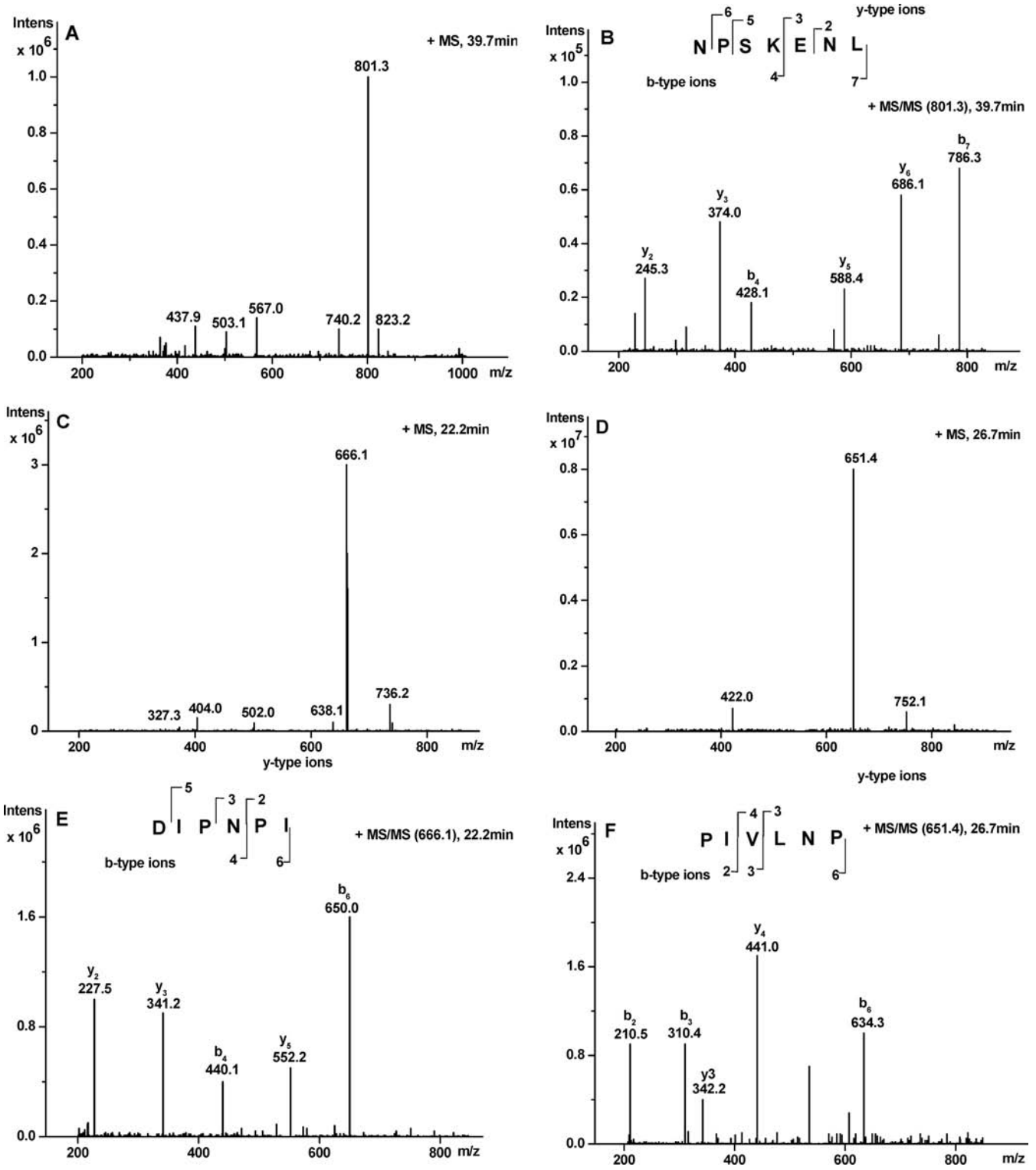

Figure 7. Identification of the purified peptides with growth-promoting activity: (A) Mass spectrum of chromatographic fraction H-2 shown in Figure 6A. (B) MS/MS spectrum of singly charged ion *m*/*z* 801.3. The sequence is displayed with the fragment ions observed in the MS/MS spectrum. For clarity, only *b* and *y* ions are labeled. (C) Mass spectrum of chromatographic fraction F2-b in Figure 6B. (D) Mass spectrum of chromatographic fraction F2-c in Figure 6B. (E) MS/MS spectrum of singly charged ion *m*/*z* 666.1. The sequence is displayed with the fragment ions observed in the MS/MS spectrum. For clarity, only *b* and *y* ions are labeled. (F) MS/MS spectrum of singly charged ion *m*/*z* 651.4. The sequence is displayed with the fragment ions observed in the MS/MS spectrum. For clarity, only *b* and *y* ions are labeled.

microorganisms use growth stimulators up to a maximum concentration, beyond which additional growth stimulators are ineffective.[41]

Three mixtures of free amino acids (M-a, Asn, Glu, Leu, Lys, Pro, and Ser, 2:1:1:1:1:1:1; M-b, Asp, Ile, Pro, and Asn, 1:2:2:1;

Table 2. pH and OD_{622nm} Values of MRS Broth after 24 h of Incubation Supplemented with the Purified Peptide at Different Protein Concentrations (0.1, 0.3, and 0.5%)

sample	addition (%)	OD_{622nm}[a]	pH[a]
control		0.60 ± 0.02	4.26 ± 0.03
H2-A	0.1	0.96 ± 0.01*	3.96 ± 0.02*
	0.3	1.76 ± 0.01**	3.75 ± 0.04**
	0.5	1.25 ± 0.02**	3.82 ± 0.05**
F2-b	0.1	0.90 ± 0.01*	3.99 ± 0.02*
	0.3	1.60 ± 0.01**	3.78 ± 0.03**
	0.5	1.21 ± 0.01**	3.85 ± 0.04**
F2-c	0.1	0.88 ± 0.02*	3.99 ± 0.04*
	0.3	1.51 ± 0.01**	3.80 ± 0.03**
	0.5	1.14 ± 0.02**	3.87 ± 0.02**

[a] The values represent the mean of three independent experiment ± SD. (*) $p < 0.05$ versus the control; (**) $p < 0.01$ versus the control.

Table 3. Effect of Purified Peptides and Free Amino Acids on the Viable Counts and Lactic Acid Yield of LAB

sample[a]	Lb counts log (CFU/mL)	St counts log[b] (CFU/mL)	latic acid concn[b] (g/L)
control	7.8 ± 0.1	8.2 ± 0.1	12.4 ± 0.9
H2-A	7.8 ± 0.0	10.0 ± 0.1**	25.4 ± 1.0**
M-a	7.7 ± 0.0	8.8 ± 0.0*	16.7 ± 0.8*
F2-b	7.7 ± 0.1	9.5 ± 0.0**	24.20 ± 0.9**
M-b	7.7 ± 0.0	8.8 ± 0.0*	17.0 ± 0.8*
F2-c	7.8 ± 0.0	9.6 ± 0.0**	23.6 ± 0.3**
M-c	7.7 ± 0.0	8.9 ± 0.1*	17.3 ± 0.8*

[a] H2-A, Asn-Pro-Ser-Lys-Glu-Asn-Leu; M-a, Asn, Glu, Leu, Lys, Pro, and Ser, 1:1:1:1:1:1; F2-b, Asp-Ile-Pro-Asn-Pro-Ile; M-b, Asp, Ile, Pro, and Asn, 1:2:2:1; F2-c, Pro-Ile-Val-Leu-Asn-Pro; M-c, Pro, Ile, Val, Leu, and Asn, 2:1:1:1:1:1. [b] The values represent the mean of three independent experiment ± SD. (*) $p < 0.05$ versus the control; (**) $p < 0.01$ versus the control.

M-c, Pro, Ile, Val, Leu, and Asn, 2:1:1:1:1) were respectively the same in amino acid composition with the amino acid residues of H2-A, F2-b, and F2-c. As shown in Table 3, the St counts were higher than Lb in all cases, which could be explained due to the fact that St was much more competitive in using the nutrients than Lb.[8] In addition, the number of St was significantly ($p < 0.05$) enhanced in the presence of H2-A, F2-b, or F2-c compared with the control, which could be attributed to the fact that St could grow better on the media with the addition of peptides because of its limited proteolytic activity.[7] However, there was little difference in Lb counts with or without the purified peptide supplement because Lb is much more proteolytic than St and can degrade casein with the liberation of low molecular weight peptides and free amino acids, which are required for their growth and survival.[8] Oliveira et al. also found that the effect of casein protein hydrolysates on the viability of different LAB varied.[20] The higher pH value and higher population of the bacteria were obtained with the addition of F2-c in comparison with F2-b (Figure 6B and Table 3), indicating that the pH decrease had no correlation with the viable enumeration of the bacteria, which was previously observed by Zotta et al.[42] Lactic acid fermentation with St and Lb was found to be homolactic and primarily growth associated. As shown in Table 3, with the supplementation of H2-A, F2-b, or F2-c lactic acid yield of the strains was respectively increased by 104.8, 95.2, or 90.3% compared with the control. Because these fermentations were under uncontrolled conditions, pH values decreased with lactic acid accumulation. In addition, the count of St was obviously promoted by the addition of the peptides and the Lb counts changed little. Thus, it could be concluded that the rise in lactic acid yield was mainly caused by the metabolism of St. As shown in Table 3, the addition of H2-A, F2-b, or F2-c resulted in significantly ($p < 0.05$) higher lactic acid concentration and bacterial counts than those of free amino acids. This could be explained by the idea that the peptides can be absorbed more rapidly and extensively by the LAB than the free amino acids themselves.[7,26] On the basis of the results obtained from this work, more studies need to be done to clarify the growth-stimulating mechanisms by which these peptides exert their effect.

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ABBREVIATIONS USED

Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; CFU, colony-forming units; Cys, cysteine; Gln, glutamine; Glu, glutamic acid; Gly, glycine; His, histidine; Ile, isoleucine; LAB, lactic acid bacteria; Lb, *Lactobacillus delbrueckii* subsp. *bulgaricus*; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; St, *Streptococcus thermophilus*; TFA, trifluoroacetic acid; Trp, tryptophan; Tyr, tyrosine; Val, valine.

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