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Research Article

A Novel Lactic Acid Bacteria Growth-stimulating Peptide from Broad Bean (*Vicia faba* L.) Protein Hydrolysates

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Abstract: In this study, broad bean protein hydrolysates (BPH) produced by alcalase with strong-stimulating activity for lactic acid bacteria (LAB) was first time reported. In order to obtain the key peptide that have growth-stimulating activity for lactic acid bacteria (LAB), gel filtration chromatography and Reverse Phase High Performance Liquid Chromatography (RP-HPLC) were applied to isolate and purify the peptides from BPH. Finally, F_{4-2} elicited the highest activity for LAB, corresponding to amino acid sequence Ser-Ala-Gln (304.10Da) was identified by Matrix-Assisted Laser Desorption/Ionization Time of Flight Mass Spectrometry (MALDI-TOF/TOF MS/MS). Thus, this study shows that broad bean peptide is a good source to promote the LAB growth and this function is reported for the first time.

Keywords: Broad bean peptide, growth-stimulating, identification, lactic acid bacteria (LAB), purification

INTRODUCTION

The broad bean (*Vicia faba* L.) originated in the Mediterranean, is now grown in many parts of the world. China is the largest grower today, producing about 60% of the world's supply (Bashir *et al*., 2013). Due to their high nutritive and biological value (Lisiewska *et al*., 2007), broad bean is ranked among the world's most competitive grain legume crops, nutritionally equivalent to soybean products (Arogundade *et al*., 2006). Besides a plentiful supply of starch, broad bean is a protein rich legume. However, broad bean proteins are usually used in the snack food industry or animal feed (Al-Kaisey *et al*., 2003; Giménez *et al*., 2013). So novel processing methods are required for broad bean proteins under-utilized potentials to be fully exploited. An interesting alternative would be to transform proteins into biologically active peptides by protease treatments.

In our study, we found that broad bean protein hydrolysates (BPH) can stimulate the LAB growth. The LAB have long been used for fermentation of foods such as milk, vegetables, meat and so on. Furthermore, many research reported that the consumption of LAB may affect the composition of indigenous microflora and have beneficial effects on the human health which mainly depends on the number of viable microbial cells that reach the human gut (Champagne *et al*., 2005; Zhang *et al*., 2011a). So the amount of LAB present in yogurt or fermented milk must be high (Robitaille, 2013). 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. However, commercial products often contain less LAB than the minimum number required (Zhang *et al*., 2011a). In recent years, a large number of biologically active peptides with growth-promoting activity for LAB have been isolated from milk and soy bean, including milk hydrolysates (Gomes *et al*., 1998), whey protein (Bury *et al*., 1998), casein hydrolysates (Desmazeaud and Hermier, 1972; Zhang *et al*., 2010, 2011a, b), casein Glycomacro-Peptide (GMP) (Brück *et al*., 2006), soy protein hydrolysates (Bai *et al*., 2012) and other sources (Desmazeaud and Hermier, 1973; Lazzi *et al*., 2013). Although there have been many reports on growth-promoting factors for LAB, however very little is known about the growth-stimulating factors from broad bean origin. Therefore, in this study, we report the effect of the broad bean protein hydrolysates (BPH) as enhancers for LAB growth and furthermore, a novel lactic acid bacteria (LAB) Growth-Stimulating Peptide (LGSP) was isolated and its amino acid sequence was determined.

MATERIALS AND METHODS

Material: Broad bean (*Vicia faba* L.) was purchased from local market. Alcalase (2×10^5 U/g) was purchased

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from Tianjin Noao Science and Technology Development Co., Ltd.

Preparation of broad bean protein hydrolysates (BPH): Broad bean protein (BBP) was prepared according to the method of Wang *et al*. (2010). Broad bean protein was resuspened in the distilled water to obtain a solution containing 6% (w/v) of protein, then homogenised at 90°C for 15 min. The samples were digested with alcalase (2%, w/v of substrate) at 37°C for 1 h. During the reaction, the pH of the mixture was maintained at pH 7 by continuous addition of 2 N NaOH solution. The enzymatic hydrolysis was stopped by heating the solutions at 85°C during 20 min. The protein hydrolysates were then centrifuged at 5000 g for 20 min to separate soluble and insoluble fractions. Finally, the soluble fraction was freeze-dried. The powders were used as broad bean protein hydrolysates (BPH).

Determination of the degree of hydrolysis: The degree of hydrolysis (DH), defined as the percent ratio of the number of peptide bonds broken (h) to the total number of peptide bonds per unit weight (h_{tot}), in each case, was calculated from the amount of base (NaOH) added to keep the pH constant during the hydrolysis (Adler-Nissen, 1986) as given below:

$$DH(\%) = \frac{h}{h_{tot}} \times 100 = \frac{B \times Nb}{Mp} \times \frac{1}{\alpha} \times \frac{1}{h_{tot}} \times 100$$

where, B is the amount of NaOH consumed (mL) to keep the pH constant during the reaction, Nb is the normality of the base, MP is the mass (g) of protein (N×6.25) and α is the average degree of dissociation of the α-NH_2 groups released during hydrolysis expressed as:

$$\alpha = \frac{10^{pH-pK}}{1+10^{pH-pK}}$$

where, pH and pK are the values at which the proteolysis was conducted. The total number of peptide bonds (h_{tot}) in a broad bean protein concentrate was assumed to be 7.75 meq/g (Adler-Nissen, 1986).

Growth-promoting activity test: Before the fermentation experiments, in order to get an inoculum, *Lactobacillus delbrueckii* subsp., *bulgaricus* (Lb) was fermented in the seed cultivation medium (12% skim milk, w/v) until it coagulated. Then the fermentation medium (MRS medium) was prepared by inoculating 8 mL of culture medium with 3~5% (v/v) seed culture. The incubation of Lb was performed in an anaerobic incubator at 37°C, 3~5% (v/v) of inoculum was transferred to the MRS medium containing BBP or BPH. The growth curve of Lb was obtained by plotting the OD_{600} nm and pH values of the fermentation medium at 0, 4, 8, 12, 16, 20 and 24 h, respectively. The control sample consisted of Lb incubated in MRS medium without any additives. Fermentation experiments were conducted in triplicate. The optimum broad bean protein hydrolysates concentration was selected for further experiments.

The number of viable Lb count was assessed using MRS agar plates. Culture aliquots (1 mL) were serially diluted with 0.85 g/L of physiological saline and then 0.1 mL of the diluted aliquots were poured onto MRS agar plates. The MRS agar plates were incubated in an anaerobic incubator at 37°C for 48 h. Following the 48 h incubation, plates with 30~300 single bacterial colonies were counted.

Purification of LAB Growth-Stimulating Peptide (LGSP) from BPH: The freeze-dried hydrolysates were dissolved in distilled water at a concentration of 5% (w/v), then 2 mL solution was fractionated on a Sephadex G-25 gel filtration column (1.6×40 cm), pre-equilibrated and eluted with distlled water. Fractions (1.5 mL each) were collected at a flow rate of 0.3 mL/min. The elution was monitored at 220 nm. Fractions were collected, freeze-dried and growth promoting activity of fractions was determined. The peptide mixture in fraction F_4 from Sephadex G-25, which exhibited the highest growth-stimulating activity, was dissolved in distilled water, filtered through 0.22 μm filters and then separated by RP-HPLC on a Waters μB ondapak C_{18} column (5 μm, 250×4.6 mm) attached with Shimadzu LC-10ATVP HPLC system. Instrumental control, data collection and data processing were carried out by SPD-10AVP Diode Array Detector (DAD). Fraction F_4 was eluted with an isocratic elution with eluting solvent 90% A (distlled water) and 10% B (acetonitrile) for 20 min at a flow rate of 1 mL/min. The elution was detected at 215 nm. The fractions were concentrated by vacuum-rotary evaporator at 35°C, peptides concentration and growth promoting activity was determined.

Identification of the LAB Growth-Stimulating Peptide (LGSP) by MALDI-TOF/TOF MS/MS analysis of amino acid sequence: The purified peptide was co-crystallised in a matrix of α-cyano-4-hydroxycinnamic acid and analysed by a Matrix-Assisted Laser Desorption Ionisation (MALDI) mass spectrometer with an ABI 4700 TOF-TOF Proteomics Analyser (Applied Biosystems, Foster City, CA). Spectra were recorded over the mass/charge (m/z) range of 300 to 1000 Da. The MALDI-TOF/TOF MS/MS was run in the positive mode. The amino acid sequence was confirmed by GPS Explorer ^{TM} software as well as manual calculation.

Statistical analysis: All the tests were conducted in triplicate. Data were expressed as means±standard errors. The statistical analysis was performed using SPSS 18.0 software (SPSS Inc., Chicago, IL, USA).

RESULTS

The growth-stimulating activity of BPH: In this study, the effects of BBP and BPH (DH 15.55%) on the growth performance of the LAB were assessed. Figure 1 shows the growth curves of Lb in the MRS medium in the presence or absence of BBP and BPH. The addition of the BPH to MRS medium resulted in the lowest pH and the highest OD_{600} compared with the BBP and control, which increased the viable number of Lb count by 1.0 log cycles (Table 1). This indicates that the BPH was the growth-promoting factor for LAB. In addition, the changes in pH of the fermentation medium were in agreement with number of Lb count while the number of Lb count and pH were remained stable at 20 h. In the course of our experiments, Lb had a 4 h lag phase and stationary phase in BPH medium, which was in consistent with the control MRS medium. This concludes that the BPH enhanced the biomass yield only, but do not have effect on the growth rate.

The optimum BPH concentration on the growth of LAB: The effect of BPH at different concentrations on the growth of Lb is depicted in Fig. 2. The MRS medium supplemented with all of the BPH concentrations exhibited significantly ($p<0.01$) higher OD_{600} values than the control. Furthermore, the addition of the BPH at a concentration of 2 mg/mL resulted in the highest OD_{600} in comparison with other concentrations. However, the OD_{600} values of the fermentation medium decreased when the concentration of the BPH was 3 mg/mL. Previous studies also found that microorganisms use growth stimulators up to a maximum concentration, beyond which additional growth stimulators are ineffective (Etoh *et al*., 2000; Gomes *et al*., 1998; Zhang *et al*., 2010, 2011a). This phenomenon could be due to the following reasons: the increase in short peptides might cause an off-balance in the cell transfer system or might result in a high osmotic pressure which negatively affects for LAB (Gomes *et al*., 1998; Zhang *et al*., 2013).

Isolation and purification of LAB growth-stimulating peptide: Since the BPH exhibited obvious growth-promoting activity for LAB, it was then separated sequentially with Sephadex G-25 gel filtration column and reversed-phase HPLC. Throughout the purification process, the growth-stimulating activities of all fractions were estimated by turbidimetric measurements (OD_{600} nm).

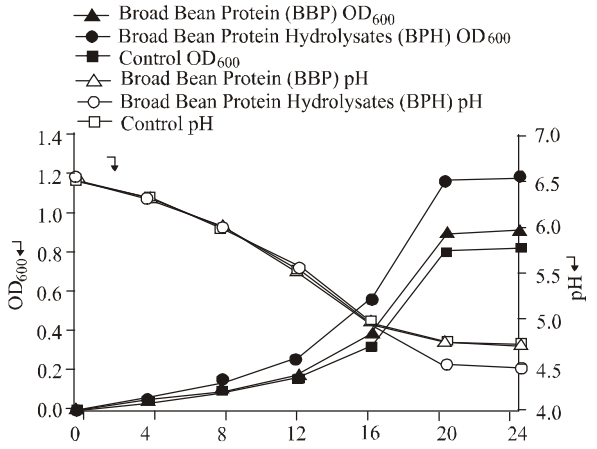


Fig. 1: Growth curves of *Lactobacillus delbrueckii* subsp., *bulgaricus* incubated in MRS medium with 2 mg/mL of broad bean protein (BBP) and broad bean protein hydrolysates (BPH) for 24 h
▲: Broad bean protein (BBP) OD_{600}; ●: Broad bean protein hydrolysates (BPH) OD_{600}; ■: Control OD_{600}; △: Broad bean protein (BBP) pH; ○: Broad bean protein hydrolysates (BPH) pH; □: Control pH

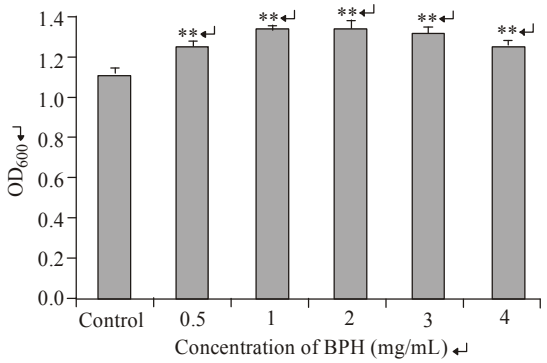


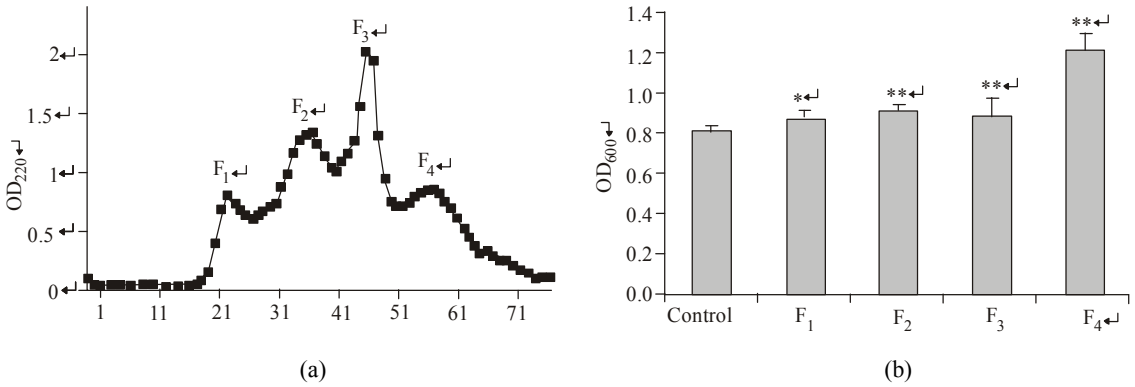
Fig. 2: OD_{600} values of MRS medium containing different concentrations of BPH after 20 h of fermentation
Values presented are the mean of triplicate analyses; **: $p<0.01$ versus the control

Table 1: The viable number of *Lactobacillus delbrueckii* subsp., *bulgaricus* with different additives in MRS medium

	Control	BBP	BPH
Viable number of *Lactobacillus delbrueckii* subsp. *bulgaricus* (log (CFU/mL))	8.04±0.1	8.06±0.0	9.35±0.0

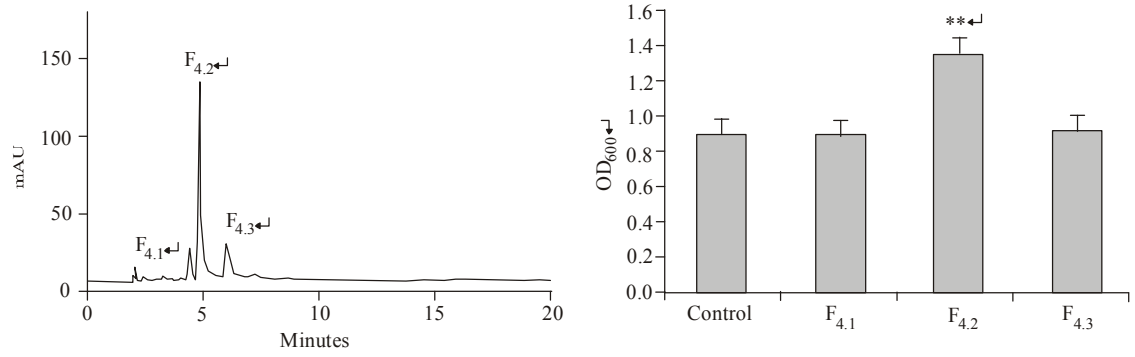
The MRS agar plates were incubated in an anaerobic incubator at 37°C for 48 h; Following the 48 h incubation, plates with 30~300 single bacterial colonies were counted; The bacterial counts were expressed as log (CFU/mL)

Isolation and purification of LAB growth-stimulating peptide by sephadex G-25 column: Figure 3 illustrates the column chromatographic profiles of the BPH and their promoting effects. The BPH was initially separated using size exclusion



(a) (b)

Fig. 3: Purification of BPH; (a): the sephadex G-25 column chromatographic profiles of the BPH. The column (1.6×40 cm) was equilibrated and eluted with distilled water at a flow rate of 0.3 mL/min; (b): lactic acid bacteria growth-stimulating activities of the separated fractions
Values presented are the mean of triplicate analyses; *: p<0.05 versus the control; **: p<0.01 versus the control



(a) (b)

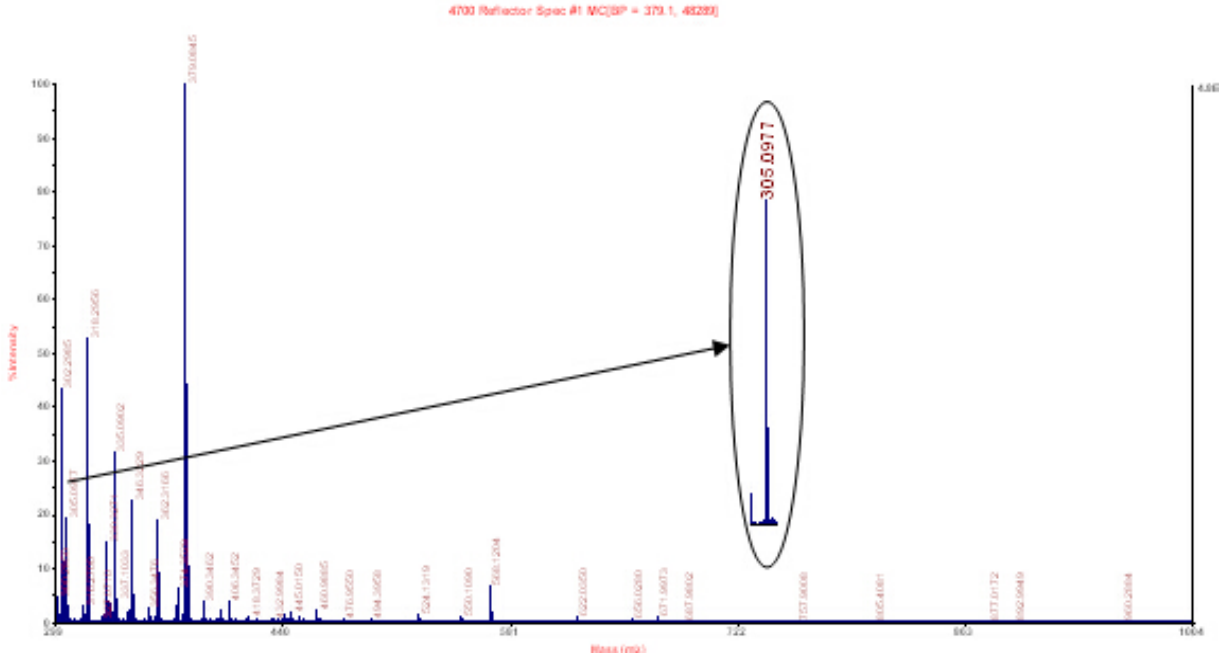
Fig. 4: Purification of F_4; (a): reversed-phase HPLC pattern on a C_{18} column of the F_4 fraction obtained from sephadex G-25 gel filtration. Flow rate, 1 mL/min; monitoring absorbance, 215 nm; (b): lactic acid bacteria growth-stimulating activities of the eluted peaks was measured
Values presented are the mean of triplicate analyses; **: p<0.01 versus the control

chromatography on Sephadex G-25 column and fractionated into four portions (F_1-F_4) (Fig. 3a). As shown in Fig. 3b, all fractions (at 0.5 mg/mL) exhibited significantly ($p<0.05$) higher OD_{600} values than the control. Furthermore, F_4 showed the highest OD_{600}, thus promoting a higher growth of Lb and was selected for further purification.

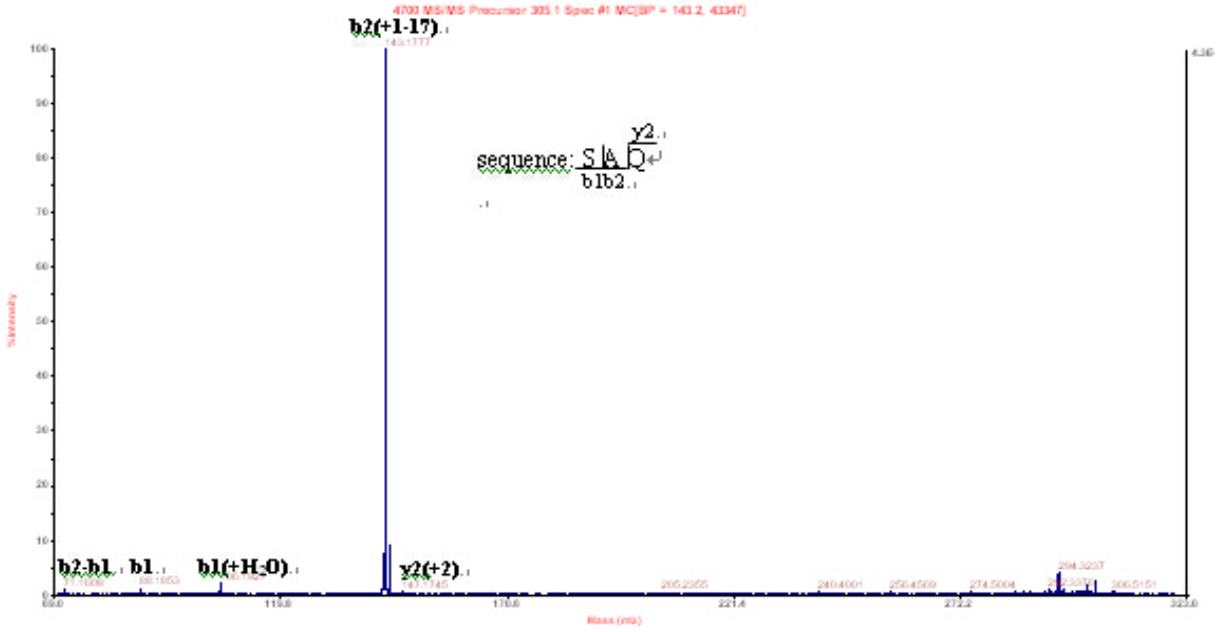
Purification and characterization of LAB growth-stimulating peptide by RP-HPLC: The lyophilized F_4 fraction was further separated by reversed-phase HPLC and fractionated into three major sub-fractions (F_{4-1}, F_{4-2}, F_{4-3}). In this study, the performance conditions of the RP-HPLC were very suitable for commercial use as the isocratic elution system could be used. The elution profile of peptides is depicted in Fig. 4a. Fractions were collected separately through repeated chromatography using reversed-phase HPLC column and concentrated in vacuum prior to testing the growth-stimulating activities. As shown in Fig. 4b, sub-fraction F_{4-2} had a significantly higher growth-stimulating activity in comparison with the other two fractions. Therefore, the molecular weights and amino acid sequence of F_{4-2} by MALDI-TOF/TOF MS/MS was finally identified.

Determination of the amino acid sequence of the purified peptide: Peptide in sub-fractions (F_{4-2}) was analyzed by MALDI mass spectrometer for molecular mass determination and MS/MS for the characterization of peptide (Fig. 5). The mass spectrum showed the molecular mass of the singly charged ions ($[M + H]^+$) was 305.0977. Therefore, the molecular mass of the peptide was determined to be 304.10 Da (Fig. 5a).

Because the software peptide sequencer failed to gain direct amino acid sequences from the MS/MS spectrum (Fig. 5b), each mass signal and corresponding fragmentation spectrum were matched to a single peptide fragment by manual calculation. Therefore, the amino acid sequence of purified peptide F_{4-2} was identified as tripeptide Ser-Ala-Gln by manual analysis and this tripeptide is a novel peptide with growth-



(a)



(b)

Fig. 5: Identification of the purified peptide (F_{4-2}) with growth-promoting activity; (a): mass spectrum of chromatographic fraction F_{4-2}; (b): MS/MS spectrum of singly charged ion m/z 305.10

stimulating activity for LAB that had never been reported.

DISCUSSION

Although there have been many reports on growth-promoting factors for LAB in recent years, very little is known about the growth-stimulating factors from broad bean origin. Therefore, the objective of this study was to analyze the growth-promoting activity of BPH and identify a novel lactic acid bacteria (LAB) Growth-Stimulating Peptide (LGSP) from BPH.

This study presents an interesting analysis about the growth-promoting activity of broad bean peptide.

The results indicated in Fig. 1 and in Table 1 clearly demonstrated that in a BBP or BPH supplemented synthetic MRS medium, BPH possessed an efficient growth promoter for *Lactobacillus bulgaricus* but BBP did not have such effect. The result was in accordance with the general finding that short peptides with 2-10 amino acids exhibit greater bioactive properties than their parent native proteins (Wang *et al*., 2013). Poch and Bezkorovainy (1991) also reported that intact caseins could not stimulate *Bifidobactrium* growth, but κ-casein from caseins by trypsin digestion has significantly growth-promoting activity. The reason for these discrepancies is that the peptides of certain sizes may be transported more readily than their parent native proteins, such as caseins or BBP.

Furthermore, a novel LGSP from BPH was isolated and identified by using chromatographic methods and MALDI mass spectrometer. The growth-stimulating peptide was identified to be a tripeptide, Ser-Ala-Gln. Since this bioactive peptide (Ser-Ala-Gln) has not been reported yet to our best knowledge, it is thought to be a novel active peptide having stimulating effect.

Several stimulatory peptides for bacteria from protein hydrolysates had also been reported and their stimulating activities depended upon their amino acid sequences. They were respectively NPSKENL, DIPNPI and PIVLNP form casein (Zhang *et al*., 2011a), heptapeptide HSQGTFT from glucagon (Desmazeaud and Hermier, 1973) and STADA from β chain of bovine hemoglobin (Zhao *et al*., 1996). Previous works on growth-promoting peptides had also concluded that peptides with 4-8 amino acid residues exhibited stronger growth-stimulating activity for LAB (Zhang *et al*., 2011a). But in the present work, LGSP containing three amino acid is different from other report. The effect of the different peptides depends on the ability of LAB proteolytic enzymes and peptide transport systems (Lazzi *et al*., 2013). LAB have three different systems for transport of free amino acids, di- and tripeptides and oligopeptides (Kunji *et al*., 1996). Although, all these systems are involved into the uptake of essential amino acids from the nutritional medium, the highest rate of uptake is provided by the transporting di- and tripeptides system (Kunji *et al*., 1993; Lazzi *et al*., 2013). Therefore, it could be concluded that tripeptides (SAQ) could be expected to exhibit strong stimulating effect on growth of LAB.

Moreover, in the previous works that *Streptococcus thermophilus* (St) could grow better on the medium with the addition of Lactic acid bacteria growth-stimulating peptide than *Lactobacillus delbrueckii* subsp., *bulgaricus* (Lb) (Zhang *et al*., 2010, 2011a, b). Unlike Lb, the response of St biomass was decreased by addition of BPH or F_{4-2} in our study. The reasons for this adverse effect can be the peptide origin, amino acid composition and the length of peptides differences. LAB showed large species and strain variations in the utilization of amino acids. Therefore, more studies need to be done to clarify the growth-stimulating mechanisms by studying more different LAB in the further research.

CONCLUSION

In the present study, broad bean proteins (BBP) was digested by alcalase to obtain Lactic acid bacteria growth-stimulating hydrolysates (BPH) and a novel tripeptide, SAQ was confirmed to contribute to the growth-stimulating activity. To our knowledge, it is the first record on the promoter potential of the Lactic acid bacteria Growth-Stimulating Peptide (LGSP) from the broad bean, which may be potent for the future food industry. Despite the need for further research in vivo, BPH or LGSP could be considered as suitable natural promoter to stimulate growth for Lactic acid bacteria.

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