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Production of Bioactive Peptides in Milk Using Two Native Strains of *Levilactobacillus brevis*

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Abstract

Background and Objective: Milk proteins are precursors of several biologically active peptides. One of the methods of producing these peptides is fermentation using lactic acid bacteria. The aim of this study was to investigate production of antioxidant and angiotensin-I converting enzyme inhibitory bioactive peptides in cow milk fermented by two strains of *Levilactobacillus brevis*.

Material and Methods: Two strains of *Levilactobacillus brevis* KX572376 (M2) and *Levilactobacillus brevis* KX572382 (M8) were used in fermentation of low-fat cow milk. Moreover, pH changes, proteolytic activity, water-soluble extract biological activity (antioxidant activity and angiotensin-I converting enzyme inhibition) of the samples and peptide fraction less than 3 kDa were investigated at 24 and 48 h of fermentation (30 °C). Peptide profile of the superior sample was analyzed as well. Statistical analysis was carried out using one-way of variance, Tukey test and SPSS software v.25.

Results and Conclusion: The two strains decreased milk pH to a similar level in the first 24 h. Quantities of free amine groups in the samples treated with M2 and M8 strains within 24 and 48 h of fermentation were significantly different ($p \leq 0.05$), compared to the control sample. In the first 24 h of fermentation, no difference was observed in the quantity of free amines of M2 and M8 samples. In the second 24 h, further free amine groups were produced due to the activity of M8 strain in milk. Antioxidant activity of the water-soluble extracts of M2 and M8 samples was significantly ($p \leq 0.05$) higher than that of the control sample during fermentation. Antioxidant activity in fractions less than 3 kDa did not show significant differences in M2 and M8 samples at 24 and 48 h of fermentation. In the control sample, no antioxidant activity was observed in fractions less than 3 kDa. The highest ACE inhibitory activity in fractions less than 3 kDa of M8 was observed after 48 h. No angiotensin-I converting enzyme inhibition was seen in fractions less than 3 kDa of M2 and control sample. The RP-HPLC peptide patterns of the fraction less than 3 kDa of M8 and control sample were different, which was a justification for the biological activity in this sample.

Conflict of interest: The authors declare no conflict of interest.

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1. Introduction

Nowadays, bioactive peptides with functional characteristics are especially interested. Bioactive peptides are special sequences with a length of 2-20 amino acids (AAs), including health effects on the consumers. Several biological activities such as antihypertensive, antioxidant, opioid, antimicrobial, anti-proliferative and immune system modulation activities have been reported for bioactive peptides [1-5]. Size of the peptides and their AA sequences, which are affected by the origin of the protein and conditions of hydrolysis, assess their activities [6]. Role of free radicals in the progression of various diseases has been verified and they are effective factors in food spoilage. Antioxidant bioactive peptides inhibit free radicals by various mecha-nisms such as hydrogen transfer and electron donation [7,8].

Hypertension is a disease that affect many people worldwide. Synthetic antihypertensive drugs include several side effects [9]. Blood pressure in the body is majorly regulated by the renin-angiotensin system (RAS). Angiotensin-I converting enzyme (ACE) is a key enzyme in RAS. The enzyme controls blood pressure by converting inactive

decapeptide angiotensin I to the potent vasoconstrictor angiotensin II. Bioactive peptides can be effective in regulating blood pressure by inhibiting ACE [10]. Most of these peptides include a molecular weight (MW) of less than 3 kDa [11].

Milk and its products are valuable sources of bioactive peptides. These peptides are sequences with potential biological activity in the structure of milk proteins, released during the secretion, storage, digestion and processing of milk by the activity of milk indigenous and digestive enzymes as well as microbial enzymes of starter and non-starter Lactic acid Bacteria (LAB) [12,13] Naturally, LAB produce various metabolites and strain-specific enzymes due to habitat diversity and culture conditions. They can affect protein substrates and release bioactive peptides by producing proteolytic enzymes [14]. Microbial fermentation is a cost-effective process for the production of bioactive peptides. Studies have shown that bioactive peptides in food hydrolysates are more effective than when used as isolated peptides alone [15]. Therefore, finding LAB strains that can produce bioactive peptides during the fermentation process and turn products into functional foods is a target of several studies.

Rubak et al. [16] assessed ACE inhibitory activity in goat milk fermented by LAB isolated from fermented foods and breast milk. A total of 21 peptides were identified as ACE inhibitors. In a study, *Leuconostoc lactis* PTCC1899 was used to ferment camel milk. Moreover, ACE inhibitory and antioxidant activity in the fermented milk was investigated after 24 h. A peptide with AA sequence of Met-Val-Pro-Tyr-Pro-Gln-Arg was identified with antioxidant and ACE inhibitory activities [17].

Panchal et al. [18] used *Limosilactobacillus fermentum* in goat milk. After 48 h of fermentation, the strain was able to produce bioactive peptides with antioxidant activity. The purpose of this study was to use two lactic isolates of traditional Motal cheese in the preparation of fermented cow milk for the production of bioactive peptides. In previous study, these isolates were known as superior strains in production of antioxidant and ACE inhibitor bioactive peptides in ultra-filtered white cheese [19]. Physicochemical characteristics (changes in pH, acidity and proteolytic activity), ACE inhibition and antioxidant activity of fermented milks were investigated at 24 and 48 h. Furthermore, RP-HPLC peptide pattern analysis was carried out for sample with high bioactivity (ACE inhibition and antioxidant activity).

2. Materials and Methods

2.1. Materials and Equipment

In this study, ACE (EC 3.4.15.1), N-[3-(2-furyl)acryloyl]-L-phenylalanylglycyl-glycine (FAPGG), 2,2-diphenyl-1-picryl hydrazyl (DPPH), ortho-phthalaldehyde (OPA) and ferrozin reagent were purchased from Sigma, St. Louis, MO, USA. De Man, Rogosa and Sharpe (MRS) media was purchased from Hi-Media, India. Pre-stained protein marker was purchased from SMOBIO, Taiwan. Moreover, RP-HPLC peptide profile analysis was carried out using Alliance 2695 HPLC system (Waters, Milford, MA, USA). Spectrophotometer (Perkin-Elmer, USA), citation imaging multi-mode reader (BioTek, Germany) and biophotometer (Eppendorf, Germany) were used to measure absorbance of the samples in antioxidant activity, ACE inhibitory activity and proteolytic activity assays, respectively. Furthermore, pH was assessed with pH meter (Metrohm, Germany).

2.2. Culture preparation

In this study, two Levi *Lactobacillus* strains, including *L. brevis* KX572376 (M2) and *L. brevis* KX572382 (M8), were used in preparation of fermented milk. These strains were previously isolated from Iranian raw milk Motal cheese [20] and were selected as the potential strains in production of antioxidant and ACE-inhibitor bioactive peptides in UF cheeses [19]. Pre-inocula for the strains were prepared in MRS media and overnight incubated at 30 °C.

2.3. Milk fermentation

Prepared cultures were centrifuged at 5000× g for 10 min and cells were isolated. Microbial suspensions adjusted to 3 McFarland standard (9.0×10^8 CFU ml^{-1}) were added to sterile low-fat milk (1.5%) (Kalleh, Iran, Tehran) at a ratio of 1% (v v^{-1}) and then incubated at 30 °C for 48 h. Sampling was carried out at 24 and 48 h to perform the analysis as described later. Control sample without bacterial inoculation was transferred into an incubator with other samples**.**

2.4. Preparation of water-soluble extract from the fermented milk

To prepare water-soluble extract (WSE) of the samples, which contained protein, peptides and AAs, pH of the fermented milk and control was set at 4.6. These were centrifuged at 20,000× g for 20 min. Supernatant was filtered using Whatman No. 42 filter papers. The resulting WSEs were passed through ultrafiltration membranes (Amicon, USA) with a MW cut-off of 3 kDa by centrifugation at 7000× g for 45 min at 4 °C [17].

2.5. Physicochemical assessment

Assessment of physicochemical characteristics was carried out based on assessment of pH and acidity changes as well as proteolytic activity in the samples.

2.5.1. Assessment of pH and acidity

Fermented milks were assessed for pH and acidity based on the Iranian National Standard No. 2852 [21]. Briefly, pH of the samples was measured using pH meter and their acidity was assessed by titrating 10 ml of the milk sample with N/9 sodium hydroxide (NaOH) solution in presence of

1 ml of phenolphthalein indicator until a pink color produced. Acidity of the samples was calculated based on the quantity of NaOH used.

2.5.2. Proteolytic activity assay

Protein concentration in WSEs was measured using Bradford assay as described by Marshall and Williams [22]. Peptide content of the experimental fermented milks was assessed using OPA method according to Church et al. [23]. Briefly, 50 µl of the prepared WSE were mixed with 1 ml of OPA reagent. After 2-min incubation at room temperature (RT), absorbance was measured at 340 nm. Serine served as standard and peptide content was calculated using standard curve.

2.6. Antioxidant activity assay

Antioxidant activity was assessed based on DPPH radical inhibition based on a protocol described by Apostolidis et al. [24]. Briefly, 900 µl of DPPH (60 µM) in ethanol were mixed with 100 µl of the sample or deionized water (blank). Each solution in 1 ml volume was centrifuged at 6400× g for 2 min. Absorbance (A) was measured spectrophotometric ally at 517 nm. The DPPH radical scavenging activity was calculated by Eq. 1:

$$\text{DPPH Radical scavenging activity (\%)} = \frac{A.\text{blank} - A.sample}{A.\text{blank}} \times 100 \quad \text{Eq. 1}$$

2.7. ACE-inhibitory activity assay

The ACE-inhibitory activity assay was carried out based on the method of Holmquist et al. [25] with mild modifications. Briefly, 30 µl of ACE (0.1 U ml^{-1}), 50 µl of peptide extract or water (blank) and 160 µl of ACE buffer [50 mM Tris-HCl (pH 7.5), 0.3 M NaCl and 1 mM $ZnCl_2$] were added to each well of ELISA microplate and pre-incubated at 37 °C for 5 min and then 65 µl of FAPGG (0.5 mM) as substrate for ACE were added to the mixture. Reaction was set at 37 °C for 30 min. Then, absorbance was measured at 340 nm. The ACE inhibitory activity was calculated by Eq. 2:

$$\text{ACE inhibitory activity (\%)} = 1 - \frac{\Delta A.\text{sample}}{\Delta A.\text{control}} \times 100 \quad \text{Eq. 2}$$

2.8. RP-HPLC peptide profile analysis

In this study, RP-HPLC was carried out based on the method of Rodriguez-Figurod et al.[26] to investigate the peptide profile of the samples. 20 µl of the fraction less than3 kDa was injected into an analytical C18 column (4.6 × 250, 5 µm, 100 Å). Eluent A with the composition of 0.04% TFA in distilled water (DW) and eluent B with the composition of 0.04% TFA in acetonitrile were used. Elution was carried out using linear gradient of 0-55% eluent B at a flow rate of 0.5 ml/min for 50 min and peptide profile was analyzed at 214 nm.

2.9. Statistical analysis

Data were analyzed using one-way of variance and SPSS software v.25. Results were expressed as mean ±SD (standard deviation) of triplicate. Tukey comparison test was used to assess statistically significant differences between the means.

3. Results and Discussion

3.1. Physicochemical characteristics

3.1.1. pH and acidity changes

As previously statedexpected, the two strains acidified milk (Table 1), which was linked to their ability to break 6-C sugars, converting them into lactic acid [27]. The M2 and M8 decreased milk pH from 6.47 ±0.04 to a similar level in the first 24 h (ΔpH = 0.76).

Table 1. pH and acidity of the fermented milks with M2 (*Levilactobacillus brevis* KX572376) and M8 (*Levilacto-bacillus brevis* KX572382) at 24 and 48 h of fermentation

Sample	pH		%(acidity)	
	24	48	24	48
M2	0.00^{a}±5.71	0.01^{b}±5.36	0.01^{d}±28.0	0.01^{e}±41.0
M8	0.01^{a}±5.71	0.01^{c}±5.46	0.00^{d}±0.28	0.00^{f}±35.0

Values are expressed as mean±standard deviations. Values with the same lower case letters in a column are not statistically significant by the ANOVA test ($P \leq 0.05$).

These results were similar to those reported by Ebadi Nezhad et al. [28] in a study on technological characteristics of the lactic strains of Motal cheese. They demonstrated that pH changes by the lactic strains in sterile skimmed milk ranged 0.67-2.06. In the second 24 h, pH decrease for the two strains proceeded at a slower rate; however, M2 strain was able to decrease pH more than that M8 strain did, producing a higher proportion of organic acids. Differences in pH values and produced acids after 48 h for the two strains might be due to differences in growth rates and metabolic pathways of the two strains [29]. None of the two strains were fast acid producers because they could not decrease pH to less than 5.3 in the first 6 h. in general, this characteristic is not appropriate for using LAB as starters [30].

3.1.2. Proteolytic activity

Proteolytic activity of M2 and M8 strains was assessed using OPA assay. The principle of this method includes measuring free amine groups produced in the fermentation process. Significant increases in quantity of free amine groups in the samples treated with M2 and M8 strains indicated enzymatic hydrolysis of milks within 24 and 48 h of fermentation by the two strains, compared to the control (Fig. 1). In the first 24 h of fermentation, no significant differences were observed in the quantity of free amines of M2 and M8 samples. However, more free amine groups were

produced in M8 sample within the second 24 h, ($p \leq 0.05$), which indicated a stronger proteolytic activity of this strain due to the need of essential amino acids (EAAs) [31]. In the investigation of proteolytic activity of lactic strains isolated from Motal cheese by using skim milk agar-well diffusion method, diameters of the clear zones around the wells created by M8 strain were significantly greater than those created by M2 strain, showing a higher proteolytic activity of this strain, compared to that of M2 strain [28].

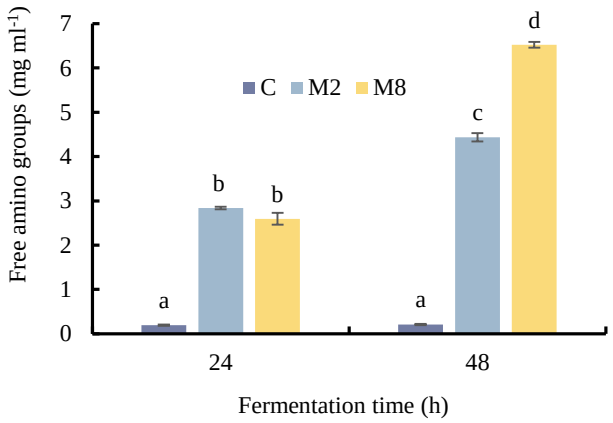

Figure 1. Free amino groups (mg ml^{-1}) of the fermented milks with M2 (*Levilactobacillus brevis* KX572376), M8 (*Levilactobacillus brevis* KX572382) and control at 24 and 48 h of fermentation. Error bars show standard deviations. Different letters above the bars indicate significant differences between the samples ($p \leq 0.05$)

3.2. Antioxidant activity

Figures 2 and 3 respectively show proportions of DPPH radical inhibition in WSE of M2 and M8 samples and fractions less than 3 kDa of WSEs, compared to the control sample. Antioxidant activity of WSEs of M2 and M8 samples was significantly ($p \leq 0.05$) higher than the control sample, demonstrating ability of these strains to produce antioxidant protein hydrolysates and peptides under conditions provided for the fermentation. No significant differences were reported between the WSEs of M2 and M8 samples in DPPH radical scavenging at 24 and 48 h.

In the first 24 h of fermentation, M2 and M8 samples were able to inhibit 89.05%±4.94 and 82% ±1.41 of DPPH radicals, respectively, and this activity decreased significantly ($p \leq 0.05$) in the two samples within the second 24 h. Antioxidant activity in the fraction less than 3 kDa did not include significant differences in M2 and M8 samples at 24 and 48 h of fermentation ($p \leq 0.05$). In the control sample, no activities were seen in fractions smaller than 3 kDa. Soleimanzadeh et al. [32] reported antioxidant activities in camel and bovin milks fermented by nine LAB strains during 24 h of fermentation. Decrease of antioxidant activity in WSE of the samples and insignificant increase of antioxidant

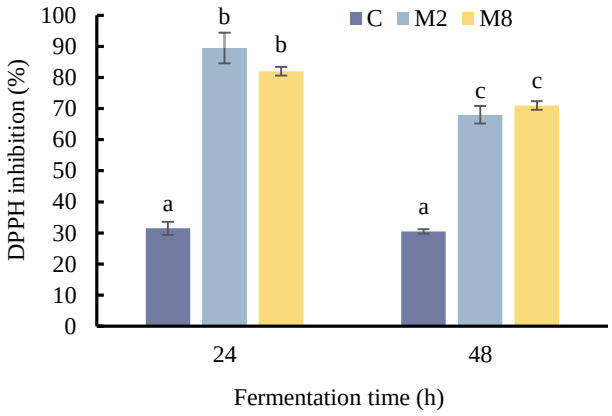

Figure 2. The DPPH radical inhibitions (%) of water-soluble extract (WSE) of the fermented milks with M2 (*Levilactobacillus brevis* KX572376), M8 (*Levilactobacillus brevis* KX572382) and control at 24 and 48 h of fermentation. Error bars show standard deviations. Different letters above the bars indicate significant differences between the samples ($p \leq 0.05$)

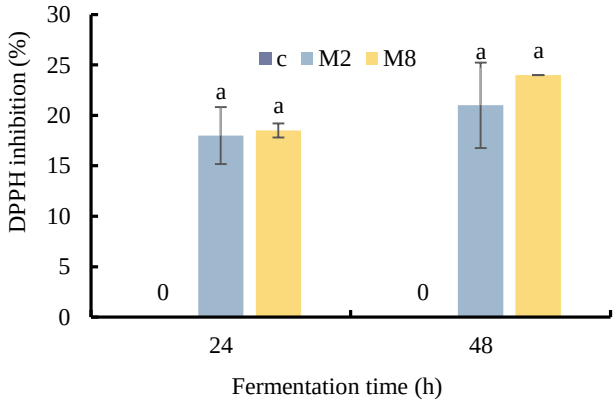

Figure 3. The DPPH radical inhibitions (%) of fractions less than 3 kDa of the fermented milks with M2 (*Levilactobacillus brevis* KX572376), M8 (*Levilactobacillus brevis* KX572382) and control at 24 and 48 h of fermentation. Error bars show standard deviations. Different letters above the bars indicate significant differences between the samples ($p \leq 0.05$)

Activity in fractions less than 3 kDa after 24 h indicated that development of proteolysis by the strains might cause hydrolysis or changes in structure of the peptides or hydrolysates of milk proteins with antioxidant activity [33]. In a study by Virtanen et al. [34], it was detected that antioxidant activity did not necessarily increase with the progress of proteolysis. In another study, two strains of *L. helveticus* and *L. fermentum* were used for milk fermentation. Despite the higher proteolytic activity of *L. helveticus*, antioxidant activity of the samples containing *L. fermentum* was 1.5-1.7× higher. The highest antioxidant activity was reported in all the samples within the first 24 h [29].

3.3. ACE-inhibitory activity

The ACE inhibitory activity in WSE of the control sample and milks fermented by M2 and M8 strains after 24 and 48 h of fermentation is shown in Fig. 4.

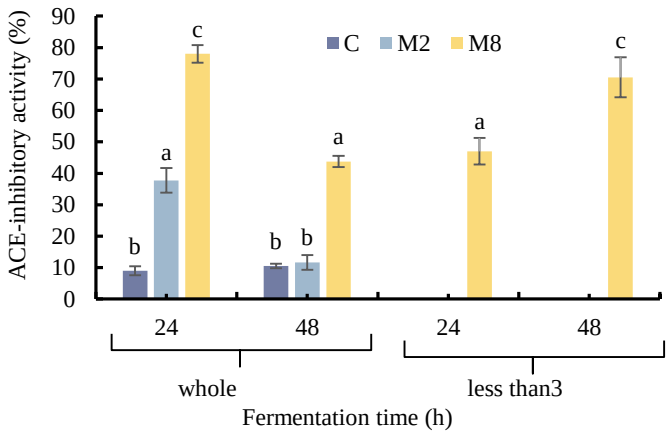

Figure 4. Angiotensin-I converting enzyme inhibitory activities (%) of the whole water-soluble extract (WSE) and fraction less than 3 kDa of the fermented milks with M2 (*Levilactobacillus brevis* KX572376), M8 (*Levilactobacillus brevis* KX572382) and control at 24 and 48 h of fermentation. Error bars show standard deviations. Different letters above the bars indicate significant differences between the samples ($p \leq 0.05$).

The ACE inhibitory activities in samples were significantly different ($p \leq 0.05$). The highest activity was observed in the sample inoculated with M8 strain within the first 24 h at a rate of 78% ±2.82. Moreover, M2 sample and control included activities of 37.75% ±3.88 and 9% ±1.41, respectively. Technically, ACE inhibitory activity in the control might be associated to the denaturation and partial decomposition of milk proteins during heat treatment and homogenization [35]. Inhibitory activity of M8 and M2 samples decreased within the second 24 h ($p \leq 0.05$), showing degradation of hydrolysates and eptides effective in inhibiting ACE into ineffective factors under the affection of the strain's enzyme system. Similar to the antioxidant activity, ACE inhibition decreased with the progress of proteolysis. Inhibitory activity was not reported in fractions less than 3 kDa of M2 and control samples. In M8 sample, ACE inhibition included 47.47% ± 4.24 within the first 24 h and 70.5% ±6.36 within the second 24 h, showing differences in proteolysis patterns and enzyme systems between the M2 and M8 strains. Regarding decrease of the ACE inhibitory activity in WSE of M8 sample after 24 h and it's increase in fractions less than 3 kDa, it could be concluded that inhibitory peptides with a MW of less than 3 kDa were formed with the development of proteolysis and the loss of hydrolysates effective in the ACE inhibition that concentrated by the membrane caused increases in their quantities in the samples. In a study of Li et al. [36], ACE inhibitory activity was reported in milks fermented by two lactic strains of *L. helveticus* KLDS.31 and *Lacticaseibacillus casei* KLDS.105. UF fractions with a MW of less than 3 kDa included the strongest inhibitory activity.

3.4. RP-HPLC peptide profile

Fractions less than 3 kDa of M8 sample were selected for RP-HPLC peptide pattern analysis due to their antioxidant and ACE inhibition activities within 48 h of fermentation. Figure 5 shows RP-HPLC chromatograms of fractions less than 3 kDa of M8 and control samples.

Chromatograms of M8 and control samples were different. This difference was clearly evident in the hydrophobic (with a retention time of 33-45 min) and the hydrophilic (with a retention time of 5-11 min) regions. Early peaks in RP-HPLC chromatograms were mostly linked to low molecular weight (LMW) hydrophilic peptides and AAs and the later peaks consisted of hydrophobic peptides [37]. Typically, peptides with biological activity such as ACE inhibitory peptides include hydrophobic characteristics [38]. Biological activity of the M8 sample could be linked to compounds, which peaks were seen in the hydrophobic region of M8 chromatogram but not present in the chromatogram of the control.

4. Conclusion

Results of this study showed that M8 strain included inhibitory effects on ACE in addition to its antioxidant activity. Peptide profile of the fractions less than 3 kDa of M8 sample verified their biological activity. Since M8 strain was not a fast acid producer, it included the potency to be used as an adjunct culture for the production of functional fermented dairy products. Purification and identification of the effective compounds in the biological activity of this strain can be targets of further studies.

5. Acknowledgements

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6. Conflict of Interest

The authors report no conflicts of interest.

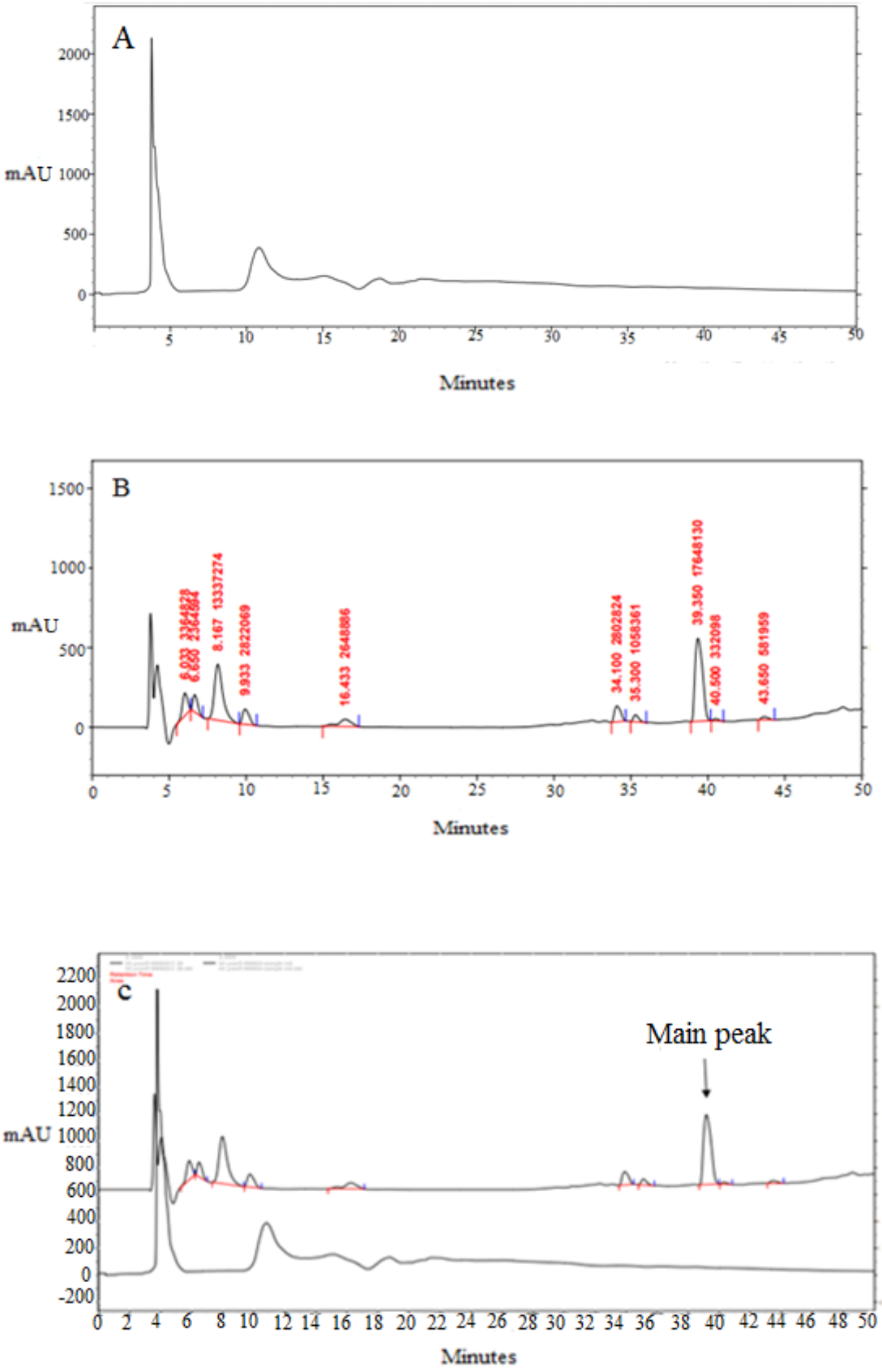

Figure 5. The RP-HPLC chromatograms of fractions less than 3 kDa of the control (A), M8 (fermented milk with *Levilactobacillus brevis* KX572382) (B) and chromatograms of the two samples (C) after 48 h at 214 nm

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تولید پپتیدهای زیست‌فعال در شیر با استفاده از دو سویه بومی *لوی لاکتوباسیلوس برویس*

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چکیده

سابقه و هدف: پروتئین های شیر پیش‌ساز بسیاری از پپتیدهای فعال بیولوژیکی هستند. یکی از روش‌های تولید این پپتیدها، تخمیر با استفاده از باکتری‌های اسید لاکتیک است. هدف از این تحقیق، بررسی تولید پپتیدهای زیست‌فعال آنتی اکسیدان و پپتیدهای زیست فعال مهارکننده آنزیم مبدل آنژیوتانسین-I در شیر گاو تخمیر شده با دو سویه *لوی‌لاکتوباسیلوس برویس* بود.

مواد و روش ها: دو سویه *لوی‌لاکتوباسیلوس برویس* (M2) KX572376 و *لوی‌لاکتوباسیلوس برویس* (M8) KX57238 در تخمیر شیر گاو کم چرب مورد استفاده قرار گرفت. تغییرات pH، فعالیت پروتئولیتیکی، فعالیت بیولوژیکی عصاره محلول در آب (فعالیت آنتی اکسیدانی و مهار آنزیم مبدل آنژیوتانسین-I) نمونه ها و بخش پپتیدی با وزن مولکولی کمتر از ۳ کیلودالتون در ۲۴ و ۴۸ ساعت تخمیر (۳۰ درجه سلسیوس) مورد بررسی قرار گرفت. در نهایت الگوی پپتیدی نمونه برتر مورد تجزیه و تحلیل قرار گرفت. بررسی آماری با نرم افزار SPSS نسخه ۲۵ و با استفاده از آزمون واریانس یک طرفه و آزمون توکی انجام شد.

یافته‌ها و نتیجه‌گیری: هر دو سویه، pH شیر را در ۲۴ ساعت اول به یک میزان کاهش دادند. میزان گروه‌های آمین آزاد در نمونه‌های تیمار شده با دو سویه‌ی M2 و M8 ، در طول ۲۴ و ۴۸ ساعت تخمیر با نمونه شاهد تفاوت معنی داری داشت($p \leq 0.05$). در ۲۴ ساعت نخست تخمیر، تفاوتی در مقدار آمین‌های آزاد دو نمونه M2 و M8 مشاهده نشد ولی در ۲۴ ساعت دوم گروه‌های آمین آزاد بیشتری در اثر فعالیت سویه‌ی M8 در شیر تولید شد. فعالیت آنتی اکسیدانی عصاره محلول در آب نمونه‌های M2 و M8 به طور معنی‌داری ($p \leq 0.05$) بیشتر از نمونه شاهد در طول تخمیر بود. فعالیت آنتی اکسیدانی در بخش با وزن مولکولی کمتر از ۳ کیلو دالتون در نمونه‌های M2 و M8 در ۲۴ و ۴۸ ساعت تخمیر تفاوت معنی‌داری نشان نداد. بیشترین فعالیت مهار آنزیم مبدل آنژیوتانسین-I در بخش کمتر از ۳ کیلو دالتون نمونه M8 ، بعد از ۴۸ ساعت مشاهده شد. در بخش دارای وزن مولکولی کمتر از ۳ کیلو دالتون نمونه M2 و نمونه‌های شاهد فعالیت بازدارندگی مشاهده نشد. الگوی پپتیدی RP-HPLC بخش دارای وزن مولکولی کمتر از ۳ کیلو دالتون نمونه M8 و شاهد با یکدیگر متفاوت بود که فعالیت بیولوژیکی مشاهده شده در این نمونه را تایید می‌کرد.

تعارض منافع: نویسندگان اعلام می‌کنند که هیچ نوع تعارض منافعی مرتبط با انتشار این مقاله ندارند.

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- فعالیت مهار کنندگی آنزیم مبدل آنژیوتانسین-I
- فعالیت آنتی اکسیدانی
- شیر تخمیری
- *لوی‌لاکتوباسیلوس برویس*

***نویسندگان مسئول**

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