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Research Article

Selectively screen the antibacterial peptide from the hydrolysates of highland barley

Highland barley is one of the most important industrial crops in Tibetan plateau. Previous research indicated that highland barley has many medical functions. In this work, the antibacterial abilities of highland barley were investigated. The protein solutions hydrolyzed by trypsin for 4 h exhibited the highest antibacterial activity. An antibacterial peptide, barleycin, was screened and purified by magnetic liposome extraction combining with the protein profiles of reversed-phase high-performance liquid chromatography (RP-HPLC). Structure, characterization, and safety evaluation of barleycin were further investigated. Amino acids sequence was determined as Lys-Ile-Ile-Ile-Pro-Pro-Leu-Phe-His by N-sequencing. Circular dichroism spectra indicated the α -helix conformation of barleycin. The activity spectrum included *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria innocua* and *Escherichia coli* and the MICs were from 4 to 16 $\mu\text{g}/\text{mL}$. Safety evaluations with cytotoxicity and hemolytic suggested this antibacterial peptide could be considered as safe at MICs. Finally, mode of action of barleycin on sensitive cells was primarily studied. The results suggested the damage of cell membrane.

Keywords: Antibacterial peptide / Characterization / Highland barley / Magnetic liposome / Purification

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1 Introduction

As the results of excessive use of antibiotics, there are more and more antibiotic bacteria emerging. The growing problem has resulted in a search for novel antibacterial alternatives. Antibacterial peptides mostly have the advantages of nontoxin, high antibacterial activity and good selectivity [1]. Thus, they may be used as a kind of new anti-microbial agents in food or medical industry [2].

Highland barley or Tibetan barley is the principal cereal cultivated on the Tibetan plateau. It is rich in starch and protein and is used mainly to make milk tea, tsampa, and alcohol. Many ancient Chinese medicine books recorded that the highland barley has many medical functions including antidiarrheal, antioxidants, anticancer and inhibiting cholesterol level and anti-inflammatory [3, 4]. Although antibacterial peptides have been reported in many resources [5, 6], there are no reports about isolation of antibacterial peptide from highland

barley. As highland barley is one of the most important industrial crops in Tibetan plateau, deep developments of highland barley play the significant role on the improvement of local economy.

The screening and isolation of antibacterial peptides from natural sources is a challenging task because these complex samples can contain varied ingredients [7, 8]. Rapid and efficient methods to screen and separate antibacterial peptides from natural sources for safe application are thus needed. Selective interaction between antibacterial peptides and sensitive microbial cell membrane is the precondition and basis of antibacterial peptides to activate against sensitive cells [9–11]. This principle could be used to isolate and purify antibacterial peptides. In this study, highland barley proteins were subjected to different enzymes to obtain the antibacterial fragments. Further, antibacterial peptide, barley, was screened and purified by an efficient two-step method. Another essential objective of this study is characterization of this antibacterial peptide.

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2 Materials and methods

2.1 Enzymatic hydrolysis of highland barley protein

After grounding to achieve the uniformity, 20 g of highland barley were dissolved in 100 mL HCl solution (0.01 M). The protein section of highland barley was extracted with 3-fold volume ethanol solution as described in [12] and then subjected to hydrolysis by pepsin, trypsin, neutrase, papain, and alcalase, respectively, as described in [3]. Each hydrolysis was performed for 1, 2, 3, 4, and 5 h. The hydrolysis was stopped by heating at 100°C for 10 min. The hydrolysate was clarified by centrifugation (5000 × g, 4°C, 15 min), and then the supernatant was 10-fold concentrated to get the hydrolysis of highland barley protein. The spot-on-lawn method was applied to determine the antibacterial activity [13] with *E.coli* CICC 10300 as indicator stains. The concentration of proteins was determined by Bradford method [13].

2.2 Preparation of the magnetic nanoliposomes

Ferromagnetic Fe₃O₄ nanoparticles were prepared by hydrothermal method with 3.5 g FeCl₃·6H₂O, 2.3 g sodium acetate, 40 mL glycol and 40 mL oleic acid [14]. Lipid of *E. coli* CICC 10300 was extracted with 1:2 (v/v) chloroform-methanol, chloroform, and ultra-pure water [15]. Nanomagnetic liposome particles were prepared by the thin film dispersion method [16]. Size, polydispersity index (PDI), and zeta potential were measured using Zeta sizer analysis equipment (DelsaMax PRO, Beckman, USA). Magnetic characterization was tested by an alternating gradient magnetometer (MicroMag™ Model 2900, Princeton Measurements Corporation, USA).

2.3 Screen and purification of antibacterial peptide from highland barley

The hydrolysate of highland barley protein was first filtered through 10 kDa cut-off membranes (Millipore, Los Angeles, CA, USA) and then was dialyzed with 100 Da cut-off membranes (Millipore). The magnetic liposome was equilibrated with PBS buffer (10 mM, pH 7.2) containing 50 mM NaCl. The hydrolysis of highland barley protein was shacked with the magnetic liposome. After 24 h of co-culture, the mixture was centrifuged (10 000, 4°C, 20 min) and the supernatant was adjusted to the original volume. RP-HPLC was used to detect the liquid chromatographic fingerprint of the supernatants of hydrolysate of highland barley protein solution before and after incubation with magnetic liposome. Chromatographic conditions: column: reversed-phase C₁₈ 250 × 4.6 mM; column temperature: 25°C; sample volume: 10 μL; flow rate of mobile phase: 1 mL/min; mobile phase: solvent A containing aqueous solution of 0.1% (V/V) TFA; solvent B is the 80% (V/V) acetonitrile solution containing 0.1% (V/V) TFA; procedures: 0–30 min, 0–100% solvent B linear gradient elution. Detection wavelength: 215 nm. The different elution was collected and adjusted to the same concentration to test for the antibacterial activity with *E.coli* CICC 10300 as the indicator strain. The concentration of proteins was determined

Table 1. Entrapment encapsulation (EE), size, polydispersity (PDI) and zeta potential of the magnetic liposomes

EE%	Size (nm)	PDI	Zeta potential (mV)	MS (emu/g)
94.28	271.5 ± 8.9	0.33 ± 0.04	-49.2 ± 3.2	21.7 ± 2.8

by Bradford method [13]. The elution with the highest activity was further analyzed and purified by C₁₈ reverse phase column (0.5 mL/min, min 0–40, 10–100% B).

2.4 Structural characterization of barleycin

Purified antibacterial peptide was tested by N-amino acids sequence (Proclise491, ABI, USA) in Beijing Huada Protein innovation Co. Ltd. Homology search of amino acid sequence was searched in NCBI (<http://www.ncbi.nlm.nih.gov/>). Physico-chemical properties of barleycin were predicted by bioinformatics tool (ProtParam in ExPasy ProtParam). CD spectrum was used to analyze the structure of barleycin. Software Hyperchem 8.0 was used to calculate and predict the 3D model of barleycin structure under the lowest energy state.

2.5 Antibacterial activity of barleycin

Activity spectrum of barleycin (100 μg/mL) was determined with the selected indicator strains within Table 1. Minimum inhibitory concentrations (MICs) were defined as the lowest barleycin concentration that completely inhibited the growth of bacteria [13] and determined by testing the OD₆₀₀ of bacteria suspensions treated with different dilutions of barleycin.

2.6 Hemolytic test n

Hemolytic test of barleycin on the human red blood cells (provided by Shaanxi human hospital, Shaanxi, China) with PBS (10 mM, pH 7.2) as negative control and 0.1% Triton X-100 used as the positive control. Cytotoxicity test (MTT test) on hepatocyte and renal cells of human beings (HL7702 and HREpiC from ATCC) was performed.

2.7 Leakages of cytoplasmic contents of *Escherichia coli*

Mid-exponential phase cells of *E.coli* CICC 10300 were washed and re-suspended in sterile saline water. Barleycin at 2 MIC was added in. Changes in K⁺_{out}, OD₂₆₀ and OD₂₈₀ were recorded as previous study [13]. Sample without the barleycin was used as the control.

2.8 Statistics

SPSS 18.0 software was used to analyze date. All data were averagely represented for three times and are shown as mean ± standard deviation.

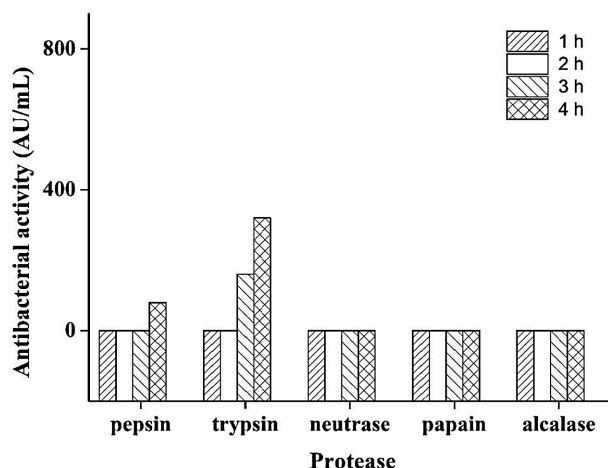


Figure 1. Antibacterial activities of highland barley fragments hydrolyzed by different enzymes for 4 h.

3 Results and discussion

3.1 Antibacterial activity of highland barley hydrolysates

Fragments hydrolyzed by trypsin for 4 h exhibited the highest antibacterial activity (Fig. 1). Hydrolysis time influences the cleavage degree of the peptide chain. The fact that the pepsin 4-h treatment of hydrolysate suggested the highest antibacterial activity was a cooperative result of amino acid composition and structures. Similar results were reported by other researchers [3, 12, 17]. In the following experiments, highland barley fragments hydrolyzed by pepsin for 4 h were used for antibacterial peptide purification in the following experiments.

3.2 Characterization of magnetic nanoliposome

The characterizations of magnetic nanoliposome were investigated (Table 1). The encapsulation efficiency was 96.73%. The encapsulation efficiency normally ranged from 80 to 100% [16–18]. The average diameter of magnetic liposome was 271.5 nm and a polydispersity index of 0.33, indicating a low liposome polydispersity [18]. Nanoliposome showed a zeta potential of -49.2 mV. High zeta-potential means more stability of liposome because the repulsive interactions would increase, reducing the frequency of liposome collisions [18]. The saturation magnetization (M_S) was 21.7 emu/g and the superparamagnetism was observed. This means that when the external magnetic field was removed, the magnetization becomes 0. It is very important for application in magnetic separation process [19].

3.3 Purification of barleycin

After adsorption, the peaks 1, 2, 3, and 4 almost disappeared. The peak area and retention time of other peaks before and after adsorption were consistent (Fig. 2A). Peak 4 showed the

highest antibacterial activity. In order to verify the method of target direction, experiments also detected the antibacterial effect of the peak which had no change in peak shape and peak area and these peaks showed no antibacterial activity, indicating that this method had better selection and targeting. It was able to quickly locate the potential antibacterial components in chromatographic fingerprints.

Traditional methods of peptide purification are always more than three steps including organic solvent extraction, gel filtration chromatography, and RP-HPLC etc. [20–23]. For example, Martinez et al. used n-propanol extraction, ion-exchange chromatography, and HPLC to get two antibacterial peptides [24]. The drawbacks are too complex with loss of the activities. In this study, the magnetic liposome adsorptions combined with protein profiles of RP-HPLC are more convenient and efficient.

3.4 Structural characterization of barleycin

The amino acid sequence of the nine amino acids was successfully identified as Lys-Ile-Ile-Ile-Pro-Pro-Leu-Phe-His. The molecular mass is calculated as 1077.38Da. A similarity search was performed for comparing the sequence of amino acids. The result suggested “no hits found” (<http://web.expasy.org/blast/>). The antibacterial peptide hence is safely assigned as a novel peptide and named barleycin. The theoretical isoelectric point is 8.76. Total number of positively charged residues is 1. The positive charge of antibacterial peptide played an important role on the mode of action [5, 25]. Bovicin HC5 was reported to two positive charges, which played an important role in interacting with the cell membrane of the sensitive bacteria [10]. Aliphatic index is 173.33. Grand average of hydrophobicity (GRAVY) is 1.089 > 0, indicating it is a hydrophobic peptide. Antibacterial peptides usually have one or more hydrophobic centers, and the mechanism of antibacterial peptides is related to its hydrophobic nature [24, 26, 27]. The minimum energy state structure (Energy = -9.843087; Gradient = 0.096196) of barleycin indicated that it is in a-helix conformation (Fig. 3A). This conformation was consistent with the results of CD spectrum (Fig. 3B). This well-defined conformation is good in protecting against some adverse conditions in the environment [10, 12]. Tang et al. reported that MDpep9 is in a-helix which is very stable at the acid environment as well as Pediocin 34 which is also in the a-helix conformation [2, 15].

3.5 Antibacterial activities and characterizations of barleycin

The activity spectrum and MIC of barleycin were listed in Table 2. Barleycin had inhibitory activities toward *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria innocua*, *E. coli*, and *Shigella dysenteriae*. However, barleycin was not able to inhibit the growth of fungus, such as *Penicillium italicum* and *Saccharomyces cerevisiae*. The MICs were from 4 to 16 $\mu\text{g}/\text{mL}$ which were low. Heat and freeze-thaw treatment did not affect the activity of barleycin and it was effective within the pH range 3–10.

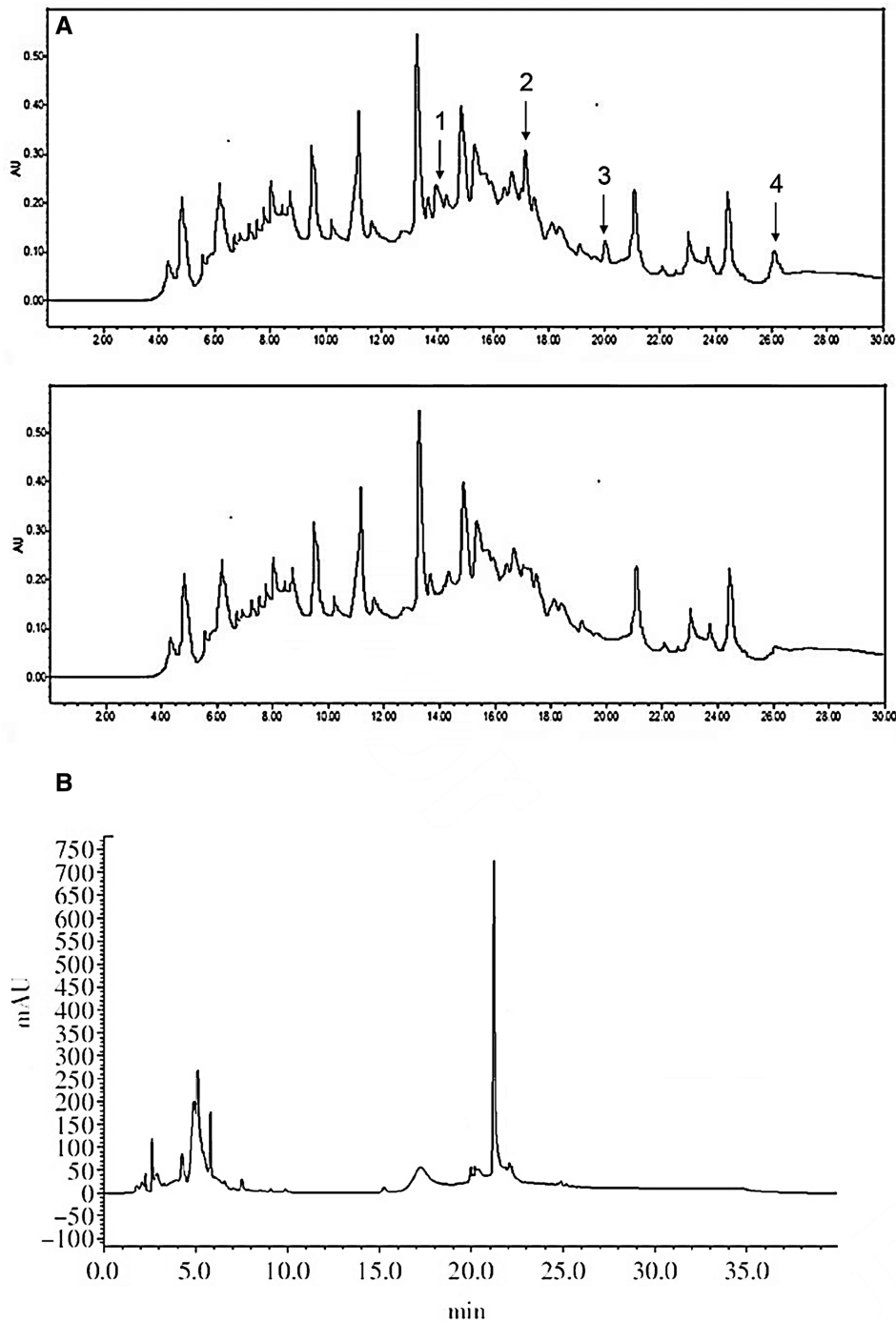


Figure 2. RP-HPLC fingerprint profiles of highland barley hydrolysates (A) before and (B) after extraction by magnetic liposome solid-phase.

Compared with other antibacterial peptides reported recently [5, 25], for example, JCpep8 b and MDpep9, barleycin MICs against *E.coli* were found to have higher potency. This might be because the liposomes used to screen barleycin were scoured from *E.coli* cell membranes. Negatively charged phospholipids on the outer surface of the negatively charged lipid A of lipopolysaccharide (in Gram-negative bacteria) might also contribute to this [5, 12, 25].

3.6 Safety evaluation

Cytotoxicity of barleycin by MTT assay showed that the viability of HL7702 and HREpiC cells was 100.2 and 100.3%, respectively in the presence of 256 $\mu\text{g/mL}$ of barleycin (Table 3). The results indicated that barleycin had no cytotoxicity. When barleycin was less than 256 $\mu\text{g/mL}$, the human blood cell did not show hemolysis phenomenon. Combined with the MIC of tested bacteria,

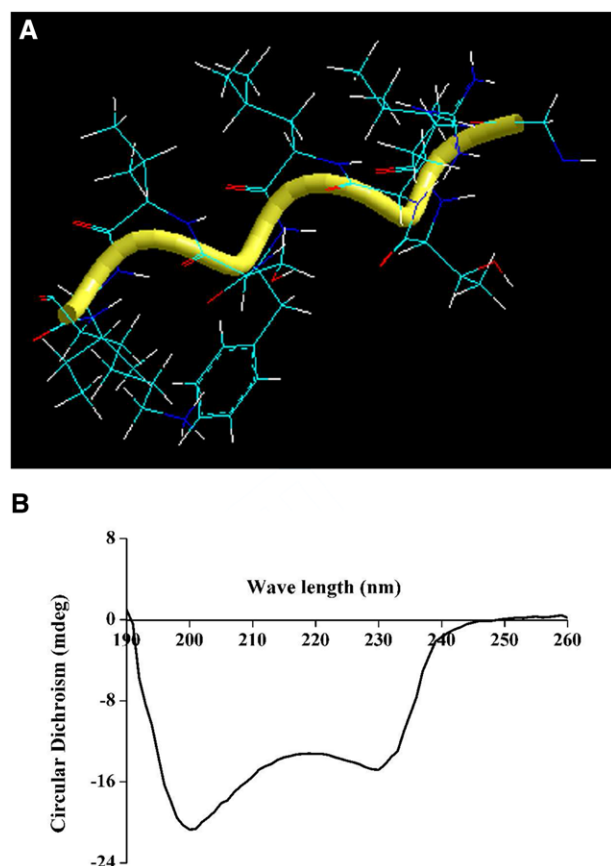


Figure 3. Structure of barleycin. (A) The three-dimensional structure of barleycin predicted with Hyperchem 8.0 software; (B) CD spectrum of barleycin.

Table 2. Characterizations of barleycin

Conditions	Activity	MIC ($\mu\text{g/mL}$)
<i>Bacillus subtilis</i> (CICC 10034, CICC 10732, CICC 10012)	3/3	8
<i>Staphylococcus aureus</i> (CICC 10384, CICC 10307, CICC 10786, CICC 10201)	4/4	4–8
<i>Listeria innocua</i> (CICC 10416, CICC 21529)	2/2	16
<i>Escherichia coli</i> (CICC 10003, CICC 10032, CICC 10004, CICC 10361)	4/4	4
<i>Shigella dysenteriae</i> ATCC 51302	1/1	8
<i>Penicillium italicum</i> (CICC 40659, CICC 40946)	0/2	
<i>Saccharomyces cerevisiae</i> (CICC 1002, CICC 1010, CICC 1016, CICC 1023, CICC 1043)	0/5	
60°C, 80°C, 100°C	+	
Freeze-thaw	+	
pH3–10	+	

Table 3. Safety evaluation of barleycin

		Viability
MTT	HL7702	100.2% \pm 0.4%
	HREpiC	100.4% \pm 0.7%
Hemolytic assay	64 $\mu\text{g/mL}$	0
	128 $\mu\text{g/mL}$	0
	256 $\mu\text{g/mL}$	3.2% \pm 1.7%
	512 $\mu\text{g/mL}$	19.5% \pm 3.5%

the barleycin at the concentration to inhibit spoilage bacteria was primarily considered as safe. Therefore, it has potential application value. These data indicated that barleycin may be a promising candidate for antibacterial drug and food preservative. Normally, the antibacterial peptides from animals are not active against animal cells (safe to human cells) because they are secreted by animals to protect from harmful microorganisms in the environment [12, 13].

3.7 Leakage of *E. coli* cytoplasmic content

Escherichia coli CICC 10300 cells treated with 2 MIC barleycin displayed obviously changes in the leakage of K^+ . About 1.1 $\mu\text{mol K}^+$ were lost in 2 h (Fig 4A). The leakages of cytoplasmic proteins and nucleic acid were increased as the treatment time elongated (Fig. 4B and C). These results indicated that barleycin would damage the permeability of cell membrane and cause the leak out of cytoplasmic content and even the death of sensitive bacteria. Other researchers also reported that membrane permeability played a very important role on the mode of action of cationic antibacterial peptides [5, 9, 11].

4 Concluding remarks

Fragments hydrolyzed by trypsin for 4 h exhibited the highest antibacterial activity. A novel antibacterial peptide named as barleycin was successfully purified from the hydrolysate of highland barley proteins by magnetic liposome adsorption combined with the protein profiles of RP-HPCL. The average diameter of magnetic liposomes was 271.5 nm and the saturation magnetization (MS) is 21.7 emu/g with superparamagnetism. Amino acids sequence of barleycin was determined as Lys-Ile-Ile-Ile-Pro-Pro-Leu-Phe-His by N-sequencing. The CD spectra suggested that the barleycin is in α -helix conformation. Barleycin exhibits the inhibitory activities toward *Bacillus subtilis*, *Staphylococcus aureus*, *Listeria innocua*, *E. coli*, and *Shigella dysenteriae*. The MICs were from 4 to 16 $\mu\text{g/mL}$ which were low. *E. coli* is very sensitive to barleycin, this might be because the liposomes used to screen barleycin were scoured from *E. coli*. Heat and freeze-thaw treatment did not affect the activity of barleycin and it was effective within the pH range 3–10. Barleycin was estimated as safe by cytotoxicity and hemolytic test. The primary mode of action of barleycin is to change the permeability of the bacterial cell membrane and cause the leak out of cytoplasmic content and even the death of sensitive bacteria. In summary, barleycin can

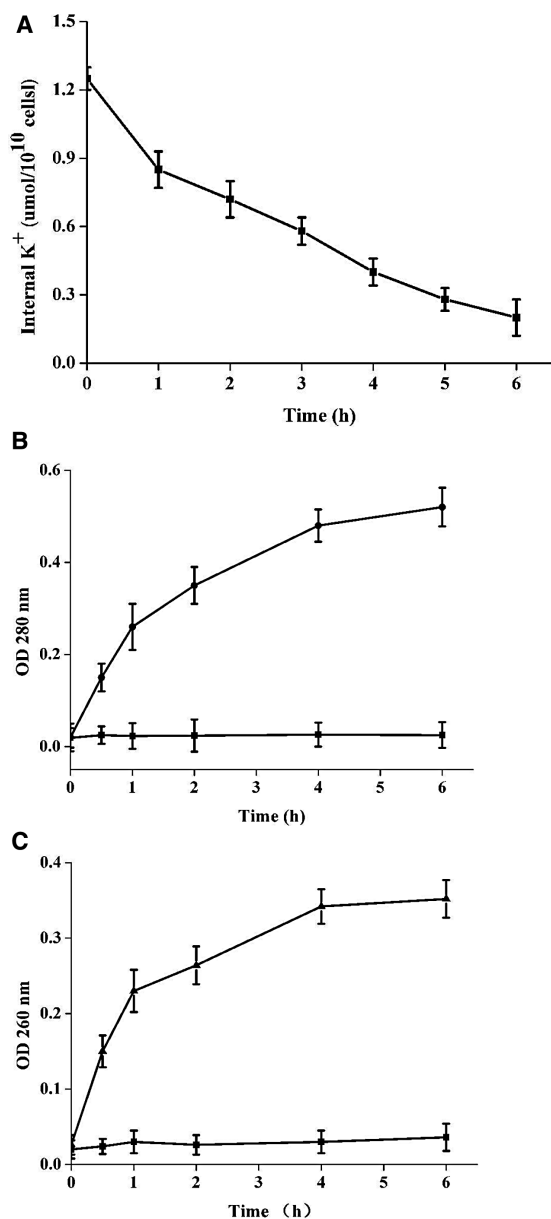


Figure 4. Leakage of *E. coli* CICC 10300 cytoplasmic content after treatment of barleycin. (A) Leakage of K^+ ; (B) leakages of enzymes; (C) leakages of nucleic acids.

be considered to have the potential value for application against *E. coli* in medical industry or food preservatives.

Practical application

An innovative method, magnetic liposome adsorptions combining the protein profiles of RP-HPLC, was developed to target screen and efficiently purify the antibacterial peptide; the antibacterial peptide purified in this study, barleycin, has the great potential for application in food/medical industry.

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The authors have declared no conflict of interest.

5 References

- [1] Zhang, Y., Guo, B. Y., Cao, Y., Huang, Q. R., Antibacterial effects of a cell-penetrating peptide isolated from Kefir. *J. Agri. Food Chem.* 2016, 64, 3234–3242.
- [2] Kaur, G., Singh, T. P., Malik, R. K., Antibacterial efficacy of Nisin, Pediocin 34 and Enterocin FH99 against *Listeria monocytogenes* and cross resistance of its bacteriocin resistant variants to common food preservatives. *Brazil J. Microbiol.* 2013, 14, 63–71.
- [3] Tomasinsig, L., Skerlavaj, B., Scarsini, M., Guida, F. et al., Comparative activity and mechanism of action of three types of bovine antibacterial peptides against pathogenic *Prototheca* spp. *J. Peptide Sci.* 2012, 18, 105–113.
- [4] Lin, M. C., Hui, C. F., Chen, J. Y., Wu, J. L., Truncated antibacterial peptides from marine organisms retain anti-cancer activity and antibacterial activity against multidrug-resistant *Staphylococcus aureus*. *Peptides* 2013, 44, 139–148.
- [5] Goh, H.F., Philip, K., Isolation and mode of action of bacteriocin BacC1 produced by nonpathogenic *Enterococcus faecium* C1. *J. Dairy Sci.* 2015, 98, 5080–5090.
- [6] Xiao, J. H., Zhang, H., Antibacterial peptide isolated from ovalbumin hydrolysate by immobilized liposome-binding extraction. *J. Biomed. Screen.* 2012, 17, 752–760.
- [7] Fiori, S., Urgeghe, P. P., Hammami, W., Razzu, S. et al., Bio-control activity of four non- and low-fermenting yeast strains against *Aspergillus carbonarius* and their ability to remove ochratoxin A from grape juice. *Int. J. Food Microbiol.* 2014, 189, 45–50.
- [8] Zhao, S. G., Han, J.Z., Bie, X. M., Lu, Z. X. et al., Purification and characterization of Plantaricin JLA-9: a novel bacteriocin against *Bacillus* spp. produced by *Lactobacillus plantarum* JLA-9 from Suan-Tsai, a Traditional Chinese fermented cabbage. *J. Agri. Food Chem.* 2016, 64, 2754–2764.
- [9] Hawrani, A., Howe, R. A., Walsh, T. R., Dempsey, C. E., Thermodynamics of RTA3 peptide binding to membranes and consequences for antibacterial activity. *Biochim. Biophys. Acta-Biomembrane* 2010, 1798, 1254–1262.
- [10] Paiva, A. D., Irving, N., Breukink, E., Mantovani, H. C., Interaction with lipid II induces conformational changes in bovicin HC5 Structure. *Antimicrob. Agents Chemother.* 2012, 56, 4586–4593.
- [11] Panagiota, K., Kyriakou, E. B., Kristianse, n P. E., Kaznessis, Y. N., Interactions of a class IIb bacteriocin with a model lipid

- bilayer, investigated through molecular dynamics simulations. 2016. *Biochim. Biophys. Acta (BBA) – Biomembranes*, 2016, 1858, 824–835.
- [12] Tang, W. T., Zhang, H., Wang, L., New cationic antimicrobial peptide screened from boiled-dried anchovies by immobilized bacterial membrane liposome chromatography. *J. Agr. Food Chem.* 2014, 62, 1564–1571.
- [13] Yue T. L., Pei J. J., Yuan Y. H., Purification and Characterization of anti-*Alicyclobacillus* bacteriocin produced by *Lactobacillus rhamnosus*. *J. Food Protect.* 2013, 76, 1575–1581.
- [14] Duan, L., Jia, S., Wang, Y. Lianfeng Duan, S. et al., Synthesis of Fe₃O₄ polyhedra by hydrothermal method: using l-arginine as precipitator. *J. Mater. Sci.* 2009, 44, 4407.
- [15] Tang, Y. L., Shi, Y. H., Zhao, W., Hao, G. et al., Interaction of MDpep9, a novel antimicrobial peptide from Chinese traditional edible larvae of housefly, with *Escherichia coli* genomic DNA. *Food Chem.*, 2009, 115, 867–872.
- [16] Malheiros, P. S., Micheletto, Y. M. S., Silveira, N. P., Brandelli, A., Development and characterization of phosphatidylcholine nanovesicles containing the antibacterial peptide nisin. *Food Res. Int.* 2010, 43, 1198–1203.
- [17] Xiao, J. H., Zhang, H., Ding, S. D., Thermodynamics of antimicrobial peptide JCpep8 binding to living staphylococcus aureus as pseudostationary phase in capillary electrochromatography and consequences for antimicrobial activity. *J. Agri. Food Chem.* 2012, 60, 4535–4541.
- [18] Boelter, J. F., Brandelli, A., Innovative bionanocomposite films of edible proteins containing liposome-encapsulated nisin and halloysite nanoclay. *Colloid Surface B*, 2016, 145, 740–747.
- [19] Qin, Z., Du, S., Luo, Y., Liao, Z. et al., Hydrothermal synthesis of superparamagnetic and red luminescent bifunctional Fe₃O₄@Mn²⁺-doped NaYF₄:Yb/Er core@shell monodisperse nanoparticles and their subsequent ligand exchange in water. *Appli. Surface Sci.* 2016, 378, 15, 174–180.
- [20] Jeong, H. Y., Deok, H. W., Mi-Sook, C., Myung-Suk, C., Microfluidic based biosensing for *Escherichia coli* detection by embedding antibacterial peptide-labeled beads. *Sens. Actuators, B: Chemical*, 2014, 191, 211–218.
- [21] Ma, Y., Liu, C., Liu, X., Wu, J. et al., Peptidomics and genomics analysis of novel antibacterial peptides from the frog, *Rana nigrovittata*. *Genomics* 2010, 95, 66–71.
- [22] Zhang, S., Guo, H., Shi, F., Wang, H. et al., Hainaninins: A novel family of antibacterial peptides with strong activity from Hainan cascade-frog, *Amolops Hainanensis*. *Peptides*, 2012, 33, 251–257.
- [23] Wang, Y., Zhang, Y., Lee, W. H., Zhang, Y. et al., Novel Peptides from skins of amphibians showed broad-spectrum antibacterial activities. *Chem. Biol. & Drug Des.*, 2016, 87, 419–424.
- [24] Martinez, R. C. R., Staliano, C. D., Vieira, A. D. S., Villarreal, M. L. M. et al., Gombosy de Melo Franco, B. D., Bacteriocin production and inhibition of *Listeria monocytogenes* by *Lactobacillus sakei subsp. sakei* 2a in a potentially symbiotic cheese spread. *Food Microbiol.* 2015, 48, 143–152.
- [25] Miao, J. Y., Guo, H. X., Chen, F. L., Zhao, L. C. et al., Antibacterial effects of a cell-penetrating peptide isolated from Kefir. *J. Agri. Food Chem.* 2016, 64, 3234–3242.
- [26] Chamorro, C., Boerman, M.A., Arnusch, C.J., Breukink, E., Pieters, R.J. Enhancing membrane disruption by targeting and multivalent presentation of antimicrobial peptides. *Biochim. Biophys. Acta.* 2012, 18, 2171–2174.
- [27] Li, L., Shi, Y., Cheserek, M.J., Su, G. et al., Antibacterial activity and dual mechanisms of peptide analog derived from cell-penetrating peptide against *Salmonella typhimurium* and *Streptococcus pyogenes*. *Appli. Microbiol. Biotechnol.* 2013, 97, 1711–1723.