Characterization of the extracellular proteases from *Bacillus inaquosorum* strain E1-8 and its application in the preparation of hydrolysates from plant and animal proteins with antioxidant, antifreeze, and anti-browning properties

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Abstract:

BACKGROUND: *Bacillus inaquosorum* strains is widely recognized for their plant-growth-promoting and biocontrol capabilities, yet their roles in protease production remain unclear. This study aims to comprehensively assess the protease-producing performance of *B. inaquosorum* strain E1-8, while also exploring the novel application of agricultural *Bacillus* proteases in the preparation of protein hydrolysates for fresh-cut fruits preservation.

RESULTS: Firstly, genomic sequencing revealed the diversity of E1-8 proteases, indicating 15 putative extracellular proteases. Subsequently, the fermentation conditions for E1-8 protease production were optimized, with sweet potato powder and soybean meal identified as the most suitable carbon and nitrogen sources, respectively, resulting in a maximum protease activity of 321.48 U/ml. Upon culturing the strain under these optimized conditions, only an S8 family serine protease and an M48 family metalloprotease were revealed by secretomic analysis and protease inhibitor assays. Additionally, the optimal protease conditions for generating protein hydrolysates from soy, pea, fish, and porcine proteins were determined. The molecular weight of the hydrolysates primarily ranged from 2000 to 180 Da, with a total of 17 amino acids identified. The application of these hydrolysates demonstrated a DPPH scavenging activity ranging from 58.64% to 84.12%, significantly reducing of the melting peaks and the freezing points. Furthermore, the browning index of apple slices stored at 4°C decreased by 14.81% to 22.15% on the second day, and similar effects were observed in fresh-cut banana stored at 4°C for 7 days.

CONCLUSION: The protein hydrolysates obtained exhibit remarkable antioxidant, antifreeze, and anti-browning properties for fresh-cut fruits.

Keywords: agricultural microorganism; extracellular proteases; animal and plant protein; protein hydrolysates; fresh-cut fruits; fresh-keeping activity

INTRODUCTION

The consumption of fresh-cut fruits is rapidly increasing, particularly among young consumers, due to their ease of carrying and consumption.[1] Surface oxidation and cold damage diminish the appearance and freshness of fresh-cut fruits, which are typically stored on refrigerated shelves.[2] Chemical agents, including sodium bisulfite, sodium metabisulfite, sodium erythorbate, sodium chloride, kojic acid, citric acid, and cysteine, have been extensively used as preservatives for fresh-cut fruits,[3] along with antifreeze agents such as sugar, alcohol, and their compounds.[4] The use of these chemical additives in fresh-cut fruits not only disrupts their natural tissue structure, taste, and nutritional content but also raises concerns about toxicity and high calorie content.[3-4] Consequently, the development of novel biological preservatives, particularly those with antioxidant properties, holds significant promise in the fresh-cut fruit processing industry.

Peptide-rich hydrolysates from both animal and plant proteins have garnered significant attention for their natural abundance, straightforward preparation, and superior performance.[5] Hydrolysates from soy and pea plant proteins have been documented as possessing antioxidant, antimicrobial, antihypertensive, and anti-fatigue properties.[6-8] Additionally, hydrolysates from fish and porcine animal proteins showcase considerable promise in the food industry, offering antioxidant, free radical-fighting, anti-aging, and weight management benefits.[9-10] In contrast to acid and alkali hydrolysis, enzymatic methods, with the assistance of proteases, especially microbial proteases, offer heightened specificity, gentler reaction condition, and fewer residual

organic solvents and toxic chemicals to produce protein hydrolysates.[11-12] *Bacillus* protease, extensively researched and commercialized, is a bacteria-derived enzyme. For instance, the *B. licheniformis* LBA46 protease generates antioxidant peptides from pea protein,[13] while the *B. subtilis* A26 protease is employed to create sardine protein hydrolysates boasting antioxidant, antibacterial, and ACE inhibitory activities.[14] As highlighted, antioxidant properties are a common feature of protein hydrolysates. To our knowledge, reports on the application of protein hydrolysates in fresh-cut fruits are relatively scarce.

Sea rice is extensively cultivated on coastal mudflats of China, particularly in Jiaozhou Bay.[15] Soil protease and the protease-producing microorganisms significantly influence the transformation of organic nitrogen in cultivation fields.[16, 17] Reports indicate that sea rice cultivation enhances soil protease activity and the richness of protease-producing microorganisms.[18] *Bacillus*-like bacterial communities dominate the soil microbiota in sea rice cultivation.[18] Among the 129 protease-producing strains previously isolated from the coastal mudflats of Jiaozhou Bay,[18, 19] *B. inaquosorum* E1-8 was found to exhibit the highest protease production. *B. inaquosorum* is frequently noted for its broad-spectrum antibacterial properties, including strain T1's resistance to *Vibrio Parahaemolyticus*,[20] strain 76-1's resistance to *B. cereus*, *Staphylococcus aureus*, and *Salmonella enterica*,[21] and strain HU Biol-II's resistance to various fungi.[22] Additionally, *B. inaquosorum* strains were regarded as potential agents for plant-growth promotion and biocontrol, such as strain KR2-7, effective against Fusarium wilt in tomatoes,[23] and strain NL1, resistant to invasive harmful weed species.[24]

Although numerous studies have examined the antibacterial and plant growth-promoting effects of *B. inaquosorum*, information regarding its protease production capabilities remains scarce. Consequently, this study presents the protease diversity of strain E1-8, derived from genome sequencing and analysis. Additionally, fermentation optimization was carried out, and secreted proteases from the optimized medium were identified using secretomic analysis and protease inhibitor assays. Furthermore, the proteases were applied to four distinct plant and animal proteins to produce protein hydrolysates, whose antioxidant, antifreeze, and anti-browning properties on fresh-cut fruits were subsequently evaluated. The findings of this research are anticipated to offer insights for novel applications of the proteases of *B. inaquosorum* in manufacturing protein hydrolysates for the preservation of fresh-cut fruits.

MATERIALS AND METHODS

Bacterial strain

Strain E1-8, isolated from the coastal beach of Jiaozhou Bay in China, was identified as *B. inaquosorum* based on its 16S sequence (GenBank accession No. OQ618960). This strain is deposited at the China Center for Type Culture Collection under the accession No. M 20232071. Utilizing MEGA 11 and the neighbor-joining method, a phylogenetic tree was constructed based on the 16S rRNA gene sequences of strain E1-8. The protease producing ability of strain E1-8 was assayed following a protocol in previous studies with minor modifications.[18, 19] In brief, solid culture media containing various protein substrates (1.0% milk powder, 0.5% casein, 2% gelatin, 0.5% soy protein, 0.5% pea protein, 2% fish protein, or 2% porcine protein) were prepared

by mixing them with 0.2% yeast extract, 1.5% agar, and artificial seawater, and adjusting the final pH to 8.0. The strain was streaked onto these media and incubated at 37°C for four days. Subsequently, the hydrolysis zones were observed, and the diameters of the hydrolysis zones and colonies were measured.

Genomic sequencing and analysis

Strain E1-8 was cultivated in marine broth 2216 (BD Difco™, USA) at 25°C. Genomic DNA was extracted using a Genomic DNA Extraction Kit (Thermo Scientific™, USA). Genomic sequencing was performed by Biomarker Biotechnology Co. Ltd., China, on the Illumina Hiseq platform and the PacBio RS platform. Coding gene sequences were predicted and annotated using Prodigal v2.6.3 [25]and RAST v2.0.[26] The genomic data of E1-8 has been submitted to GenBank with the accession No. JAXOUB000000000. SignalP was used to predict the signal peptides of the proteases (http://www.cbs.dtu.dk/services/SignalP).[27] The peptidase family was analyzed by conducting a Blastp search in the MEROPS peptidase database v11.0, with an E-value threshold of 1e-5 (http://merops.sanger.ac.uk). [28]

Optimization of fermentation medium and culture conditions

Yeast extract (0.2%), gelatin (0.5%) and casein (0.3%) in the basal medium was replaced by seven kinds of carbon sources, namely sweet potato powder, rice bran, mung bean, bran, xanthan gum, sucrose, sorbitol, or seven kinds of nitrogen sources, including soybean meal, peanut cake meal, fish meal, soy protein, pea protein, peptone, skim milk powder. In addition, two single factors, the medium pH (6-10) and the fermentation temperature (20-45°C) were investigated for their effects on protease

production. Strain E1-8 was cultured in each modified medium, and the extracellular protease production was detected at the 36th hour. The sweet potato powder concentration, soybean meal concentration, medium pH, and fermentation temperature were selected as experimental factors for a further three-level orthogonal experiment.

Secretomic sequencing and analysis

Strain E1-8 was cultured at 37ºC for 36 hours in a fermentation medium consisting of 3.5% sweet potato powder, 0.5% soybean meal, and artificial seawater, maintaining a pH of 7.0. Subsequently, the culture was centrifuged at 18,000 g, for 30 minutes at 4ºC. The supernatant underwent precipitation, denaturation, and desalination, using a previously described method with minor modifications.[29] MS spectra were recorded using a mass spectrometer Orbitrap Elite in conjunction with an Easy-nLC 1000 (ThermoFisher, Germany). The scanning protocol involved a full MS scan with a mass resolution of 120,000 across the 250 – 1450 m/z, followed by repeated CID MS/MS scans for the 20 most intense ions selected from the previous full MS scan with an isolation window. The normalized collision energy was set at 30%, and the activation time was maintained at 50 ms. Finally, the secreted proteins were analyzed using the Proteome Discoverer™ software v1.4.

Characterization of the extracellular proteases

Extracellular protease activity was assessed using the Folin-Ciocalteu method with casein, soy protein and pea protein as substrates, or with ninhydrin and sodium citrate colorimetry method using fish protein and porcine protein as substrates.[30, 31] The impact of protease inhibitors on extracellular protease activity was examined using previously

157 reported procedure with minor modifications.[32] Inhibitors included
158 phenylmethylsulfonyl fluoride (PMSF, Sigma), 1,10-phenanthroline (OP, Sigma), E64
159 (Merck), and Pepstatin A (PA, Merck). The influence of metal ions on extracellular
160 protease activity was investigated using previously reported method.[33] Metal ions tested
161 included Mn^{2+}, K^{+}, Co^{2+}, Ba^{2+}, Li^{+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, and Fe^{2+}. Extracellular proteases
162 were pre-incubated with each inhibitor and metal ion at 4ºC for 1 hour, followed by
163 measuring the residual enzyme activity using the Folin-Ciocalteu method with casein
164 as substrate. Enzyme activity in the absence of inhibitors or metal ions was designated
165 as 100%.

166 **Optimization of enzymatic hydrolysis parameters**

167 During the enzymatic hydrolysis of soy, pea, fish, and porcine proteins, single-
168 factor experiments were carried out to optimize four key parameters: hydrolysis
169 temperature, pH value, E/S ratio, and reaction time. Briefly, enzymatic hydrolysis was
170 conducted across a range of temperatures from 10 to 80ºC, pH levels from 2 to13, E/S
171 ratios between 200 and 1200 U/g, and hydrolysis durations from 10 to 70 minutes. The
172 hydrolysis reaction was terminated by heating the reaction mixture at 90ºC for 15
173 minutes, followed by centrifugation of the resultant slurry. For each parameter, the
174 outcome was determined by the quantity of amino acids or short peptides released into
175 the supernatant.

176 **Characterization of the enzymatic hydrolysates**

177 The enzymatic hydrolysis products of E1-8 extracellular proteases were obtained
178 by optimizing hydrolysis conditions specifically for various protein substrates,

including soy protein (60°C, pH 12, 800 U/g, 60 min), pea protein (60°C, pH 11, 800 U/g, 50 min), fish protein (50°C, pH 9, 1000 U/g, 50 min), and porcine protein (60°C, pH 11, 600 U/g, 50 min). The reaction mixture was centrifuged at 18,000 g for 30 minutes at 4°C, after which the supernatant was collected and freeze-dried. The hydrolysis products, as reported by He et al., contained peptides and amino acids.[34] The type and content of the fully hydrolyzed amino acids, obtained post-treatment with 6 M HCl, were analyzed using an Amino acid analyzer (L-8900, Hitachi, Japan). The molecular weight distribution of peptides in the enzymatic hydrolysis products was analyzed according to the previously reported procedures, with minor modifications.[35] Briefly, the protein hydrolysate sample was diluted, dissolved by ultrasonic treatment for 5 min, centrifuged, and then injected using microporous filtration. RP-HPLC (Waters alliance 2695, USA), equipped with a 2487 UV detector, an empower workstation GPC software, and a symmetry C18 column (Waters, Milford, USA), was utilized. The chromatographic conditions were set at a flow rate of 0.5 ml/min, a column temperature of 30°C, and a detection wavelength of 280 nm. The mobile phase system was water plus acetonitrile, and trifluoroacetic acid (TFA) was used as an ion-pair reagent.

Detection of antioxidant and antifreeze activity

The antioxidant activity of the hydrolyzed product was assessed using a previously reported procedure, with slight modifications.[36] In brief, 2 ml of DPPH (100 μM) was combined with 1 ml of hydrolyzed product at concentrations ranging from 1 to 20

mg/ml, and incubated in the dark at 25°C for 40 minutes. Subsequently, the absorbance of the reaction mixtures was measured at 525 nm.

The antifreeze activity of the hydrolysis products was analyzed via differential scanning calorimetry (DSC), with minor modifications to the protocol reported by Sui et al.[37] In brief, each hydrolysis product (10-50 mg/ml) was introduced into an aluminium pans, cooled from 20°C to -25°C at the rate of -5°C/min, maintained at -25°C for 5 minutes, and subsequently heated to 10°C using a DSC system (TAQ2000, METTLER TOLEDO, China) at a rate of 5°C/min. Heat flow was measured during both the cooling and heating phases. The freezing point of each hydrolysis product was identified as the onset of the heat flow curve during the heating phase.

Browning index detection of fresh-cut fruits

The impact of protein hydrolysates on browning of fruit slices was assessed following the protocol outlined by Wang et al.[38] In brief, freshly cut apples and bananas were coated with each protein hydrolysate and subsequently exposed to air at either room temperature or 4°C. The browning extent of the fruit slices was observed and photographed every 24 hours. To assess the color of the fruit slices, parameters L*, a* and b* were obtained using a CR-400 automatic colorimeter (Konica Minolta Holdings, Japan) . Subsequently, the browning index (BI) value was calculated using the provided formula.

$$BI = 100[(a^*+1.75L^*)/(5.645L^*+a^*-3.012b^*)-0.31]/0.172$$

Statistical analysis

The experiments were conducted in triplicate, and the values are presented as mean ± standard deviation. The significance of differences between groups was evaluated using a paired sampling t-test. The statistical significance level was set at 5% (p-value < 0.05).

RESULTS AND DISCUSSION

Diversity analysis of the proteases of strain E1-8 by genome sequencing and gene annotation

In our prior research focused on the community structure of protease-producing bacteria strains along the coastal mudflats of Jiaozhou Bay, which encompassed sea rice fields, clam growing zones, and adjacent regions, we isolated 129 strains of such bacteria, with strain E1-8 being the one with the highest protease output. [18, 19] Neighbor-joining phylogenetic analysis identified this strain as *B. inaquosorum* (Fig. S1). As depicted in Fig. 1A, employing skim milk powder, casein, gelatin, soy protein, pea protein, fish protein and porcine protein as substrates, varied hydrolysis halos were observed around strain E1-8 on these protein plates, demonstrating its capability to break down diverse protein types.

Sequencing of strain E1-8's genomic DNA revealed a total size of 3,722,538 bp and an average GC content of 43.93%, with 4,125 predicted genes. Fig. S2 illustrates the genome's circular representation. Next, we commenced analyzing the diversity of proteases within strain E1-8 and its family by genes annotation . According to Fig. 1B, strain E1-8 harbors 58 putative proteases, categorized into nine metalloprotease (M) peptidase families, eight serine (S) peptidase families, one cysteine (C) peptidase family,

one aspartic (A) peptidase family, one threonine (T) peptidase family, and three of unknown (U) catalytic type. Based on signal peptide predictions, 14 proteases with putative signal peptides were classified as extracellular, while the remaining 44 are considered intracellular proteases (Fig. 1B).

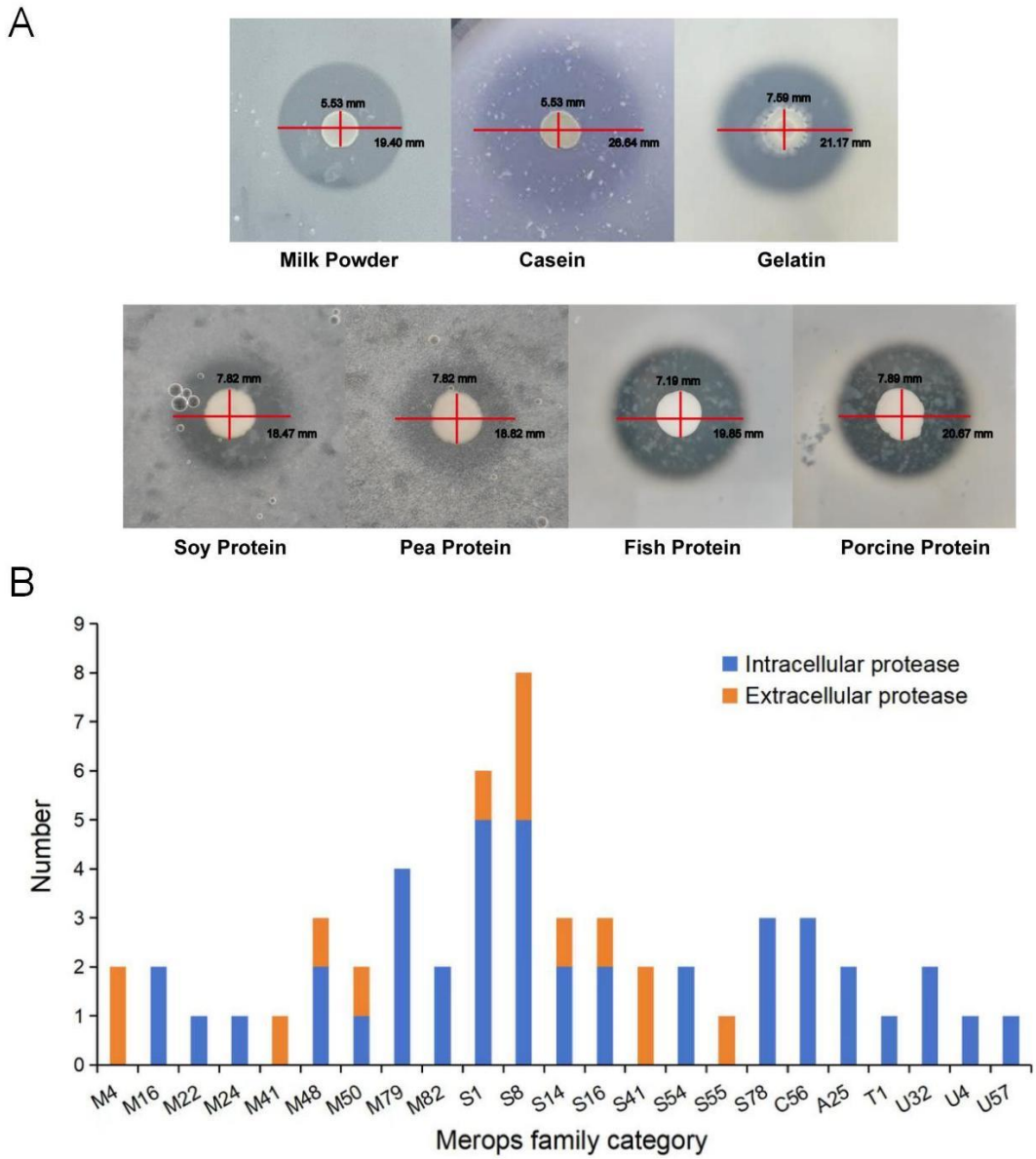

Figure 1. Proteases production capacity of the strain E1-8. (A) Hydrolytic halos of strain E1-8 on protein substrate plates; (B) Putative number of the proteases of strain E1-8 in each Merops family based on genome sequencing and gene annotation. The putative number of extracellular proteases is displayed in orange and the number of

intracellular proteases in blue.

As shown in Fig. 1B and Table 1, 15 putative extracellular proteases are categorized into 10 families. These families include two thermostable neutral proteases from the M4 family, one FtsH-like peptidase from the M41 family, two YhfN-like peptidases from the M48 family, one S2P-like peptidase from the M50 family, one chymotrypsin-like peptidase from the S1 family, three Subtilisin-like peptidases from the S8 family, two types of peptidases from the S41 family, and one each peptidase from the S14, S16, and S55 families. The M4 and S8 protease families are secreted to decompose extracellular proteins and peptides, serving as a nutritional source for the bacteria.[39, 40] Notably, the S8 protease may process the capability to degrade animal collagen.[41] The M48 protease is potentially involved in the hydrolysis of proteins.[42] The predicted functions of other extracellular proteases from strain E1-8 include protein quality control, especially the degradation of unfolded or incorrectly synthesized proteins (Table 1).

Table 1. The extracellular proteases of strain E1-8 predicted by genome sequencing and gene annotation.

Gene ID	Family	Holotype Protease	Predicted functions
GE000896, GE002676	M4	Thermolysin	Most are secreted enzymes that degrade extracellular proteins and peptides for bacterial nutrition.
GE004061	M41	FtsH	Putative membrane metalloprotease
GE002630	M48	YhfN	Involved in proteolysis
GE000270	M50	S2P	Involved in sporulation
GE002185	S1	Chymotrypsin	Involved in intestinal digestion, IgA-mediated immune response
GE000323, GE000355, GE000864	S8	Subtilisin	Involved in nutrition
GE003904	S14	Clp endopeptidase	Involved in both protein quality

			control and the regulatory degradation of specific proteins
GE002711	S16	Lon-A	Degradation of unfolded proteins
GE000017, GE003523	S41	C-terminal processing peptidase-1	Degradation of incorrectly synthesized proteins
GE003786	S55	SpoIVB	Involved in sporulation

Fermentation optimization of E1-8 for protease production

The previously established flask fermentation medium for the protease-producing strain E1-8 comprised 0.2% yeast extract, 0.5% gelatin, 0.3% casein, and artificial sea water, maintained at a pH of 8.0.[18, 19] In this research, this medium was termed the basic medium. To enhance protease production and minimize fermentation costs, sweet potato powder, rice bran, mung bean, bran, xanthan gum, sucrose, and sorbitol were employed as carbon sources, whereas soybean meal, peanut cake meal, fish meal, soy protein, pea protein, peptone, and skim milk powder served as nitrogen sources, all being more cost-effective alternatives to yeast extract, gelatin, and casein (Table S1). Sweet potato powder and soybean meal are demonstrated as the optimal carbon and nitrogen sources, respectively, because they enhanced protease production and minimized fermentation costs meanwhile (Fig. 2A and B, Table S1). Similar studies have shown that potato pulp powder boosts the production of acid protease in *Aspergillus oryzae*,[1] whereas soybean meal emerges as the preferred nitrogen source for protease production and secretion in *Bacillus amyloliquefaciens* 35M.[43]

Subsequently, the effects of concentrations of these two carbon and nitrogen sources were assessed through single-factor experiments. Fig. 2C indicates that the most effective concentrations of sweet potato powder and soybean meal were 3% and 1%, respectively. Additionally, the study examined the impact of medium pH and

fermentation temperature, revealing that the most suitable pH and temperature were 7 and 37^{o}C, respectively (Fig. 2D).

Based on the results of single-factor experiments, an orthogonal experiment was conducted to optimize the fermentation medium. As shown in Table S1, nine combinations of four single factors, including carbon source concentration, nitrogen source concentration, medium pH, and fermentation temperature, were generated using an L9 (3^4) design. The protease activity of strain E1-8 was assessed under each fermentation condition. Among these, combinations 2, 6, and 7 demonstrated the highest enzyme activity, with combination 6 reaching 315.80 U/ml (Fig. 2E, Table S2). The optimal fermentation combination was 3.5% sweet potato powder, 0.5% soybean meal, pH 7.0, and a temperature of 37^{o}C (Table S2). The cost of the optimized medium is 0.54 yuan/L, significantly lower than that of the basal medium, which is 3.47 yuan/L, (Table S3). Finally, the verified optimal fermentation medium and culture condition combination achieved a maximum protease activity of 321.48 U/ml at the 60th hour (Fig. 2F). Overall, we successfully optimized the fermentation conditions, reduced the fermentation cost of producing protease by strain E1-8, and paved the way for the industrial application of E1-8 extracellular protease.

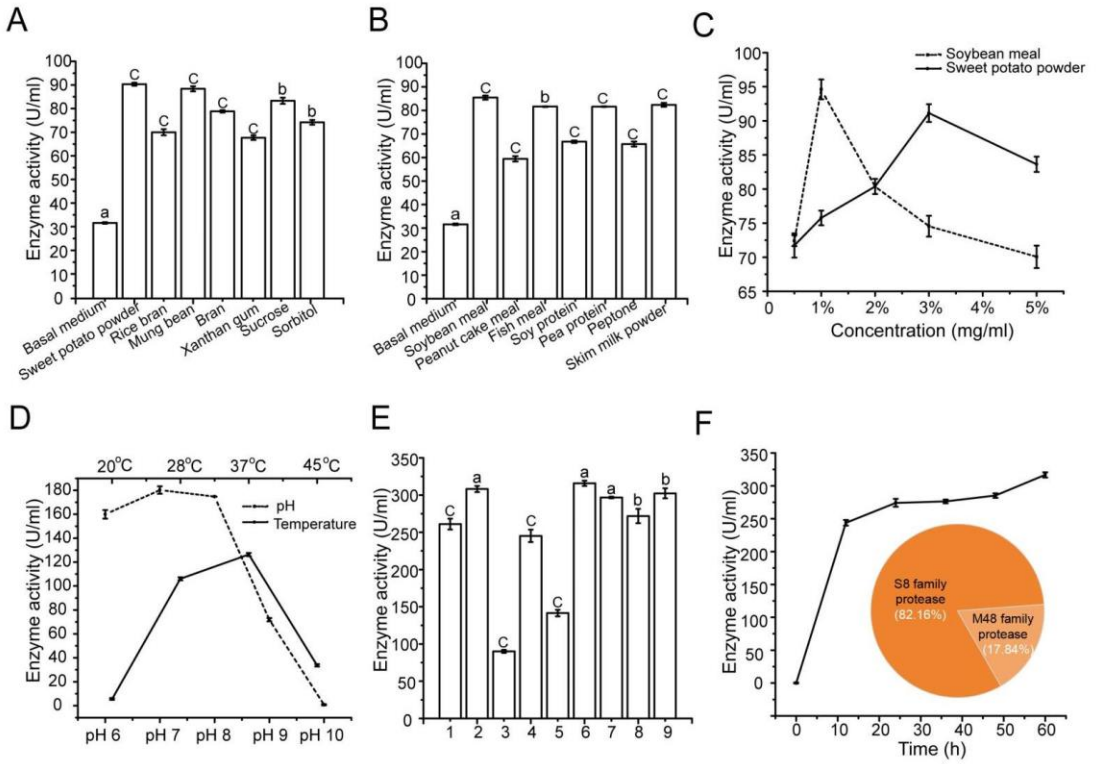

Figure 2. Optimization of the fermentation conditions of *B. inaquosorum* E1-8 to produce protease. (A) Impact of carbon source type on protease production; (B) Impact of nitrogen source type on protease production; (C) Impact of concentrations of sweet potato powder and soybean meal on protease production; (D) Impact of pH and fermentation temperature on protease production; (E) The protease production of strain E1-8 cultured under the fermentation conditions designed by orthogonal test; (F) The protease production of strain E1-8 cultured in the optimized fermentation medium over time; (insert) The abundance of strain E1-8 extracellular protease predicted by secretomic sequencing and analysis. The graphs show data from treatments conducted in triplicate (mean ± S.D.). Statistical analysis of enzyme activity was conducted by t-test. The lowercase letter b and c represented significant differences between each group at $p < 0.05$ and $p < 0.01$, respectively.

Identification and characterization of the extracellular proteases of strain E1-8

The secreted proteome of strain E1-8 cultured in a medium composed of sweet potato powder and soybean meal was analyzed to ascertain the presence of secreted proteases. Interestingly, the secreted proteome comprises only two types of proteases: the S8 family serine protease (GE000323) and the M48 family metalloprotease

(GE000819). The S8 protease predominates with an abundance of 82.16%, followed by the M48 protease with 17.84% (Fig. 2F insert). We observed that only the S8 family proteases possess putative signal peptides, which are likely responsible for their extracellular secretion via signal peptide-mediated translocation. Conversely, the M48 family proteases, lacking putative signal peptides, may be secreted via alternative secretion systems, such as non-classical secretion system.[44] In strain E1-8, 14 predicted proteases have putative signal peptides, as detailed in Table 1. However, aside from the S8 family proteases, the other 13 proteases with putative signal peptides were not identified via secretomic analysis. A possible explanation is that these proteases were either not expressed or secreted below the detection threshold of the secreted proteome sequencing when strain E1-8 was cultured with sweet potato powder and soybean meal.

The effects of protease inhibitors on the extracellular proteases of strain E1-8 were assessed to further study the types of extracellular proteases. Protease inhibitors include specific inhibitors for serine protease, metalloprotease, cysteine protease, and aspartic protease, namely PMSF, OP, E64, and PepA, respectively. PMSF and OP significantly inhibited the extracellular protease activity of E1-8 by 80.53% and 41.60%, respectively, whereas PepA and E64 demonstrated minimal inhibitory effects (Table 2). The findings indicate that the extracellular protease of strain E1-8 primarily consists of serine protease and metalloprotease, aligning with the aforementioned secretomic findings. Additionally, the study evaluated the impact of metal ions on the extracellular protease activity of strain E1-8. The presence of Mn^{2+} significantly increased the activity of extracellular protease in E1-8 by 242%, whereas Mg^{2+}, Ni^{2+}, and Fe^{2+} exhibited

inhibitory effects ranging from 61.86 to 88.07% (Table 2).

Table 2. The impact of inhibitors and metal ions on the activity of E1-8 extracellular proteases [a].

Inhibitors	Inhibition ratio (%) [b]	Metal ions	Relative activity (%)	Metal ions	Relative activity (%)
Control	0.00 [c] a	Control	100.00 a	Li^{+}	89.29±8.77 a
PMSF	80.53 ± 0.92 c	Mn^{2+}	242.36 ± 5.73 c	Ca^{2+}	89.00 ± 7.81 a
OP	41.60 ± 1.70 c	K^{+}	99.21 ± 7.41 a	Mg^{2+}	88.07± 4.61 b
E64	ND a	Co^{2+}	92.47±3.89 a	Ni^{2+}	80.32 ± 4.84 b
PepA	ND a	Ba^{2+}	91.77 ±7.95 a	Fe^{2+}	61.86 ± 8.84 b

[a] The group without the addition of any metal ion was designated as the control. Each data is a representative of at least three repeats. Statistical difference was evaluated by T-test. The lowercase letter b and c stand for significant differences between the control and the inhibitor/metal ion treatments at $p < 0.05$ and $p < 0.01$, respectively.

[b] Inhibition ratio refers to the difference in relative residue activity between samples incubated with and without the addition of an inhibitor.

[c] ND indicates that the inhibitory effect was not detectable.

Enzymolysis optimization of E1-8 extracellular proteases

To determine the potential application of E1-8 extracellular protease in the preparation of plant and animal protein hydrolysates, the activity of E1-8 protease on soy protein, pea protein, fish protein, and porcine protein was tested. Fig. S3 demonstrates that the extracellular protease of strain E1-8 exhibits significant hydrolytic activity on both plant and animal proteins. To enhance the production of protein hydrolysates with the E1-8 extracellular protease, single-factor experiments were conducted to examine the effects of hydrolysis temperature, pH value, enzyme-to-substrate (E/S) ratio, and reaction time on the released amino acids and peptides. The results indicated that the optimal hydrolysis temperature for soy protein, pea protein, and porcine protein substrates was 60°C, while the optimal hydrolysis temperature for fish protein substrates was 50°C (Fig. 3A). The extracellular protease from strain E1-8

366 demonstrates a broad pH tolerance ranging from 3 to 12. When pea protein and porcine
367 protein as substrates, the enzyme's activity peaked at pH 11, while at pH 12 with soy
368 protein and pH 9 with fish protein (Fig. 3B). Under the optimized hydrolysis
369 temperature and pH conditions, the optimal E/S ratio and reaction time for the E1-8
370 extracellular protease were further determined. The optimal E/S ratios were found to be
371 800 U/g for soy protein and pea protein, 1000 U/g for fish protein, and 600 U/g for
372 porcine protein (Fig. 3C). The yield of the protein hydrolysates increased dramatically
373 within the first hour, followed by minor fluctuations thereafter (Fig. 3D). Based on
374 these results, a method for preparing plant and animal protein hydrolysates using E1-8
375 extracellular protease was established, as illustrated in Fig. 3E. The freeze-dried powder
376 of the prepared protein hydrolysate is completely soluble in water, yielding a clear,
377 bitter-free solution that appears light yellow (Fig. 3F-I).

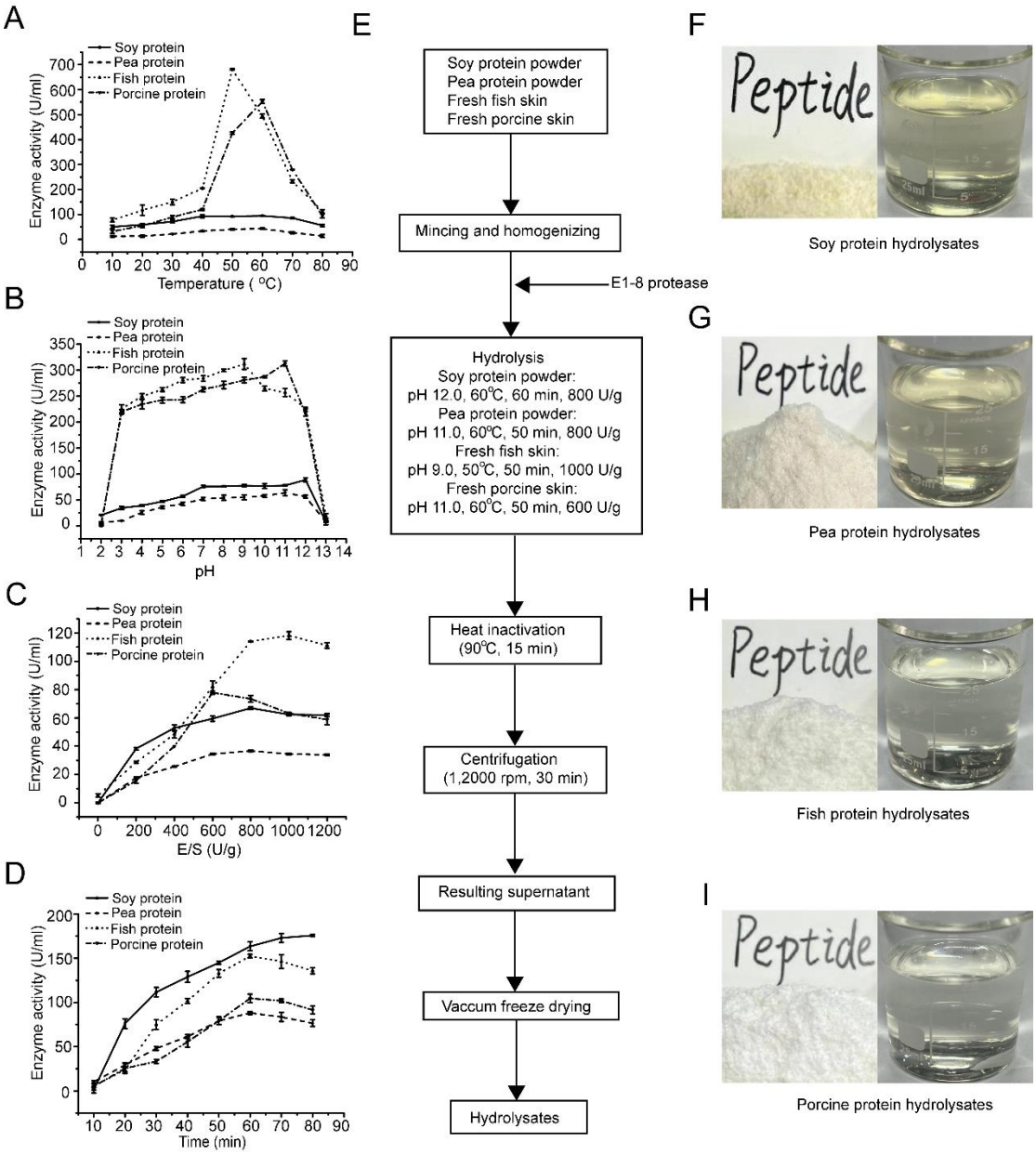

Figure 3. Optimization of the hydrolysis parameters of E1-8 extracellular protease towards soy protein, pea protein, fish protein, and porcine protein. (A) Effects of hydrolysis temperature; (B) Effects of pH value; (C) Effects of E/S ratio; (D) Effects of reaction time. The graphs show data from triplicate experiments (mean ± SD). (E) Flow sheet for the preparation of protein hydrolysates with E1-8 extracellular proteases; (F-I) Lyophilized powder and solution (20 mg/ml) of the prepared protein hydrolysates.

Analysis of amino acids and peptides in the protein hydrolysates

The molecular weight distribution and amino acid composition are two critical indicators of the physicochemical and biological properties of protein hydrolysis.[45]

Initially, RP-HPLC was employed to assess the molecular weight distribution of the prepared protein hydrolysate. As illustrated in Fig. S4 and quantified in Table 3, the molecular weights of the hydrolysates from soy protein, pea protein, fish protein, and porcine protein primarily fall within the 2000 ~ 180 Da range, with respective proportions of 72.36%, 83.88%, 81.93%, and 75.18%. These findings demonstrate that the peptides in these hydrolysates are predominantly oligopeptides, consisting of 20 ~ 2 amino acids.

Table 3. The molecular weight distribution of the protein hydrolysates produced by E1-8 extracellular proteases.

MW range (Da)	Number of amino acids	Percentage of peak area (%)			
		soy protein hydrolysate	pea protein hydrolysate	fish protein hydrolysate	porcine protein hydrolysate
>10000	>100	0.7	0.17	0.19	0.23
10000~5000	100~50	3.55	1.71	2.28	2.7
5000~3000	50~30	2.77	3.52	5.19	9.93
3000~2000	30~20	3.1	5.52	7.95	9.63
2000～1000	20~10	10.3	16.57	19.64	19.41
1000~500	10~5	23.96	33.22	35.78	31.12
500～180	5~2	38.1	34.09	26.51	24.65
＜180	1	17.54	5.21	2.45	2.33

We conducted a detailed analysis of amino acid composition and content within the prepared hydrolysates. These hydrolysates contained 17 different amino acids, including seven essential amino acids for humans: Thr, Val, Met, Ile, Leu, Phe, and Lys (Table 4), suggesting their potential as functional foods. Additionally, plant protein hydrolysates are particularly rich in Glu, comprising 9.63% of soy protein hydrolysates and 8.89% of pea protein hydrolysates. The predominant amino acid in animal protein hydrolysates is glycine, making up 14.31% of fish hydrolysates and 14.29% of porcine protein hydrolysates. The variations between the plant and animal protein hydrolysates

405 are attributable to amino acid composition of the respective protein sources.[18, 46]

406 **Table 4.** Composition and content of amino acids in protein hydrolysates prepared by
407 E1-8 extracellular proteases.

Amino acid	Soy protein hydrolysates	Pea protein hydrolysates	Fish protein hydrolysates	Porcine protein hydrolysates
Asp	5.18	5.41	3.60	3.35
Thr *	1.45	1.60	1.57	1.07
Ser	1.91	2.26	3.49	1.99
Glu	9.63	8.89	5.94	6.27
Gly	1.64	1.74	14.31	14.29
Ala	1.44	1.85	5.10	5.31
Cys	0.47	0.21	0.12	0.72
Val *	2.02	2.19	1.21	1.48
Met *	0.35	0.22	0.78	0.24
Ile *	1.85	2.02	0.80	0.73
Leu *	2.86	3.61	1.40	1.75
Tyr	1.69	1.46	0.34	0.43
Phe *	2.31	2.43	1.12	1.10
Lys *	2.76	3.50	2.10	2.27
His	1.17	1.08	0.66	0.44
Arg	2.67	3.82	4.51	4.91
Pro	2.42	1.99	6.20	8.30

408 * Human-essential amino acids.

409 **Antioxidant and antifreeze abilities of the protein hydrolysates**

410 To assess the antioxidant activity of the prepared protein hydrolysates, we
411 evaluated their free radical scavenging activity against 2, 2-Diphenyl-1-Picrylhydrazyl
412 (DPPH). DPPH scavenging activity significantly increases with the concentration of
413 each protein hydrolysis product, demonstrating a concentration-dependent pattern (Fig.
414 4A-D). At a concentration of 10.0 mg/ml, the scavenging rates of the four protein
415 hydrolysates on DPPH are as follows: soy protein hydrolysate with a rate of 68.58 ±
416 0.23% (Fig. 4A), pea protein hydrolysate with 67.31 ± 1.90 % (Fig. 4B), fish protein
417 hydrolysate at 53.33 ± 2.69 % (Fig. 4C), and porcine protein hydrolysate at 56.78 ±

418 2.33 % (Fig. 4D), which are significantly higher than the commercial free radical
419 scavenging material, HA, and the hydrolysate enriched with fish collagen oligopeptides,
420 prepared from the serine protease of *Pseudoalteromonas* sp. SM9913, with a
421 scavenging rate of 34 ± 1.91% as reported in our prior research.[47, 48] Additionally, at a
422 concentration of 20 mg/ml, the scavenging rates of these protein hydrolysates were
423 84.12%, 83.98%, 75.57%, and 58.64%, respectively, highlighting pronounced and
424 significant antioxidant activity. Reports indicate that acidic amino acids (Glu, and Asp),
425 alkaline amino acids (Lys, Arg, and His), aromatic amino acid (Tyr), and sulfur-
426 containing amino acid (Cys) possess robust antioxidant capacities.[49,50] Notably, all
427 these amino acids have been identified in the aforementioned composition and content
428 analysis of amino acids (Table 4), thereby accounting for the antioxidant activity
429 exhibited by protein hydrolysates prepared using E1-8 extracellular proteases.

430 To assess the antifreeze properties of these protein hydrolysates, their melting peak
431 and freezing point were measured. Based on the DSC experiment results, it can be
432 inferred that the melting peaks of protein hydrolysates from both plant and animal
433 sources are significantly lower than those of the PBS solution (Fig. 4E-H), indicating
434 that all four protein hydrolysates demonstrate an inhibitory effect on the icing process.
435 Additionally, the freezing points of the four protein hydrolysates were found to be
436 significantly lower than that of the PBS solution (Fig. 4I-L), highlighting their
437 antifreeze capabilities and potential as antifreeze agents. Similar finding indicated that
438 peptides with a high Gly content derived from tilapia scales possess antifreeze
439 capabilities, potentially maintaining the fluidity of cell membrane through hydrogen

bonding within phospholipid bilayer, thereby safeguarding *Streptococcus thermophilus* against low temperature.[51] Reports also indicate that the peptides abundant in Gly, Ala, Leu, and Glu exhibit notable antifreeze performance.[52,53] The protein hydrolysates produced using E1-8 extracellular protease comprise all these amino acids (Table 4), contributing to their antifreeze abilities.

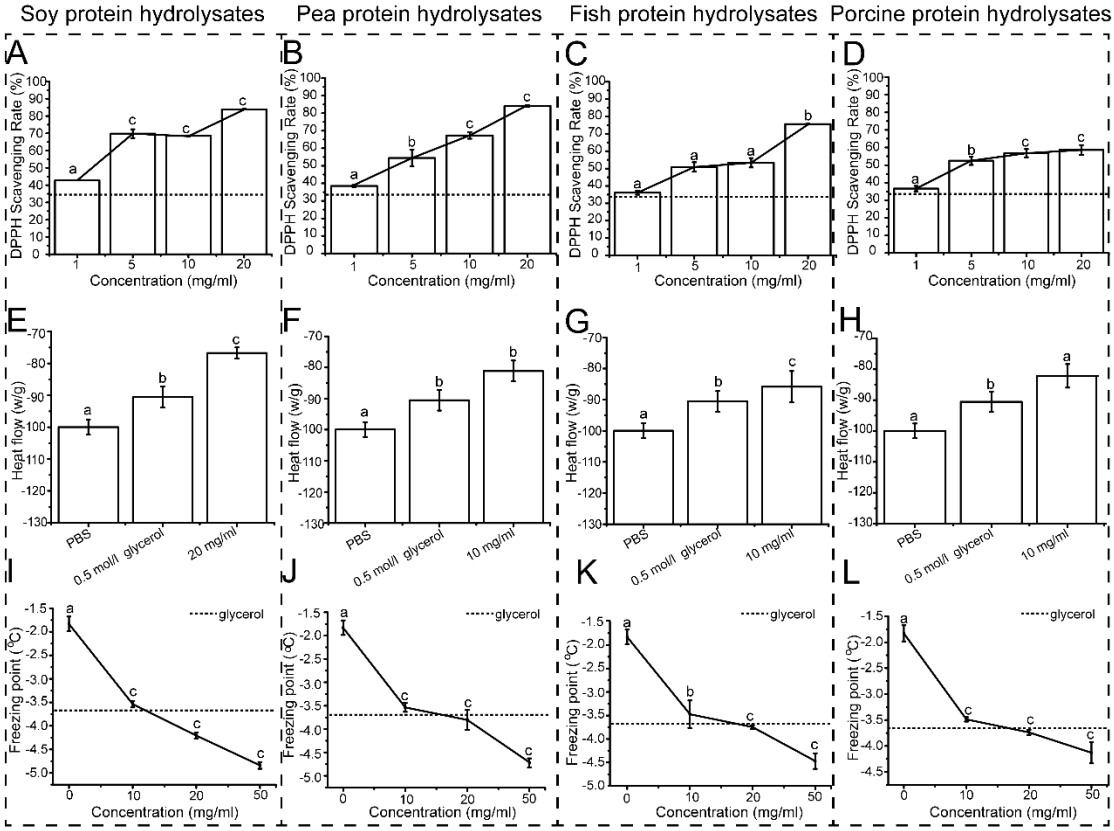

Figure 4. Antioxidant and antifreeze abilities of protein hydrolysates produced by the extracellular proteases of strain E1-8. (A-D) DPPH scavenging ability. The reference line represents the value of a hydrolysate enriched with fish collagen oligopeptides, prepared from the serine protease of *Pseudoalteromonas* sp. SM9913 [48]; (E-H) Heat flow curves and (I-L) freezing points during the warming process. The reference line represents the value of 0.5 mol/l glycerol. Each data is representative of at least three repeats. The vertical bars indicate the standard error of the means. The statistical difference was evaluated using the t-test. The lowercase letter b and c represented significant differences between the PBS and protein hydrolysates treatment at $p < 0.05$ and $p < 0.01$, respectively.

Application of the protein hydrolysates on fresh-cut fruits

Compared with whole fruits, the cut surfaces of fresh-cut fruits are prone to oxidation and browning, posing a significant challenge in their storage and preservation.[1] Additionally, refrigeration storage is a standard preservation technique for both vegetables and fruits, particularly for fresh-cut fruits. However, the cooling preservation process can also pose risks to fruits and vegetables.[54] To evaluate the practical application of prepared protein hydrolysates in fresh-cut fruits, their effectiveness in preventing browning was studied across different temperatures. Over the two-day of storage period, fresh-cut apples displayed progressively severe browning on their cut surfaces (Fig. S5A). As illustrated in Fig. 5, the groups treated with the prepared protein hydrolysates exhibited lower browning index values after two days, indicating their potential to inhibit fruit browning. On the second day, when stored at 4°C, the BI values for the soy, pea, fish, and porcine protein hydrolysate groups were, respectively, 22.15%, 16.89%, 18.30%, and 14.81% lower than the control group (Fig. 5A). Similarly, when stored at 25°C, the decrease ratios were approximately 12.61%, 11.21%, 14.46%, and 12.92%, respectively, (Fig. 5B).

In addition, as a tropical fruit, bananas are prone to chilling injury during refrigeration, particularly following mechanical cutting (Fig. S5B). By treating banana slices with a 20 mg/ml protein hydrolysate solution, the browning of fresh-cut banana was significantly inhibited after 7 days of storage at 4°C (Fig. 5C). Taken together, the protein hydrolysates we have prepared exhibits remarkable antioxidant and antifreeze

properties, effectively preventing browning in fresh-cut fruits, thus making it a highly promising biological fresh-keeping agent.

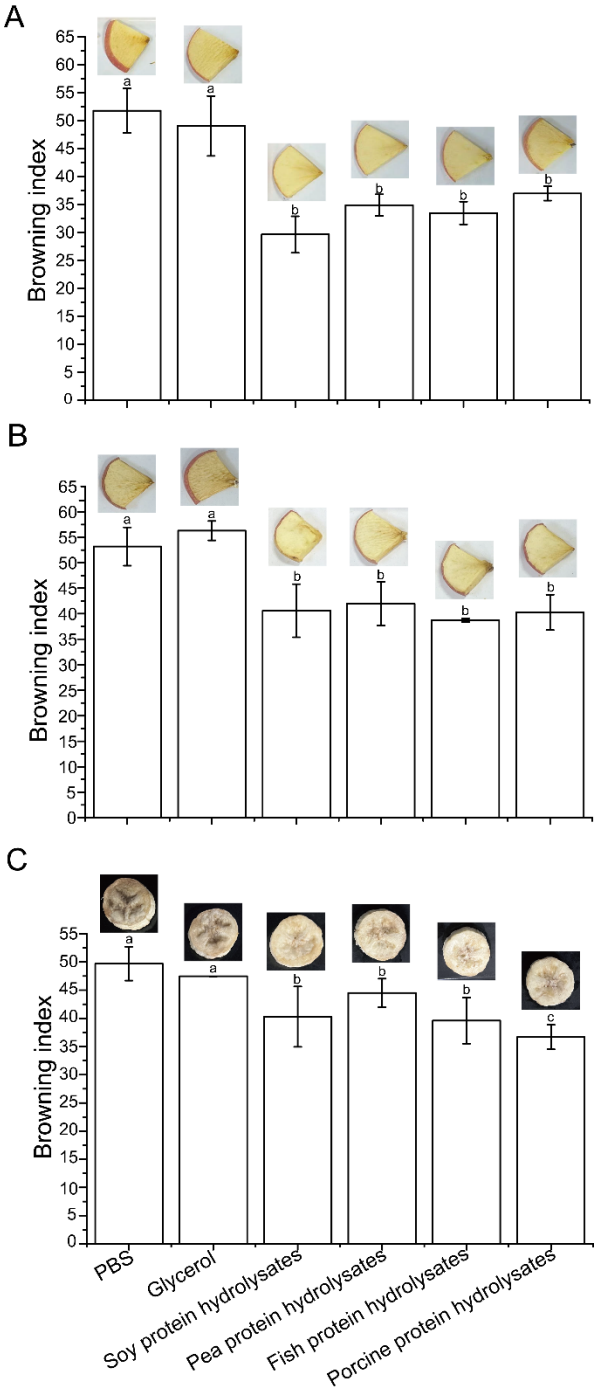

Figure 5. The impact of protein hydrolysates on the browning of fresh-cut fruits. The tissue browning phenotype and browning index of fresh-cut apples after storage for 2 days at 4°C (A) and 25°C (B), and of fresh-cut bananas after storage for 7 days at 4°C (C). Each data is representative of at least three repeats. The statistical difference was evaluated using the t-test. The lowercase letter b and c represent significant differences

between the PBS and protein hydrolysates treatment at $p < 0.05$ and $p < 0.01$, respectively.

CONCLUSIONS

The present study revealed that *B. inaquosorum* strain E1-8 possesses 58 putative proteases, and 15 of the putative proteases were noticed as extracellular proteases. It was discovered that sweet potato powder and soybean meal were most suitable carbon and nitrogen sources, respectively. The secretomic analysis revealed that two extracellular proteases, namely an S8 family serine protease and an M48 family metalloprotease, were present when strain E1-8 was cultivated in optimized fermentation medium, and were further confirmed by the protease inhibitor assay. The investigation showed that significant hydrolytic abilities against soy protein, pea protein, fish protein, and porcine protein were exhibited by the extracellular proteases of strain E1-8 . Moreover, the parameters of hydrolysis conditions for the production of protein hydrolysates using E1-8 extracellular proteases were further optimized, resulting in protein hydrolysates that exhibited notable antioxidant activity and antifreeze activity. In addition, findings revealed that the plant and animal protein hydrolysates produced were effective in preventing the occurrence of browning reactions in fresh-cut banana and apples stored under refrigerated conditions. Overall, the outcome of this study have presented new insights into the proteases of *B. inaquosorum* and some other agricultural microorganisms by highlighting their diversity and potential applications.

ACKNOWLEDGMENTS

The authors would like to thank China National Natural Science Foundation (32000004), Breeding Plan of Shandong Provincial Qingchuang Research Team-Innovation Team of Functional Plant Protein-Based Food (2021), Qingdao Agricultural University Research Fund for High-level Professionals (1120088), and Postgraduate

Innovation Program of Qingdao Agricultural University (QNYCX23043).

CONFLICT OF INTEREST

The authors declare no financial interests or personal relationships which could influence the work reported in this work.

DATA AVAILABILITY STATEMENT

Data will be made available on request.

REFERENCES:

1 Murthy P S and Kusumoto K I, Acid protease production by *Aspergillus oryzae* on potato pulp powder with emphasis on glycine releasing activity: A benefit to the food industry. *Food and Bioproducts Processing* **96**: 180-188 (2015).

2 Ma L, Zhang M, Bhandari B and Gao Z, Recent developments in novel shelf life extension technologies of fresh-cut fruits and vegetables. *Trends Food Sci. Technol* **64**: 23-38 (2017).

3 Meireles A, Giaouris E and Simões M, Alternative disinfection methods to chlorine for use in the fresh-cut industry. *Food Res Int* **82**: 71-85 (2016).

4 Chen X, Wu J, Cai X and Wang S, Production, structure-function relationships, mechanisms, and applications of antifreeze peptides. *Compr Rev Food Sci Food Saf* **20**: 542-562(2021).

5 Czelej M, Garbacz K, Czernecki T, Wawrzykowski J and Waśko A, Protein hydrolysates derived from animals and plants - a review of production methods and antioxidant activity. *Foods* **11**: 1953 (2022).

6 Ashaolu T J, Soy bioactive peptides and the gut microbiota modulation. *Appl*

533 *Microbiol Biotechnol* **104**: 9009-9017 (2020).
534 7 Feng T, Huang Y, Tang Z, Wei D and Mo J, Anti-fatigue effects of pea (*Pisum sativum*
535 L.) peptides prepared by compound protease. *J Food Sci Technol* **58**: 2265-2272
536 (2021).
537 8 Jia S, Wu Q, Wang S, Kan J, Zhang Z, Zhang X et al., Pea peptide supplementation
538 in conjunction with resistance exercise promotes gains in muscle mass and
539 strength. *Front Nutr* **9**: 878229 (2022).
540 9 Gui M, Gao L, Rao L, Li P, Zhang Y, Han J et al., Bioactive peptides identified from
541 enzymatic hydrolysates of sturgeon skin. *J Sci Food Agric* **102**: 1948-1957 (2022).
542 10 Choi D, Min S and Jo Y J, Functionality of porcine skin hydrolysates produced by
543 hydrothermal processing for liposomal delivery system. *J Food Biochem* **42**:
544 e12464 (2018).
545 11 Sun Q, Zhang X, Gao M, Zhang C and Peng B, Resource utilization of bovine neck
546 ligament: enzymatic preparation of elastin peptide and its antioxidant activity.
547 *Appl Biochem Biotechnol* **195**: 33-50 (2023).
548 12 Song P, Zhang X, Wang S, Xu W, Wang F, Fu R, et al., Microbial proteases and their
549 applications. *Front Microbiol* **14**: 1236368 (2023).
550 13 Aguilar J G S, de Castro R J S and Sato H H, Production of antioxidant peptides
551 from pea protein using protease from *Bacillus licheniformis* LBA 46. *Int J Pept*
552 *Res Ther* **26**: 435-443 (2020).
553 14 Jemil I, Abdelhedi O, Nasri R, Mora L, Jridi M, Aristoy M C et al., Novel bioactive
554 peptides from enzymatic hydrolysate of Sardinelle (*Sardinella aurita*) muscle

555 proteins hydrolysed by *Bacillus subtilis* A26 proteases. *Food Res Int* **100**: 121-133
556 (2017).
557 15 Gao J G and Zhu X G, The legacies of the “Father of Hybrid Rice” and the seven
558 representative achievements of Chinese rice research: a pioneering perspective
559 towards sustainable development. *Front in Plant Sci* **14**: 1087768 (2023).
560 16 Gao P, Guo L, Sun J, Wang Y, She Z, Gao M et al., Accelerating waste sludge
561 hydrolysis with alkyl polyglucose pretreatment coupled with biological process of
562 thermophilic bacteria: hydrolytic enzyme activity and organic matters
563 transformation. *J Environ Manage* **247**: 161-168 (2019).
564 17 López-Aizpún M, Arango-Mora C, Santamaría C, Lasheras E, Santamaría J M,
565 Ciganda V S et al., Atmospheric ammonia concentration modulates soil enzyme
566 and microbial activity in an oak forest affecting soil microbial biomass. *Soil Biol*
567 *Biochem.* **116**: 378-387 (2018).
568 18 Yang J, Liu Z, Zhang M, Zhu X, Wang M, Xu X et al., Impact of sea rice planting
569 on enzymatic activity and microbial community of coastal soils: focus on
570 proteinase. *Agronomy* **13**: 2089 (2023).
571 19 Liu Z, Liu G, Guo X, Li Y, Ji N, Xu X, et al., Diversity of the protease-producing
572 bacteria and their extracellular protease in the coastal mudflat of Jiaozhou Bay,
573 China: in response to calm naturally growing and aquaculture. *Front Microbio* **14**:
574 1164937 (2023).
575 20 Avery S E, Ruzbarsky S P, Hise A M and Schreier H J, Antibacterial activity of
576 *Bacillus inaquosorum* strain T1 against $pirAB^{Vp}$ - bearing *Vibrio parahaemolyticus*:

577 genetic and physiological characterization. *Appl Environ Microbiol* **86**: e01950-
578 20 (2020).
579 21 Zhang Y, Meng Z, Li S, Liu T, Song J, Li J et al., Two antimicrobial peptides derived
580 from *Bacillus* and their properties. *Molecules* **28**: 7899 (2023).
581 22 Knight C A, Bowman M J, Frederick L, Day A, Lee C and Dunlap C A, The first
582 report of antifungal lipopeptide production by a *Bacillus subtilis* subsp.
583 *inaquosorum* strain. *Microbiol Res* **216**: 40-46 (2018).
584 23 Kamali M, Guo D, Naeimi S and Ahmadi J, Perception of biocontrol potential of
585 *Bacillus inaquosorum* KR2-7 against tomato fusarium wilt through merging
586 genome mining with chemical analysis. *Biology* **11**: 137 (2022).
587 24 Hagaggi N S A and Abdul-Raouf U M, Phytotoxic interference of culture filtrates of
588 endophytic bacteria associated with nerium oleander leaf against seed germination
589 of the invasive noxious weed cenchrus echinatus. *Current microbiology* **80**: 67
590 (2023).
591 25 Hyatt D, Chen G L, LoCascio P F, Land M L, Larimer F W and Hauser L J, Prodigal:
592 prokaryotic gene recognition and translation initiation site identification. *BMC*
593 *Bioinf* **11**: 119 (2010).
594 26 Aziz R K, Bartels D, Best A A, DeJongh M, Disz T, Edwards R A, et al., The RAST
595 server: Rapid annotations using subsystems technology. *BMC Genomics* **9**: 75
596 (2008).
597 27 Bendtsen J D, Nielsen H, von Heijne G and Brunak S, Improved prediction of signal
598 peptides: SignalP 3.0. *J Mol Biol* **340**: 783-95 (2004).

28 Rawlings N D, Morton F R, Kok C Y, Kong J and Barrett A J, MEROPS: the peptidase database. *Nucleic Acids Res*. **38**: D320-D325 (2008).

29 Cheng J H, Wang Y, Zhang X Y, Sun M L, Zhang X, Song X Y et al., Characterization and diversity analysis of the extracellular proteases of thermophilic *Anoxybacillus caldiproteolyticus* 1A02591 from deep-sea hydrothermal vent sediment. *Front. Microbiol* **12**: 643508 (2021).

30 He H, Chen X, Li J, Zhang Y and Gao P, Taste improvement of refrigerated meat treated with cold-adapted protease. *Food Chemistry* **84**: 307-311 (2004).

31 Worthington Biochemical Co. Worthington Enzyme Manual. Collagenase. Worthington Biochemical Co., Freehold, NJ. (1993)

32 Chen X L, Zhang Y Z, Gao P J and Luan X W, Two different proteases produced by a deep - sea psychrotrophic bacterial strain, *Pseudoaltermonas* sp. SM9913. *Marine Biology* **143**: 989-993 (2003).

33 Daroit D J, Sant'Anna V and Brandelli A, Kinetic stability modelling of keratinolytic protease P45: influence of temperature and metal ions. *Appl Biochem Biotechnol* **165**: 1740-1753 (2011).

34 He H, Chen X, Sun C, Zhang Y and Gao P, Preparation and functional evaluation of oligopeptide-enriched hydrolysate from shrimp (*Acetes chinensis*) treated with crude protease from *Bacillus* sp. SM98011. *Bioresour Technol* **97**: 385-90 (2006).

35 Lin X, Lu Y, Zhang T, Liang M, Cen Y, Yuan E et al., Accuracy and precision comparison for molecular weight distribution assay of fish collagen peptides: a methodology study between two gel permeation chromatography columns. *Food*

Analytical Methods. **12**: 246-257 (2019).

36 Braca A, Sortino C, Politi M, Morelli I and Mendez J, Antioxidant activity of flavonoids from *Licania licaniaeflora*. *J Ethnopharmacol* **79**: 379-381 (2002).

37 Sui X, Guo H, Cai C, Li Q, Wen C, Zhang X, et al., Ionic conductive hydrogels with long-lasting antifreezing, water retention and self-regeneration abilities. *Chemical Engineering Journal* **419**: 129478 (2021).

38 Wang W, Cao Z, Hou F, Shi J, Jiao J, Chen L et al., Quality maintenance mechanism of oxalic acid treatment in fresh-cut apple fruit during storage based on nontarget metabolomics analysis. *Food Chem* **436**: 137685 (2024).

39 Laskar A, Rodger E J, Chatterjee A and Mandal C, Modeling and structural analysis of PA clan serine proteases. *BMC Res Notes* **5**: 256 (2012).

40 Zhao X, Cai M, Yang Z J, Luo T Q, Sarwar A, Megrous S et al., Purification and characterization of a novel milk-clotting enzyme produced by *Bacillus amyloliquefaciens* GSBa-1. *Eur Food Res Technol* **245**: 2447-2457 (2019).

41 Ran L Y, Su H N, Zhao G Y, Gao X, Zhou M, Wang P et al., Structural and mechanistic insights into collagen degradation by a bacterial collagenolytic serine protease in the subtilisin family. *Mol Microbiol* **90**: 997-1010 (2013).

42 Yomantas Y A, Abalakina E G, Golubeva L I, Gorbacheva L Y and Mashko S V, Overproduction of *Bacillus amyloliquefaciens* extracellular glutamyl-endopeptidase as a result of ectopic multi-copy insertion of an efficiently-expressed mpr gene into the *Bacillus subtilis* chromosome. *Microb Cell Fact* 10, 64 (2011).

643 43 Xie F, Feng F, Liu D, Quan S, Liu L, Zhang X et al., *Bacillus amyloliquefaciens* 35
644 M can exclusively produce and secrete proteases when cultured in soybean-meal-
645 based medium. *Colloids and Surfaces B: Biointerfaces* **209**: 112188 (2022).
646 44 Karched M, Bhardwaj R G, Tiss A and Asikainen S, Proteomic analysis and
647 virulence assessment of *Granulicatella adiacens* secretome. *Front Cell Infect*
648 *Microbiol* **9**: 104 (2019).
649 45 Chalamaiah M, Dinesh Kumar B, Hemalatha R and Jyothirmayi T, Fish protein
650 hydrolysates: proximate composition, amino acid composition, antioxidant
651 activities and applications: a review. *Food Chem.* **135**: 3020-38 (2012).
652 46 Adeva-Andany M, Souto-Adeva G, Ameneiros-Rodríguez E, Fernández-Fernández
653 C, Donapetry-García C and Domínguez-Montero A, Insulin resistance and glycine
654 metabolism in humans. *Amino Acids* **50**: 11-27 (2018).
655 47 Wang L, An X, Yang F, Xin Z, Zhao L and Hu Q, Isolation and characterisation of
656 collagens from the skin, scale and bone of deep-sea redfish (Sebastes mentella).
657 *Food Chem*. **108**: 616-23 (2008).
658 48 Chen X L, Peng M, Li J, Tang B L, Shao X, Zhao F, et al., Preparation and functional
659 evaluation of collagen oligopeptide-rich hydrolysate from fish skin with the serine
660 collagenolytic protease from *Pseudoalteromonas* sp. SM9913. *Sci Rep* **7**: 15716
661 (2017).
662 49 Li H and Aluko R E, Identification and inhibitory properties of multifunctional
663 peptides from pea protein hydrolysate. *J Agric Food Chem* **58**: 11471-11476
664 (2010).

50 Van Lancker F, Adams A and De Kimpe N, Formation of pyrazines in Maillard model systems of lysine-containing dipeptides. *J Agric Food Chem* **58**: 2470-2478 (2010).

51 Chen X, Wu J, Li L, and Wang S, Cryoprotective activity and action mechanism of antifreeze peptides obtained from tilapia scales on *Streptococcus thermophilus* during cold stress. *J Agric Food Chem* **67**: 1918-1926 (2019).

52 Zeng Y, Li W, Liu Y and Jiang W, Antifreeze peptides preparation from tilapia skin and evaluation of its cryoprotective effect on *Lacticaseibacillus rhamnosus*. *Foods* **11**: 857 (2022).

53 Zhang Y, Wang W, Liu Y, Liu X, Wang H and Zhang H, Cryoprotective effect of wheat gluten enzymatic hydrolysate on fermentation properties of frozen dough. *Journal of Cereal Science* **104**: 103423 (2022).

54 Jha P K, Xanthakis E, Chevallier S, Jury V and Le-Bail A, Assessment of freeze damage in fruits and vegetables. *Food Res Int* **121**: 479-496 (2019).

Figure Legends:

Figure 1. Proteases production capacity of the strain E1-8. (A) Hydrolytic halos of strain E1-8 on protein substrate plates; (B) Putative number of the proteases of strain E1-8 in each Merops family based on genome sequencing and gene annotation. The putative number of extracellular proteases is displayed in orange and the number of intracellular proteases in blue.

Figure 2. Optimization of the fermentation conditions of *B. inaquosorum* E1-8 to produce protease. (A) Impact of carbon source type on protease production; (B) Impact of nitrogen source type on protease production; (C) Impact of concentrations of sweet potato powder and soybean meal on protease production; (D) Impact of pH and fermentation temperature on protease production; (E) The protease production of strain E1-8 cultured under the fermentation conditions designed by orthogonal test; (F) The protease production of strain E1-8 cultured in the optimized fermentation medium over time; (insert) The abundance of strain E1-8 extracellular protease predicted by secretomic sequencing and analysis. The graphs show data from treatments conducted in triplicate (mean ± S.D.). Statistical analysis of enzyme activity was conducted by t-test. The lowercase letter b and c represented significant differences between each group at $p < 0.05$ and $p < 0.01$, respectively.

Figure 3. Optimization of the hydrolysis parameters of E1-8 extracellular protease towards soy protein, pea protein, fish protein, and porcine protein. (A) Effects of hydrolysis temperature; (B) Effects of pH value; (C) Effects of E/S ratio; (D) Effects of reaction time. The graphs show data from triplicate experiments (mean ± SD). (E) Flow sheet for the preparation of protein hydrolysates with E1-8 extracellular proteases; (F-I) Lyophilized powder and solution (20 mg/ml) of the prepared protein hydrolysates.

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