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Article

Potential of Marine Bacterial Metalloprotease A69 in the Preparation of Antarctic Krill Peptides with Multi-Bioactivities

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Abstract: Antarctic krill (*Euphausia superba*) is a nutrient-rich marine resource. Although several terrestrial proteases have been used to prepare Antarctic krill peptides (AKPs), there has been no report on the preparation of AKPs using a marine protease. Here, marine bacterial protease A69 was used to prepare AKPs with multi-bioactivities. Through optimizing hydrolysis parameters, we established a process for AKPs preparation by hydrolyzing Antarctic krill powder with A69. In the prepared AKPs, peptides less than 3000 Da and 1000 Da accounted for 99.23% and 88.37%, respectively. The scavenging ratios of the AKPs to $ABTS^+$, DPPH· and ·OH reached 93.23 ± 0.09%, 99.90 ± 0.15%, and 93.90 ± 0.47%, respectively. It also had high angiotensin-converting enzyme (ACE)-inhibitory activity, with an IC_{50} of 0.22 ± 0.04 mg/mL. At 40 mg/mL, the AKPs inhibited α-glucosidase and dipeptidyl peptidase IV (DPP-IV) activities by 7.18% and 13.62%, respectively, and displayed antibacterial activity to *Escherichia coli*. Moreover, 14 antioxidant peptides, 24 ACE-inhibitory peptides, 2 α-glucosidase-inhibitory peptides, and 10 DPP-IV-inhibitory peptides were identified from the AKPs. These results demonstrate that the prepared AKPs contains diverse bioactive peptides and has multi-bioactivities. This study indicates that marine bacterial protease A69 has promising application potential in preparing AKPs with multi-bioactivities.

Keywords: marine bacterial protease A69; Antarctic krill peptides; antioxidant activity; angiotensin-converting enzyme (ACE)-inhibitory activity; antibacterial activity; α-glucosidase-inhibitory activity; dipeptidyl peptidase IV (DPP-IV)-inhibitory activity

1. Introduction

Active peptides, consisting of 2 to 50 amino acid residues, have a variety of biological activities, and have been widely used as bioactive ingredients in the fields of food [1,2], medicine [3], healthcare products [1,4] and others. Active peptides have been shown to have a variety of biological functions, such as antioxidant [5–7], anti-inflammatory [8,9], antibacterial [10–12], immune regulation [13–15], lowering blood pressure [16,17] and lowering uric acid [18], etc. Active peptides are now commonly prepared by enzymatic hydrolysis technology. Many proteases from terrestrial animals, plants and microorganisms have been used in the preparation of active peptides, including pepsin, trypsin,

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papain, alkaline protease, neutral protease and others [19,20]. In contrast, marine-derived proteases used in the preparation of active peptides are still limited.

Protease A69 is a MEROPS M4 family metalloprotease derived from the marine bacterium *Anoxybacillus caldiproteolyticus* 1A02591 [21]. In previous studies, protease A69 was heterologously expressed in *Escherichia coli* and *Bacillus subtilis*, and recombinant protease A69 has been used to prepare bovine collagen peptides [21], soy protein peptides [22], and peanut peptides [23]. The prepared bovine collagen peptides have moisturizing ability and antioxidant activity, the prepared soybean peptides have angiotensin-converting enzyme (ACE)-inhibitory activity, and the prepared peanut peptides have antioxidant activity and ACE-inhibitory activity. These studies showed that the marine-derived protease A69 has good potential in preparing bioactive peptides.

Antarctic krill (*Euphausia superba*) is a small crustacean widely distributed in Antarctic waters. As a key species in the Antarctic marine ecosystem, Antarctic krill are not only an important part of the Antarctic food chain, but also provide a major food source for a wide variety of marine life [24]. In addition, Antarctic krill has attracted more and more attention in the food [25–28], healthcare [29,30] and pharmaceutical fields [30–32] due to its rich nutritional composition and bioactive characteristics. Antarctic krill has been used as raw materials for the production of astaxanthin [33,34], chitin [35,36], krill oil [29,37,38], krill peptides [28,29], and feed [37,39,40], etc. Although some terrestrial proteases, including neutral protease, alkaline protease, Corolase PP, trypsin, pepsin, papain, flavor protease, thermoase PC10F, protamex and animal proteolytic enzyme, have been used in the preparation of Antarctic krill peptides (AKPs) which have antioxidant, anti-hypertensive, anti-diabetic and/or antibacterial activities [41–49], there has been no report on the preparation of Antarctic krill peptides using marine-derived proteases.

The aim of this study was to evaluate the potential of marine bacterial protease A69 in the preparation of AKPs with biological activities. With Antarctic krill powder, a by-product from krill oil production, as the material, on the basis of optimizing hydrolysis parameters, a process of preparing AKPs by hydrolyzing Antarctic krill powder with protease A69 was established. The characteristics and biological activities of the prepared AKPs were further analyzed. The AKPs has a high proportion of small molecular peptides, contains 17 amino acids, and has antioxidant, ACE-inhibitory, α-glucosidase-inhibitory, dipeptidyl peptidase IV (DPP-IV)-inhibitory and antibacterial activity. The results show that the marine bacterial protease A69 has a great application potential in the preparation of AKPs with good nutritional function, and antioxidant, anti-hypertensive, anti-diabetic and antibacterial activities.

2. Results and Discussion

2.1. Optimization of the Hydrolysis Parameters of Protease A69 on Antarctic Krill Powder

Protease A69 was previously shown to achieve the highest activity at 60°C and pH 7.0 [22]. To determine the optimal conditions for A69 to hydrolyze Antarctic krill powder for AKPs production, two additional enzymatic hydrolytic parameters, enzyme/substrate (E/S) ratio and hydrolysis time, were optimized at 60°C and pH 7.0 by single factor experiments. As shown in Figure 1A, when Antarctic krill powder was hydrolyzed with A69 at the E/S ratio range from 500 U/g to 6000 U/g, the hydrolysate yield increased rapidly with the E/S ratio from 500 U/g to 5000 U/g, but only showed a slight increase from 5000 U/g to 6000 U/g. In addition, the content of oligopeptides with a molecular weight <500 Da in the hydrolysates from 5000 U/g and 6000 U/g hydrolysis was almost the same, reaching the highest (Table 1). Based on these results, the optimal E/S ratio for A69 to hydrolyze Antarctic krill powder for AKPs production was determined to be 5000 U/g. As shown in Figure 1B, the hydrolysate yield increased with the hydrolysis time and reached the peak at 5 h. The content of oligopeptides with a molecular weight <1000 Da in the hydrolysate from 6 h hydrolysis was the highest, and the contents of those <500 Da were similar in the hydrolysates from 5 h and 6 h hydrolysis (Table 2). Based on these results, the optimal hydrolysis time for A69 to hydrolyze Antarctic krill powder for AKPs production was determined to be 6 h.

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Protease A69 was previously used to prepare active peptides from bovine bone collagen, soy protein, and peanut protein. The optimal E/S ratio and hydrolysis time for recombinant A69 from *E. coli* to prepare collagen peptides were determined to be 25 U (collagenolytic activity)/g, and 2 h, respectively [21], those for recombinant A69 from *B. subtilis* to prepare soy protein peptides were 4000 U (caseinolytic activity)/g, and 3 h [22], and those for recombinant A69 from *B. subtilis* to prepare peanut protein peptides were 3000 U (caseinolytic activity) /g, and 4 h [23]. In this study, the optimal E/S ratio and hydrolysis time for recombinant A69 from *B. subtilis* to prepare AKPs from Antarctic krill powder were determined to be 5000 U (caseinolytic activity)/g, and 6 h. These differences may be attributed to the discrepancy in the sequences and structures of these protein sources.

Table 1. Molecular weight distribution of the AKPs prepared from Antarctic krill powder hydrolysis by A69 under different E/S ratios.

MW range (Da)	Content (%)						
	500 U/g	1000 U/g	2000 U/g	3000 U/g	4000 U/g	5000 U/g	6000 U/g
＞10000	0.17	0.12	0.09	0.01	0.01	0	0
5000-10000	1.78	1.25	0.89	0.74	0.62	0.5	0.49
3000-5000	3.92	3.16	2.57	2.36	2.17	1.92	1.92
1000-3000	24.91	22.97	20.15	18.95	18.43	18.81	17.07
500-1000	28.87	28.95	29.86	29.53	29.42	27.5	29.14
＜1000	69.22	72.5	76.31	77.94	78.77	78.77	80.52
＜500	40.35	43.56	46.44	48.41	49.35	51.27	51.38

Table 2. Molecular weight distribution of the AKPs prepared from Antarctic krill powder hydrolysis by A69 for different hydrolysis time durations.

MW range (Da)	Content (%)					
	1 h	2 h	3 h	4 h	5 h	6 h
＞10000	0.02	0.01	0.01	0.01	0	0.01
5000-10000	0.63	0.49	0.5	0.37	0.11	0.27
3000-5000	2.11	1.8	1.77	1.46	1.1	1.13
1000-3000	21.42	19.42	19.1	13.86	16.95	11.79
500-1000	26.56	31.7	31.28	35.08	29.08	35.14
＜1000	75.82	78.28	78.62	84.3	81.84	86.8
＜500	49.26	46.58	47.34	49.22	52.76	51.66

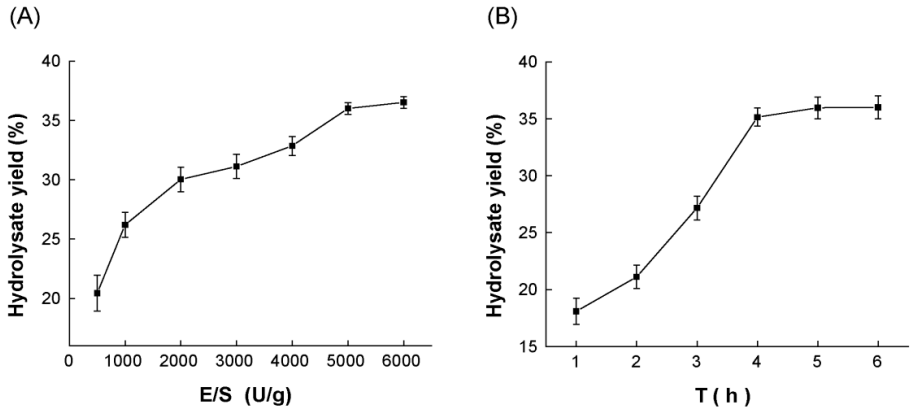

Figure 1. Optimization of the parameters for protease A69 to hydrolyze Antarctic krill powder. (A) The effect of the E/S ratio on the hydrolysate yield. Antarctic krill powder was hydrolyzed at 60 °C and pH 7.0 for 6 h by A69 under different E/S ratios. (B) The effect of hydrolysis time on the hydrolysate yield. Antarctic krill powder was

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hydrolyzed at the E/S ratio of 5000 U/g, 60 °C, and pH 7.0 for different time durations. The graphs show data from triplicate experiments (mean ± SD).

2.2. Preparation and Characterization of AKPs

Based on the optimal hydrolysis parameters determined above, we set up a process to prepare AKPs using protease A69, and a flow chart of this process was shown in Figure 2. The AKPs prepared with this process were milky white powder (Figure 3), and had good water solubility when the concentration reached 30% (w/v) (Figure 4). The enzymatic hydrolysis efficiency of the Antarctic krill powder was 35.99 ± 0.03% based on its weight before and after enzymatic hydrolysis, and 60.83 ± 0.02% based on its protein content before and after enzymatic hydrolysis. The molecular weight distribution of peptides in the AKPs prepared with the established process were further analyzed. The result showed that peptides with a molecular weight < 500 Da in the AKPs accounted for 52.37% and the content of those < 1000 Da was 88.37% (Figure 5 and Table 4), indicating that the prepared AKPs were rich in peptides composed of less than 10 amino acid residues. It has been reported that bioactive peptides usually contain 2-20 amino acid residues [1]. Thus, the prepared AKPs may contain a variety of bioactive peptides with various biological activities.

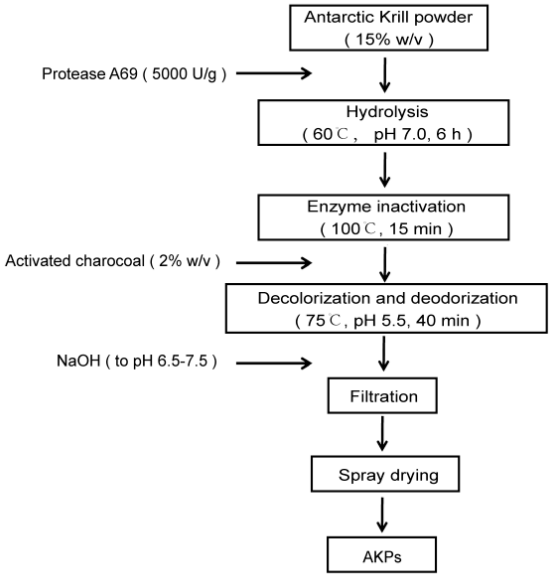

Figure 2. A flow chart of AKPs preparation with protease A69.

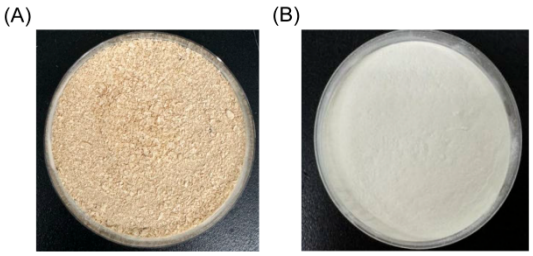

Figure 3. Antarctic krill powder (A) and the AKPs powder (B). The AKPs powder was prepared using the process shown in Figure 2.

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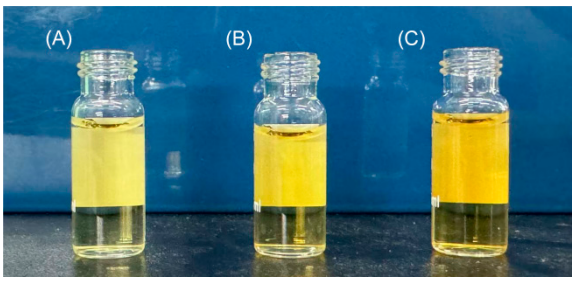

Figure 4. Solubility of the prepared AKPs powder. (A) The AKPs solution of 10% (w/v) concentration. (B) The AKPs solution of 20% (w/v) concentration. (C) The AKPs solution of 30% (w/v) concentration.

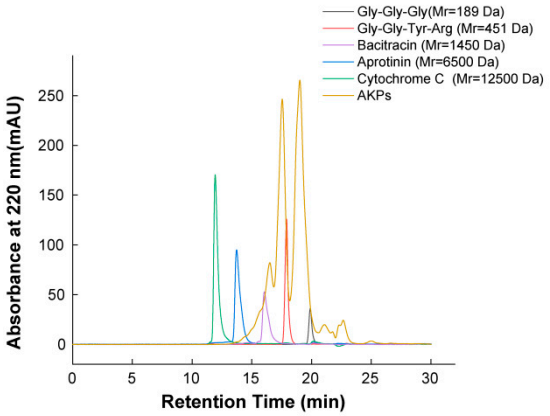

Figure 5. Molecular weight distribution of the prepared AKPs analyzed by HPLC.

Table 3. Proportions of peptides with different molecular weights in the prepared AKPs based on HPLC analysis.

MW range (Da)	Content (%)
＞10000	0
5000-10000	0.07
3000-5000	0.7
1000-3000	10.86
500-1000	36
＜1000	88.37
＜500	52.37

2.3. Amino Acid Composition of the Prepared AKPs

To evaluate the nutritional value of the prepared AKPs, the composition of free and total amino acids of the AKPs was detected. The content of free amino acids in the prepared AKPs was less than 5%, and that of total amino acids was more than 63% (Table 4). Among the total amino acids in the AKPs, the most abundant was Glu, which accounted for 9.80 ± 0.72% of the total. In addition, the contents of Pro, Asp and Lys were also high, reaching 8.11 ± 0.50%, 6.58 ± 0.54%, and 5.31 ± 0.49%, respectively. Moreover, there were seven human essential amino acids detected in the AKPs, accounting for 21.21 ± 0.27% in total. These results show that the prepared AKPs have high nutritional value.

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Table 4. The amino acid composition and content of the AKPs prepared using protease A69[a].

Amino Acids	Free Amino Acids (%)	Total Amino Acids (%)
Asp	0.072 ± 0.001	6.582 ± 0.542
Thr	**0.027 ± 0.001**	**2.507 ± 0.205**
Ser	0.034 ± 0.001	2.271 ± 0.123
Glu	0.205 ± 0.001	9.802 ± 0.718
Gly	0.196 ± 0.001	2.731 ± 0.225
Ala	0.142 ± 0.001	3.625 ± 0.296
Cys	0.103 ± 0.001	1.723 ± 0.097
Val	**0.180 ± 0.001**	**3.157 ± 0.227**
Met	**0.115 ± 0.001**	**0.951 ± 0.117**
Ile	**0.180 ± 0.002**	**2.312 ± 0.223**
Leu	**0.525 ± 0.001**	**4.029 ± 0.404**
Tyr	1.551 ± 0.003	1.825 ± 0.149
Phe	**0.659 ± 0.002**	**2.940 ± 0.224**
Lys	**0.262 ± 0.001**	**5.308 ± 0.490**
His	-	1.532 ± 0.300
Arg	0.545 ± 0.001	3.873 ± 0.310
Pro	0.150 ± 0.001	8.111 ± 0.496
Trp[b]	-	-
Total	4.948 ± 0.001	63.281 ± 0.303

[a] Human essential amino acids are shown in bold. The data shown in the table are from triplicate experiments (mean ± SD); [b] Trp was not detectable because it was destroyed in the process of acid hydrolysis.

2.4. The Antioxidant Activity of the Prepared AKPs

The *in vitro* antioxidant activity of the prepared AKPs was evaluated by determining its scavenging ratios to free radicals $ABTS^+$, DPPH·, ·OH and O_2-·. Ascorbic acid (Vc), hyaluronic acid (HA), and L-reducing glutathione were used as three positive controls in determining the $ABTS^+$ scavenging ratio, while Vc and HA as two positive controls in determining the scavenging ratios of DPPH·, ·OH and O_2-·, As shown in Figure 6, within the investigated range of the AKPs concentration, the scavenging ratios of all free radicals rose with the increase of the AKPs concentration, demonstrating a dose-effect scavenging ability of the AKPs to the radicals. The scavenging ratio to $ABTS^+$ of the AKPs at 10 mg/ml reached 93.23 ± 0.09%, approximately the same as those of Vc and L-reducing glutathione, and much higher than that of HA (Figure 6A). The scavenging ratios of the AKPs to DPPH· and ·OH were also high, reaching 99.90 ± 0.15% (at 30 mg/ml), and 93.90 ± 0.47% (at 40 mg/ml), respectively, basically equivalent to those of Vc, and much higher than those of HA (Figure 6B, C). By contrast, the scavenging ratio of the AKPs to O_2-·was much lower, reaching 47.70 ± 1.38% at 30.00 mg/mL (Figure 6D). The EC_{50} values of the AKPs were 0.93 ± 0.02 mg/mL for $ABTS^+$, 9.04 ± 0.63 mg/mL for DPPH· and 5.10 ± 0.18 mg/mL for ·OH. Altogether, these results indicated that the AKPs possesses strong scavenging ability to a variety of free radicals, showing its good antioxidant activity.

Several studies have shown that Antarctic krill hydrolysates (AKH) prepared using terrestrial enzymes have antioxidant activity. Zhang et al. reported that the ·OH and DPPH· scavenging ratios of the AKH prepared using alcalase were 65.99 ± 1.22% and 55.32 ± 1.08% at 5 mg/mL, respectively, significantly higher than those prepared using trypsin, Neutrase, pepsin or papain [48]. Lan et al. reported that the high fischer ratio oligopeptides prepared from the sequential hydrolysis of Antarctic krill powder with alcalase and flavorzyme showed scavenging activity against four free radicals, with EC_{50} values of 0.91 mg/mL for $ABTS^+$, 0.83 mg/mL for O2-·, 4.90 mg/mL for DPPH·, and 4.62 mg/mL for ·OH [45]. Our results in this study showed that the AKPs prepared using marine bacterial protease A69 had a comparable EC_{50} value for $ABTS^+$ (0.93 ± 0.02 mg/mL), and higher EC_{50} values for DPPH· (9.04 ± 0.63 mg/mL) and ·OH (5.10 ± 0.18 mg/mL).

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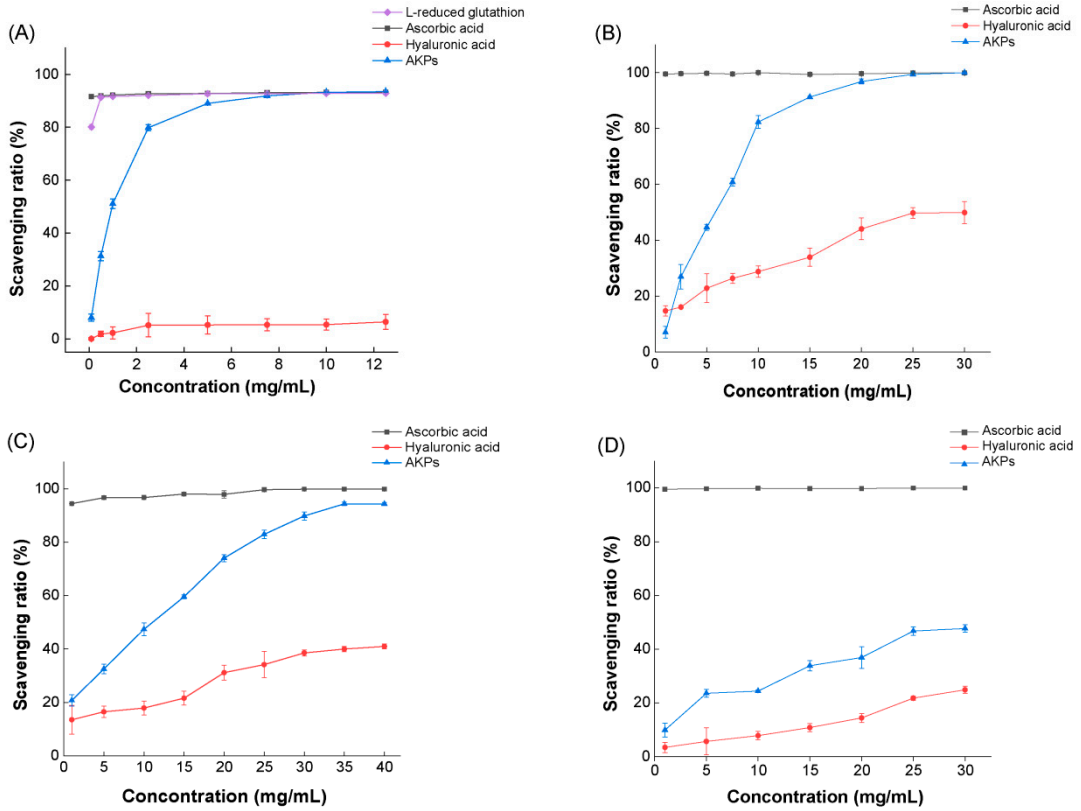

Figure 6. Antioxidant activity of the prepared AKPs. (A) $ABTS^{+}$ scavenging capacity of the AKPs. (B) •OH scavenging capacity of the AKPs. (C) DPPH• scavenging capacity of the AKPs. (D) O_2-• scavenging capacity of the AKPs. The graphs show data from triplicate experiments (mean ± SD).

2.5. The ACE-Inhibitory Activity of the Prepared AKPs

ACE inhibitors have been used to treat hypertension by blocking the activity of ACE in the renin-angiotensin-aldosterone system of human [50]. Many peptides with ACE-inhibitory activity have been reported, such as tuna muscle peptide [51], broccoli peptide [52], walnut peptide [53], etc. In order to ascertain the ACE-inhibitory activity of the AKPs prepared with protease A69, the ACE-inhibitory rates of the AKPs of different concentrations were measured. As illustrated in Figure 7, the ACE-inhibitory rate increased promptly with the AKPs concentration, and reached 94.12 ± 0.45% at 2.5 mg/mL. The IC_{50} value of the AKPs for ACE was determined to be 0.22 ± 0.04 mg/mL. Thus, the AKPs demonstrated remarkable ACE-inhibitory activity and likely contained ACE-inhibitory peptides.

It has been reported that AKHs prepared with commercial enzymes have ACE-inhibitory activity. The ACE-inhibitory rate of the AKH prepared with trypsin was 38.82 ± 0.71% when the concentration was 1 mg/mL [46]. The IC_{50} of the AKH prepared by hydrolysis of peeled Antarctic krill tail meat using Thermoase PC10F was 1.944 mg/mL [41]. Ji et al. reported that the ACE-inhibitory rate of the AKH prepared with corolase PP was approximately 48% at 10 mg/mL, which was higher than those prepared with alcalase, flavourzyme, and papain [43]. The IC_{50} value (0.22 ± 0.04 mg/mL) of the AKPs prepared with marine bacterial protease A69 in this study is much lower than those of the reported AKHs prepared with terrestrial commercial enzymes, suggesting that the AKPs has higher ACE-inhibitory activity.

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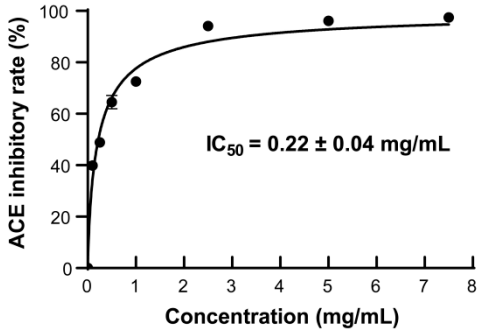

Figure 7. The ACE-inhibitory activity of the AKPs. The graph shows data from triplicate experiments (mean ± SD).

2.6. The Antibacterial Activity of the Prepared AKPs

To detect the antibacterial activity of the AKPs, the antibacterial effects of the AKPs on *E. coli* and *S. aureus* were studied by observing the inhibition zone formation on solid agar plates. As shown in Figure 8, the AKPs at 40 mg/mL formed a clear inhibition zone on the plate containing *E. coli* cells after 12 h incubation, and the diameter of the zone increased with incubation time, reaching 2.99 ± 0.08 mm at 48 h, in contrast to that of the positive control kanamycin (1 mg/mL) that retained approximately the same size (2.38 ± 0.20 mm) from 12 to 48 h (Figure 8, Table 5). On the plate containing *S. aureus* cells, the positive control kanamycin (1 mg/mL) formed a clear inhibition zone, but the AKPs not (Figure 8). This result showed that the AKPs had antibacterial activity against the Gram-negative bacterium *E. coli,* but no against the Gram-positive bacterium *S. aureus.*

Zhao et al. reported that the AKH prepared with protamex showed antibacterial activity against *S. aureus* [42]. In contrast, our study showed that the AKPs prepared with protease A69 exhibited antibacterial activity against *E. coli,* but no antibacterial activity against *S. aureus*. This difference suggests that proteases protamex and A69 likely have distinct hydrolytic sites on Antarctic krill proteins and generate bioactive peptides with differences in amino acid composition and sequence, structural characteristics and antimicrobial activity.

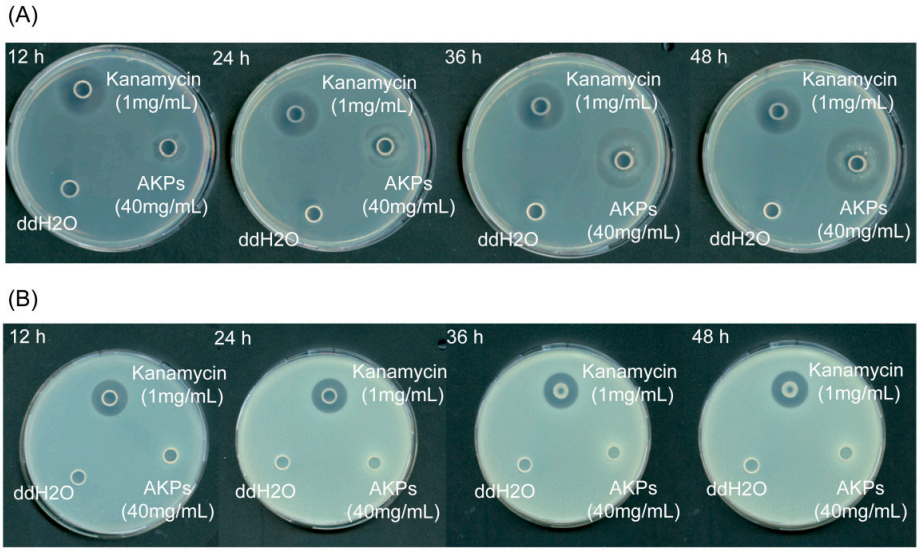

Figure 8. Antibacterial effects of the AKPs on *E. coli* (A) and *S. aureus* (B). Plates in the figure are representatives from triplicate experiments.

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Table 5. Inhibition zone diameters of the AKPs and kanamycin against *E. coli* and *S. aureus*[a].

strain	sample	Inhibition zone diameter （mm）			
		12 h	24 h	36 h	48 h
E. coli	kanamycin (1 mg/mL)	23.8 ± 2.0	23.8 ± 2.0	23.8 ± 2.0	23.8 ± 2.0
	H_2O	-	-	-	-
	AKPs（40 mg/mL)	14.8 ± 1.0	19.5 ± 0.4	27.6 ± 0.6	29.9 ± 0.8
S. aureus	kanamycin (1 mg/mL)	18.2 ± 3.0	18.2 ± 3.0	18.2 ± 3.0	18.2 ± 3.0
	H_2O	-	-	-	-
	AKPs（40 mg/mL)	-	-	-	-

[a] The data shown in the table are from triplicate experiments (mean ± SD).

2.7. The Inhibitory Activities of the AKPs to α-Amylase, α-Glucosidase and DPP-IV

To evaluate the hypoglycemic potential of the AKPs, its inhibitory effects on α-amylase, α-glucosidase and DPP-IV activities were measured. When the concentration of AKPs was 40 mg/mL, its inhibitory rates on α-glucosidase and DPP-IV activities were 7.18% and 13.62%, respectively, and it had no inhibitory effect on α-amylase activity.

There has been no report on the α-amylase-inhibitory activity of AKH, and only one report on the α-glucosidase-inhibitory activity of AKH. Zheng et al. reported that the α-glucosidase-inhibitory ratio of the AKH prepared with neutral protease was 43.82%, which was higher than that of AKH prepared with trypsin, alcalase, protamex, papain or flavoenzyme [49]. In contrast, there are more reports on the DPP-IV-inhibitory activity of AKHs prepared with different enzymes. Ji et al. reported that the AKH prepared with an animal proteolytic enzyme showed DPP-IV-inhibitory activity with an IC_{50} value of 1.6272 mg/mL [44]. They also prepared AKHs using corolase PP, alcalase, flavourzyme, and papain, and found that the AKH prepared with corolase PP had the highest inhibition rate against DPP-IV, approximately 40% at a concentration of 10 mg/mL [43]. Lang et al. reported that the AKHs prepared with compound protease, neutral protease, alkaline protease, flavor protease, and animal hydrolase all had inhibitory activity against DPP-IV at a concentration of 100 mg/mL, and that prepared with compound protease displayed the highest (66.81 ± 2.50%) [47]. Similar to these reports, our results in this study showed that the AKPs prepared with marine bacterial protease A69 had inhibition effects on α-glucosidase and DPP-IV activities, but no on α-amylase activity.

2.8. Identification of Bioactive Peptides from the Prepared AKPs

The peptide sequences in the prepared AKPs were determined by LC-MS/MS, and a total of 5657 peptide sequences from the AKPs were detected. By searching the AODB database, 14 antioxidant peptides were identified from the detected peptide sequences in the prepared AKPs, including 3 dipeptides, 5 tripeptides, 2 tetrapeptides, 1 pentapeptide, 1 hexapeptide, and 2 heptapeptides (Table 6). Similarly, 24 ACE-inhibitory peptides were identified by searching the AHTPDB database, including 8 dipeptides, 8 tripeptides, 6 tetrapeptides, and 2 pentapeptides (Table 7). By searching the BIOPEP-UWM database, 2 α-glucosidase-inhibitory peptides (1 pentapeptide and 1 heptapeptide) (Table 8) and 10 DPP-IV-inhibitory peptides (7 dipeptides and 3 tetrapeptides) (Table 9), but no α-amylase-inhibitory peptide, were identified. In addition, no antimicrobial peptide was identified by searching the APD database. These data demonstrate that the AKPs prepared with protease A69 contains diverse bioactive peptides, consistent with its multiple bioactivities determined above.

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Table 6. The antioxidant peptides identified from the prepared AKPs.

Sequence	Molecular Weight (Da)	Source	Antioxidant Activity	References
FL	278.35	Milk Protein	-	[54]
HL	268.31	Designed Peptide	-	[55]
LY	294.34	Soybean Protein	-	[56]
FSL	365.42	Egg White Protein	Oxygen radical absorbance capacity-fluorescein value, <0.022 μmol of Trolox equivalent per μmol of peptide.	[57]
TVM	349.44	Skipjack Tuna	TVM presented scavenging activity on DPPH radical (EC_{50} values of 0.537 ± 0.026), hydroxyl radical (EC_{50} values of 0.942 ± 0.0.067), and superoxide anion free radical (EC_{50} values of 1.069 ± 0.063).	[58]
WAF	422.48	Palm Kernel Cake Hydrolysates	DPPH radical dot radical scavenging activity: 71 ± 2.22%, IC_{50} (μM) = 1.360; Metal chelating activity: 41 ± 1.08%, IC_{50} (μM) = 0.002.	[59]
YMY	475.56	Synthesis Peptide	-	[60]
YYG	401.41	Synthesis Peptide	-	[61]
LVPK	455.59	Black Soybean	-	[61]
SGGY	382.37	Walnut	-	[62]
LKYPI	632.79	Casein-Derived Bioactive Peptides	-	[63]
WDDMEK	822.88	Marine Bivalve	The peptide showed scavenging activity on hydroxyl radical with IC_{50} of 182.4 μM.	[64]
IIAPPER	794.93	*Gryllodes sigillatus*	$ABTS^+$ scavenging (EC_{50} mg mL^{-1}): 15.62 ± 0.1; DPPH• scavenging (EC_{50} mg mL^{-1}): 1.01 ± 0.02; Fe^{2+} chelating activity (EC_{50} mg mL^{-1}): 0.142 ± 0.05; Reducing power (Abs700): 0.148 ± 0.01ab LOX inhibitory activity (IC_{50} mg mL^{-1}): 8.21 ± 0.04; COX inhibitory activity (IC_{50} mg mL^{-1}):8.16 ± 2.22.	[65]
NWDDMEK	936.98	*Scomberomorus niphonius*	-	[66]

Table 7. The ACE-inhibitory peptides identified from the prepared AKPs.

Sequence	Molecular Weight (Da)	Source	IC_{50} (μmol L-1)	References
PT	216.222	Milk hydrolysate	-	[67]
PT	216.24	Chicken (*Gallus gallus*)	-	-
PT	216.24	Bovine (*Bos taurus*) β-caseins	-	-
PT	216.24	Pork sarcoplasmic proteins	-	[68]
PT	216.24	Cereals storage protein	-	[69]
PT	216.24	Bovine lactoferrin (*Bos taurus*)	-	[70]

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SL	218.25	-	-	[71]
LL	244.33	-	-	[71]
LL	244.33	-	-	[72]
HL	268.302	-	3200	-
HL	268.32	Pork sarcoplasmic proteins	-	[68]
HL	268.32	Cereals storage protein	-	[69]
HL	268.32	Bovine (*Bos taurus*) β-caseins	-	[70]
HL	268.32	-	-	[73]
HL	268.32	-	-	[71]
HL	268.32	-	-	[74]
IF	278.337	-	930	[75]
FL	278.35	Rapeseed (Canola meal defatted)	1.33	[76]
FL	278.35	Rapeseed (Canola meal defatted)	1.33	[77]
FL	278.35	-	-	[71]
FL	278.35	-	<20000	[72]
IF	278.35	Chicken (*Gallus gallus*)	-	-
IF	278.35	Bovine β-caseins	-	-
IF	278.35	Pork sarcoplasmic proteins	-	[68]
IF	278.35	Cereals storage protein	-	[69]
IF	278.35	Soybean Sauce	65.8	[78]
IF	278.35	Cereals (Wheat (Gamma-gliadin from wheat))	-	[70]
IF	278.35	-	-	[73]
IF	278.35	Soybean (Salt-free soy sauce)	65.8	[79]
IF	278.35	Royal jelly	1.67-930	[79]
IF	278.35	-	-	[71]
IF	278.35	-	-	[80]
IF	278.35	-	<20000	[72]
IF	278.35	-	-	[74]
LY	294	Fish (Sardine (*Sardina pilchardus* muscle))	38.5	[81]
LY	294	Amaranth (*Amaranthus hypochondriacus*)	-	[82]
LY	294	Cereals (Maize (*Zea mays*))	-	[83]
LY	294.33	Fish (Sardine muscle)	18	[81]
LY	294.35	Bovine (*Bos taurus*) β-caseins	-	-
LY	294.35	Pork sarcoplasmic proteins	-	[68]
LY	294.35	Cereals storage protein	-	[69]
LY	294.35	Fish (Sardine)	38.5	[84]
LY	294.35	Rapeseed proteins	110	[85]
LY	294.35	Bovine lactoferrin (*Bos taurus*)	-	[70]
LY	294.35	Bovine β-caseins	-	[70]
LY	294.35	Egg proteins	6.8	[86]
LY	294.35	Milk	-	[87]

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LY	294.35	-	38.5	[88]
LY	294.35	-	-	[71]
LY	294.35	-	<20000	[72]
LY	294.35	-	-	[74]
FF	312.37	-	-	[71]
LLP	341	Cereals (Maize (*Zea mays*))	57	[89]
LLP	341	Amaranth (*Amaranthus hypochondriacus*)	-	[82]
LLP	341	Cereals (Maize (*Zea mays*))	-	[83]
LLP	341.437	Alpha-zein	57	[83]
LLP	341.45	Cereals storage protein	-	[69]
LLP	341.45	Cereals (Rye)	57	[90]
LLP	341.45	Cereals (Wheat (α/β-Wheat gliadin))	-	[70]
LLP	341.45	-	57	[88]
KLP	356.47	-	-	[74]
FGF	369.42	-	-	[80]
FGF	369.42	-	-	[72]
FGF	369.42	-	-	[74]
LNF	392.46	Soybean proteins	-	[76]
MPF	393.5	Egg	6.59 - 27.38	[79]
MPF	393.5	Egg (cooked egg protein)	17.98	[80]
GVGY	394.43	Silkworm fibroin	35	[91]
GVGY	394.43	Silkworm fibroin	-	[80]
GVGY	394.43	-	-	[74]
YYG	401.42	-	-	[80]
YYG	401.42	-	-	[72]
YYG	401.42	-	-	[74]
VRF	420.51	Soybean proteins	1.3	[76]
LSLP	429	-	-	[83]
VFPS	448.507	Cereals (Finnish)	0.46	[92]
VFPS	448.52	Synthesized	0.46	[93]
FLPP	473	Cereals (Maize (*Zea mays*))	-	[83]
LIYP	504.607	Human (Human β-casein)	10	[94]
LIYP	504.63	Human (Synthetic peptide of Human β-casein)	10	[94]
LIYP	504.63	Milk	10	[95]
LIYP	504.63	-	10	[88]
LIYP	504.63	-	-	[74]
LIYP	505	Milk (human β-casein)	10	[94]
VLPIP	537.687	Human (Human β-casein)	31	[94]
VLPIP	537.7	Human (Synthetic peptide of Human β-casein)	31	[94]
VLPIP	537.7	-	31	[94]

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VLPIP	537.7	-	31	[88]
VLPIP	538	Milk (human β-casein)	31	[94]
VRYL	549.651	-	24.1	[70]
VRYL	549.67	Milk-Cheese (Sheep milk and cheeses proteins)	24.1	[96]
VRYL	549.67	Cheese (Manchego)	24.1	[77]
VRYL	549.67	Cheese (Manchego)	24.1	[97]
VRYL	550	Cheese (Manchego cheese)	-	[98]
IYEGY	643.69	Meat protein	<10	[76]

Table 8. The α-glucosidase-inhibitory identified from the prepared AKPs.

Sequence	Chemical Mass	IC_{50} (μmol L^{-1})
LDNFR	663.7207	9.21
IIAPPER	794.936	28.75

In the AODB database, no antioxidant peptides of Antarctic krill origin were deposited. The 14 antioxidant peptides we identified from the prepared AKPs show homolog sequences to those previously reported from other animals and plants and, marking their first identification from Antarctic krill. In the AHTPDB database, there are 4 ACE-inhibitory peptides from Antarctic krill currently documented, including VW[41,79], LKY [41,79], ITRY [79], and VFER [79]. In this study, we identified 24 ACE-inhibitory peptides from the prepared AKPs that differ from known Antarctic krill-derived sequences in the database, representing their first discovery in Antarctic krill. In the APD database, no antimicrobial peptides from Antarctic krill were recorded, and none was identified from the prepared AKPs through searching this database. However, the prepared AKPs exhibited *in vitro* antibacterial activity against *E. coli* (Figure 8), suggesting that there are antimicrobial peptides in the AKPs that could not be identified through database search, which thus need future identification. By searching the BIOPEP-UWM database, no α-amylase-inhibitory peptides were identified from the prepared AKPs, consistent with the lack of *in vitro* α-amylase-inhibitory activity of the AKPs. However, α-glucosidase-inhibitory peptides and DPP-IV-inhibitory peptides were identified from the AKPs, consistent with the inhibitory effects of the AKPs on α-glucosidase DPP-IV activities detected above. Notably, the inhibitory effect of the AKPs on α-glucosidase activity was quite low, probably due to the small number and/or low abundance of α-glucosidase-inhibitory peptides in the AKPs.

Table 9. The DPP-IV-inhibitory peptides identified from the prepared AKPs.

Sequence	Chemical Mass	IC_{50} (μmol L^{-1})
PT	216.2337	-
SL	218.2495	2517.08
HA	226.2319	-
LL	244.3296	-
PM	246.3269	-
HL	268.3114	143.19
FL	278.3459	399.58
IIAP	412.5222	-
VPIP	424.5329	54.69
FDPF	524.5643	-

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3. Materials and Methods

3.1. Experimental Materials

Antarctic krill powder was kindly provided by Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences. The protease A69 was produced using recombinant *B. subtilis*, as previously reported [22]. Aprotinin, cytochrome C, salicylic acid, pyrogallol, 2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), potassium persulfate, L-reduced glutathione, ACE, hippuric acid, Hip-His-Leu (HHL), Starch, DPP-IV and Gly-Pro-para-nitroaniline hydrochloride (Gly-Pro-PNA) were purchased from Sigma (St Louis, MO, USA). α-amylase, α-glucosidase and 4-nitrophenyl-α-D-glucopyranoside (PNPG) were purchased from Shanghai yuanye Bio-Technology Co., Ltd (Shanghai, China). Bacitracin and H_2O_2 were purchased from Aladdin (Shanghai, China). Tetrapeptide GGYR and tripeptide GGG were synthesized by Qiangyao Co., Ltd. (Shanghai, China). Vc was purchased from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). HA was purchased from Shandong Freda Bioeng Co., Ltd. (Jinan, China). DPPH• was purchased from Tokyo Chemical Industry (Tokyo, Japan). Other chemicals were of analytical grade and commercially available.

3.2. Enzyme Assay

The activity of protease A69 was determined by the Folin-Ciocalteu method as previously reported [21]. One unit (1 U) was defined as the amount of enzyme that released 1 μg of tyrosine from casein at 60 °C and pH 7.0 per minute.

3.3. Optimization of the Hydrolytic Parameters of Protease A69 towards Antarctic Krill Powder

Because the optimal temperature and pH for the activity of A69 were previously determined to be 60 °C and 7.0, respectively [21], all the hydrolytic reactions for hydrolysis parameter optimization were performed at 60 °C and pH 7.0. The hydrolysis parameters of protease A69 towards Antarctic krill powder, including hydrolysis time and enzyme/substrate (E/S) ratio, were optimized based on the methods previously reported [21] with some modifications. Briefly, 5 g Antarctic krill powder in 30 mL ddH_2O was hydrolyzed by protease A69 at 60°C and pH 7.0 with a constant stir (180 rpm) for all optimization reactions. The optimal E/S ratio was determined by hydrolyzing the Antarctic krill powder for 6 h with different E/S ratios (500 U/g, 1000 U/g, 2000 U/g, 3000 U/g, 4000 U/g, 5000 U/g, 6000 U/g). The optimal hydrolysis time was determined by hydrolyzing the Antarctic krill powder under the determined optimal E/S ratio (5000 U/g) for different time (1 h-6 h). After hydrolysis, the mixtures were incubated at 100°C for 15 min to terminate the reaction, and then centrifuged (4°C, 16970 × g, 30 min). The supernatant and precipitate were, respectively, freeze-dried, and weighed. The powder from freeze-dried supernatant was prepared as 5 mg/mL solution with ddH_2O, which was subjected to peptide molecular weight distribution analysis by HPLC (Shimadzu, Kyoto, Japan) on a TSK gel G2000 SWXL column (7.8×300 mm; Tosoh, Tokyo, Japan) as previously reported [21]. The weight of the freeze-dried precipitate was used to calculate the hydrolysate yield using the formula：Hydrolysate yield (%) = $(W_a - W_b) / W_a \times 100$, where W_a and W_b were the weight of Antarctic krill powder before hydrolysis and that of the freeze-dried precipitate after hydrolysis, respectively.

3.4. Preparation of AKPs with Protease A69

According to the determined optimal hydrolytic parameters, 150 g Antarctic krill powder in 1 L ddH_2O was hydrolyzed with protease A69 (5000 U/g) at 60 °C and pH 7.0 for 6 h with constant stir (180 rpm). After hydrolysis, the AKPs powder was prepared from the mixture through activated carbon treatment, centrifugation, filtration and centrifugal spray drying as previously described [23].

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3.5. Characterization of the Prepared AKPs

The prepared AKPs was dissolved in ddH_2O to prepare 10% (w/v), 20% (w/v), and 30% (w/v) AKPs solutions to investigate the water solubility of the AKPs. Peptide molecular weight distribution of the prepared AKPs was analyzed by HPLC with a 5 mg/mL solution using the method mentioned above. The free and total amino acid composition of the prepared AKPs was determined on an automatic amino acid analyzer, HITACHI 835 (HITACHI, Tokyo, Japan), as previously described [21].

3.6. Determination of Protein Content

The protein content of the Antarctic krill powder samples before and after A69 hydrolysis was determined using the colorimetric method according to the National Standard of the People's Republic of China for determination of protein in foods (GB 5009.168-2016) [99] as previously described [23].

3.7. Bioactivity assays of the Prepared AKPs

The *in vitro* antioxidant activity of the prepared AKPs was evaluated by determining the scavenging rates of $ABTS^+$, DPPH·, ·OH and O_2-·. The scavenging rate of $ABTS^+$was determined according to the National Standard of the People's Republic of China for the Determination of antioxidant activity of peptides (GB/T 39100-2020) [100]. The clearance rates of DPPH·, ·OH and O_2-· were determined according to the previously reported methods [21]. The ACE-inhibitory activity of the prepared AKPs was determined with the previously reported method [22]. The α-amylase-inhibitory activity [101], α-glucosidase-inhibitory activity [102] and the DPP-IV-inhibitory activity [103] of the prepared AKPs were determined according to the methods previously reported.

The antibacterial effects of the prepared AKPs on *E. coli* and *S. aureus* were determined using the Oxford Cup method [104] with some modification. Briefly, 100 μL of the AKPs water solution (40 mg/mL) was added in the cup standing on the plates containing LB solid medium and *E. coli* or *S. aureus* cells. Kanamycin (1 mg/mL, 100 μL) was used as the positive control and sterile water (100 μL) as the negative control. The plates were incubated at 37°C. The clear inhibition zones were observed and their diameters were measured.

3.8. Identification of Bioactive Peptides from the Prepared AKPs

The prepared AKPs were subjected to mass spectrometry using Q Exactive™ Hybrid Quadrupole-Orbitrap™ Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) in Beijing biotech-pack Scientific Co., Ltd. (Beijing, China). The raw MS files were analyzed and searched against protein databases based on the species in the sample using Byonic. The parameters were set as follows: the protein modifications were carbamidomethylation (C) (fixed), oxidation (M) (variable), and acetyl (N-term) (variable), the enzyme specificity was set to non-specific, the maximum missed cleavages were set to 3, the precursor ion mass tolerance was set to 20 ppm, and MS/MS tolerance was 0.02 Da. Only peptides identified with high confidence were chosen for downstream protein identification analysis. In the determined peptide sequences of the prepared AKPs, peptide sequences with bioactivity deposited in databases were searched, including peptide sequences with ACE-inhibitory activity deposited in the AHTPDB database (https://webs.iiitd.edu.in/raghava/ahtpdb/, accessed on 23 March 2025), peptide sequences with antioxidant activity deposited in the AODB database (https://aodb.idruglab.cn/, accessed on 17 March 2025), peptide sequences with antimicrobial activity deposited in the APD database (https://aps.unmc.edu, accessed on 13 March 2025), peptide sequences with α-amylase-inhibitory activity, α-glucosidase-inhibitory activity, or DPP-IV-inhibitory activity deposited in BIOPEP-UWM (http://www.uwm.edu.pl/biochemia/index.php/pl/biopep, accessed on 23 March 2025).

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4. Conclusions

In this study, Antarctic krill powder derived from krill oil production was hydrolyzed by marine bacterial metalloprotease A69 to prepare AKPs with multi-bioactivities. Through optimization of hydrolysis parameters, we established a process for AKPs preparation. The prepared AKPs was milky white powder with good water solubility, containing high content of small peptides with molecular weights below 1000 Da (>85%), and 17 amino acids, including 7 human essential ones. The prepared AKPs displayed multiple bioactivities, including antioxidant activity, ACE-inhibitory activity, antibacterial activity, α-glucosidase-inhibitory activity and DPP-IV-inhibitory activity. Correspondingly, 14 antioxidant peptides, 24 ACE-inhibitory peptides, 2 α-glucosidase-inhibitory peptides, and 10 DPP-IV-inhibitory peptides were identified from the AKPs. This study indicates that marine bacterial metalloprotease A69 has a promising potential in preparing AKPs with good nutritional value and multi-bioactivities, offering a possible method for the high value-added utilization of Antarctic krill powder.

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Data Availability Statement: The original data presented in the study are included in the article; further inquiries can be directed to the corresponding author.

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