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Specificity of Carboxypeptidases from *Actinomucor elegans* and Their Debittering Effect on Soybean Protein Hydrolysates

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Abstract The specificities of carboxypeptidases from *Actinomucor elegans* were investigated by determining enzymatic activities at pH 7.0 and pH 4.0 with 16 Z-dipeptides and three Z-tripeptides as substrates. The debittering effect was evaluated and the free amino acid compositions of the soybean protein hydrolysates were analyzed before and after treatment with *A. elegans* extract at pH 7.0 and pH 4.0, with carboxypeptidases from *Aspergillus oryzae* as control. The results of the enzyme activity determinations indicated that carboxypeptidases from *A. elegans* prefer hydrophobic substrates, such as Z-Phe-Leu, Z-Phe-Tyr-Leu, and Z-Phe-Tyr. The sensory evaluation and free amino acid composition analysis showed that these carboxypeptidases are efficient tools for decreasing the bitterness of peptides because they liberated the fewest free amino acids, which consisted of 73% hydrophobic amino acids, under acidic conditions. Carboxypeptidases from *A. elegans* display promising prospects for future applications in the protein hydrolysate industry.

Keywords *A. elegans* · *A. oryzae* · Bitter taste · Carboxypeptidase · Protein hydrolysate · Sufu

Introduction

Bitter peptides are frequently generated during enzymatic production of functional, bioactive protein hydrolysates or during the aging process of fermented products such as

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cheese [1–3]. The bitterness is usually associated with peptides with a high content of hydrophobic amino acids [4], and becomes stronger as the content of hydrophobic amino acids increases or becomes more concentrated at the C terminus [5–9]. It was reported that the bitterness of the oligopeptide Arg-Pro-Phe-Phe, which has a hydrophobic Phe-Phe at the C terminus, was 25 times greater than that of caffeine. However, the bitterness of this oligopeptide completely vanished when the Phe-Phe was substituted by Gly-Gly [10]. Kim and Eunice compiled a database consisting of 224 di- to tetradecapeptides and five amino acids in order to study the quantitative structure–activity relationship of bitter peptides [11]. They found that bulky hydrophobic acids at the C terminus and bulky basic amino acids at the N terminus were highly correlated to the bitterness [11].

Many attempts have been made to decrease the bitterness of protein hydrolysates and fermented food. It has been found that incubating protein hydrolysates with exopeptidases (aminopeptidases and carboxypeptidases) is an efficient way to obtain product with no bitterness as it releases amino acids at the C or N terminus that may be contributing to the bitterness of the peptide. Carboxypeptidases from many sources, such as *Aspergillus* [12], wheat [13], and pancreatin [14, 15], have been confirmed to have the ability to decrease bitterness. The production of carboxypeptidases, usually serine carboxypeptidases (also termed acid carboxypeptidases), has been reported from several species of fungus, such as *Aspergillus* [16, 17] and *Monascus* [18–20]. Those carboxypeptidases have optimum activities in the pH range of 3–4 [21].

Actinomucor elegans has been commonly used as a starter in the manufacture of sufu, a traditional Chinese fermented soybean curd that has been served as an appetizer in China for more than 1,000 years [22]. During sufu fermentation, *A. elegans* proteases [23] catalyze the degradation of proteins into low molecular weight peptides and amino acids [24], which contribute some special flavors and textures to the products [25]. Our previous research [26] demonstrated that *A. elegans* extract is quite effective in lessening the bitter taste of the soybean protein hydrolysates at neutral pH. Meanwhile, various exopeptidase (aminopeptidase and carboxypeptidase) activities of the *A. elegans* extract against synthetic substrates were observed. In particular, the highest hydrolysis rate appeared with N-CBZ-Ile-Leu as substrate, implying that the carboxypeptidases in *A. elegans* extract may be a key factor in eliminating the bitter taste of soybean protein hydrolysates. In order to further elucidate the role of *A. elegans* carboxypeptidases in the debittering effect, this study examined the enzymatic specificity of *A. elegans* carboxypeptidases using 19 Z-dipeptides and Z-tripeptides as substrates. The debittering effect of *A. elegans* carboxypeptidases on soybean protein hydrolysates was also compared with *Aspergillus oryzae* carboxypeptidases as control under neutral and acidic conditions.

Materials and Methods

Organism and Culture Conditions

A. elegans AS 3.2778 was generously provided by Wang Zhihe Sufu Company, Beijing, China. *A. oryzae* GIM 3.31, 3042 was purchased from Guangdong Microbial Culture Collection Center. The culture conditions were the same for the two molds in this experiment. The spore suspension was prepared by adding 10 mL of sterile distilled water to a slant culture that had just been taken out of the incubator (28 °C) on the fourth day of cultivation. Wheat bran (10.0 g) and distilled water (10.0 mL) were added to 250-mL conical flasks and autoclaved for 40 min at 121 °C. After the spore suspension (1 mL;

containing about 3×10^6 viable propagules/mL) was uniformly dispensed into the wheat bran medium in the flasks, they were then incubated at 28 °C for 2 days.

Preparation of *A. elegans* and *A. oryzae* Extracts

All of the bran koji (*A. elegans* bran and *A. oryzae* bran) in the conical flasks was harvested simultaneously and kept at 4 °C. A sterile shallow pan was used as a container for mixing bran koji from the same batch for the same kind of mold. Then 10.0-g samples of bran koji were weighed out and kept individually in sterile bags at −20 °C. To determine carboxypeptidase activity, 100 mL of sterile distilled water was added to one portion (10.0 g) of bran koji, and kept at 4 °C overnight. The mixture was then filtered through fourfold sterilized gauze fabric, followed by centrifugation (RCF, 4,020×*g*, 4 °C; HITACHI CR 22G) to remove insoluble matter. The supernatants from this step were designated as *A. elegans* extract and *A. oryzae* extract and were immediately used to determine carboxypeptidase activity and in the treatment of bitter peptides.

Assay of Carboxypeptidase Activity

Nineteen N-substituted dipeptides and tripeptides were selected as substrates of the carboxypeptidases; they were regarded as bitter-tasting peptides [3, 10, 11, 27].

Peptides Z-Trp-Leu and Z-Ala-Tyr were purchased from Bachem Inc., (USA); Z-Glu-Phe, Z-Ile-Phe, and Z-Leu-Tyr were purchased from Indofine Chemical Company Inc (Somerville, USA); Z-Ala-Phe and Z-Leu-Phe were from Chem-Impex International Inc. (Wood Dale, IL, USA); Z-Phe-Tyr-Leu, Z-Gly-Pro-Leu, Z-Glu-Tyr, and Z-Phe-Tyr were obtained from Pepnet International Inc., (USA); other N-substituted peptides, N-Cbz-Ile-Leu, Z-Gly-Phe, Z-Val-Phe, N-Cbz-Gly-Tyr, N-Cbz-Ala-Leu, N-Cbz-Gly-Gly-Leu, and N-Cbz-Phe-Leu, and ninhydrin were all from Sigma Chemical Co. (USA).

Each of the above substrates was dissolved at a final concentration of 2.0 mmol/L in 100 mmol/L potassium phosphate buffer (pH 7.0) and 50 mmol/L citrate buffer (pH 4.0). Fresh *A. elegans* and *A. oryzae* extracts were diluted separately with 100 mmol/L potassium phosphate buffer (pH 7.0) and 50 mmol/L citrate buffer (pH 4.0) to 10 mL in volumetric flasks. Cd-ninhydrin reagent was prepared in advance according to the method of Doi (0.8 g ninhydrin were dissolved in a mixture of 80.0 mL of 99.5% ethanol and 10.0 mL of acetic acid while 1.0 g $CdCl_2$ was dissolved in 1.0 mL water, and the two solutions were mixed and kept at room temperature) [27].

Calibration curves were prepared as follows. Solutions of L-Leu, L-Tyr, and L-Phe (2.0 mmol/L) in distilled water served as standard solutions. They were diluted separately to concentrations in the range of 0–150 μmol/L with distilled water, and 0.2 mL of *A. elegans* or *A. oryzae* extract was added, to a final volume of 1.0 mL, then the mixture was allowed to react with 2.0 mL Cd-ninhydrin reagent at 84 °C for 5 min. The mixture was then cooled to room temperature, and the absorbance was read at 507 nm. The calibration curves for each amino acid were calculated for the two mold extracts at pH 7.0 and pH 4.0.

The reaction mixtures, consisting of 1.8 mL substrate solution (2.0 mmol/L) and 0.2 mL diluted *A. elegans* or *A. oryzae* extract (dilute multiples were 10 and 5, respectively), were incubated at 37 °C for up to 6 h [28]. Aliquots (50 μL) were taken and mixed with distilled water (950 μL) and Cd-ninhydrin reagent (2.0 mL), then incubated at 84 °C for 5 min, and the absorbance was read at 507 nm. The concentrations of amino acids liberated from the substrates were calculated from the above calibration curves. The activities were expressed as micromoles of amino acid per hour per milliliter of extract. The assays were performed in triplicate.

Preparation of Bitter Peptides

Soybean protein isolate (SPI; Jilin Bu'er Protein Co., Ltd., China) was dispersed in distilled water (5%, *w*/*v*) and stirred gently at room temperature for 2 h, then the pH of the SPI solution was adjusted to 7.0 with 1 mol/L HCl. The protein dispersion was incubated in a bath of 95 °C for 20 min, then cooled to room temperature in an ice-water bath. Protease Alcalase 2.4L (Novozyme, Bagsvaerd, Denmark) with 1,000U/1 g SPI was introduced into the preheated SPI solution and incubated at 55 °C for 3 h. At the end of the enzymatic hydrolysis, the solution was heated to 100 °C and kept at that temperature for 20 min to deactivate the enzyme. After centrifugation (RCF, 4,020×*g*, 4 °C; HITACHI CR 22G), the supernatant was used as bitter peptides.

Electrophoresis of Bitter Peptides

Polyacrylamide gel electrophoresis (PAGE) of the bitter peptides was performed by the method of Laemmli [29] with 14% polyacrylamide gels in the presence of sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (SDS-PAGE), which were stained with Coomassie Brilliant Blue. Standard molecular weight markers consisted of phosphorylase(97 kD), albumin (66 kD), glutamic dehydrogenase (53 kD), glyceraldehyde-3-phosphate(36 kD), and trypsinogen(24 kD).

Treatment of Bitter Peptides with *A. elegans* and *A. oryzae* Extracts

The bitter peptide solutions obtained were divided into six parts, each aliquot with a volume of 100 mL. The first two parts were treated with either 20 mL *A. elegans* extract or 20 mL *A. oryzae* extract at pH 7.0, the second two parts were treated with either 20 mL *A. elegans* extract or 20 mL *A. oryzae* extract at pH 4.0, and the last two parts were treated with either 20 mL inactivated *A. elegans* extract or 20 mL inactivated *A. oryzae* extract, as controls. All prepared solutions were incubated at 40 °C for 6 h and then heated to 95 °C and kept at that temperature for 20 min to deactivate the enzymes.

Sensory Evaluation

Taste evaluation was performed according to the literature [30, 31] with minor modification as follows. Non-bitter iso-electric soluble soy peptide (Jilin Bu'er Protein Co., Ltd., China) was utilized as a background for the quinine (Sigma, Chemical Co., USA) bitter-tasting standards. The laboratory's taste panel consisted of five participants, who were instructed to rank five bitter-tasting standards (the standards contained 20, 40, 80, 160, and 200 ppm quinine, and the corresponding sum of ranks (SR) was 1.0, 2.0, 3.0, 4.0, and 5.0, respectively). The bitterness value of a sample was obtained by comparison with the standards. The protein ($N \times 6.25$) concentrations in the samples and standards were 3.0% (*w*/*v*); the pH of the samples and standards was adjusted to 6.5 with 4 mol/L NaOH or 6 mol/L HC1. The test was carried out twice so that all five samples could be evaluated.

Analysis of Free Amino Acid Composition

Free amino acid compositions of the bitter peptides before and after treatment with *A. elegans* extract and *A. oryzae* extract were assayed using the HPLC method [32], with high-performance liquid chromatography equipment(Waters Ltd., Milford, MA, USA) and a

PICO.TAG amino acid analysis column. The detection conditions were as follows: wavelength 254 nm, temperature 38 °C, flow rate 1 mL/min. The concentration of each amino acid in each sample was calculated by calibrating against standard amino acid solutions (amino acid standard solution, type H, Sigma AAS18, St. Louis, MO, USA).

Statistical Analysis

Data were expressed as means±standard deviation of three parallel measurements. Statistical calculations were carried out by SAS V.9.0 software. P values <0.05 were regarded as significant.

Results and Discussion

Specificity of *A. elegans* Carboxypeptidases

Five Z-dipeptides and three Z-tripeptides with Leu at the C terminus, six Z-dipeptides with Phe at the C terminus, and five Z-dipeptides with Tyr at the C terminus were used as substrates to investigate the carboxypeptidase activities of *A. elegans* and *A. oryzae* extracts at pH 7.0 and pH 4.0. The modified colorimetric ninhydrin method C [27] was selected to monitor the amino acids released by the carboxypeptidases and, with the

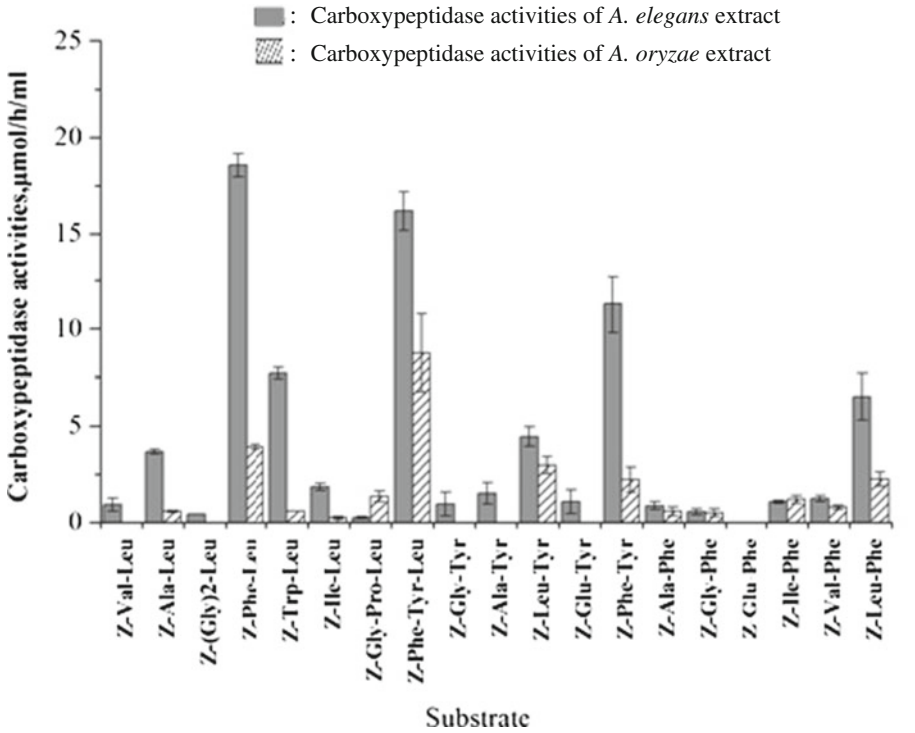

Fig. 1 Carboxypeptidase activities of *A. elegans* and *A. oryzae* extracts at pH 7.0. : carboxypeptidase activities of *A. elegans* extract. : carboxypeptidase activities of *A. oryzae* extract

addition of crude enzyme extract, standard curves were constructed to eliminate the influence of peptides, amino acids, or their derivatives and pigments from the extract on the experimental results.

Figure 1 shows carboxypeptidase activities present in *A. elegans* extract and *A. oryzae* extract at pH 7.0. The carboxypeptidases in *A. elegans* extract exhibited activities towards 18 substrates (all except Z-Glu-Phe). Compared with the carboxypeptidases in *A. oryzae* extract, those in *A. elegans* extract displayed much higher activities and broader specificities under neutral pH conditions, especially when the substrates were Z-Phe-Leu (18.56 μmol/h/mL extract), Z-Phe-Tyr-Leu (16.19 μmol/h/mL extract), and Z-Phe-Tyr (11.29 μmol/h/mL extract; Fig. 1). The carboxypeptidase activities for *A. elegans* extract also displayed a relationship with the amino acid at the C terminus and at the penultimate position from the C terminus. In particular, the carboxypeptidases in *A. elegans* extract were more active when Leu, rather than Tyr or Phe, was at the C terminus, and when a hydrophobic amino acid was at the penultimate position from the C terminus (Z-Phe-Leu, Z-Phe-Tyr-Leu, Z-Phe-Tyr, Z-Leu-Phe, and Z-Leu-Tyr) rather than when a hydrophilic amino acid was at the penultimate position from the C terminus (Z-Gly-Tyr, Z-Ala-Tyr, Z-Gly-Phe, Z-Ala-Phe, and Z-Glu-Phe).

Figure 2 shows the carboxypeptidase activities present in *A. elegans* and *A. oryzae* extracts at pH 4.0. The carboxypeptidase activities in *A. elegans* extract were generally less than 5 μmol/h/mL extract, but there was still activity towards 18 substrates (all but

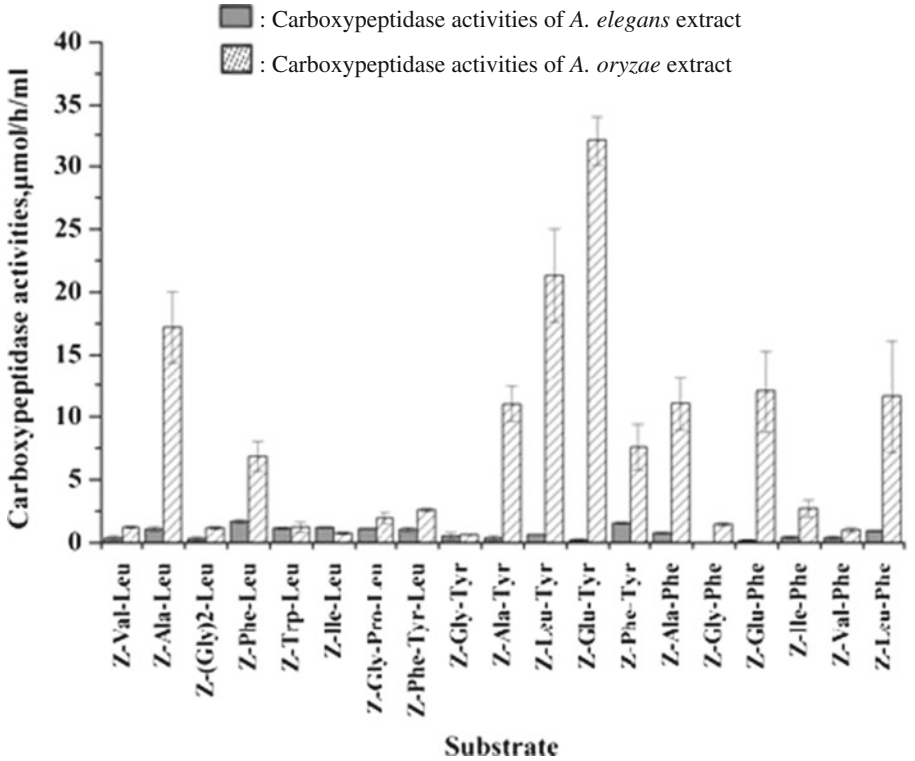

Fig. 2 Carboxypeptidase activities of *A. elegans* and *A. oryzae* extracts at pH 4.0. : carboxypeptidase activities of *A. elegans* extract. : carboxypeptidase activities of *A. oryzae* extract

Z-Glu-Phe), and in general unchanged broad specificity was displayed. On the other hand, at pH 4.0 the carboxypeptidase activities of *A. oryzae* extract were significantly greater than those of *A. elegans* extract and *A. oryzae* extract at pH 7.0, especially when the substrates were Z-Glu-Tyr (32.08 μmol/h/mL extract), Z-Leu-Tyr (21.33 μmol/h/mL extract), and Z-Ala-Leu (17.21 μmol/h/mL extract). These observations confirmed that *A. oryzae* has acidic carboxypeptidases, which is in agreement with earlier reports [17, 33–35]. The data presented here indicate that the carboxypeptidase activities in *A. oryzae* were higher when Tyr and Phe were at the C terminus than when Leu was at the C terminus.

The carboxypeptidases from *A. oryzae* were acidic carboxypeptidases, whereas the carboxypeptidases from *A. elegans* were not (Figs. 1 and 2) but were similar to a carboxypeptidase (CP3) from *Mucor racemosus* (*A. elegans* and *M. racemosus* belong to the same genus) [36]. The carboxypeptidase activities of *A. elegans* were much higher at pH 7.0 than at pH 4.0; the enzyme from *M. racemosus* has a pH optimum of 6.5 for N-CBZ-Phe-Leu hydrolysis. The carboxypeptidases from *A. elegans* had a strong preference for cleaving peptide bonds when a hydrophobic amino acid was at the C terminus and when another hydrophobic amino acid was at the penultimate position. The enzyme from *M. racemosus* is able to hydrolyze peptides with aliphatic or aromatic side chains, but is not able to degrade peptides containing Gly in the penultimate position.

Debittering Effect of Carboxypeptidases from *A. elegans*

Figure 3 shows that the main bands of soybean protein completely disappeared after enzymatic hydrolysis by alcalase; the bitterness value of this peptide solution was 4.4±0.42 (Table 1). Alcalase is a well-known endoproteinase with broad specificity that usually cleaves the peptide bond at the carboxylic side of hydrophobic amino acids [37, 38]. It cleaved the peptide bonds at Gln^{4}-His^{5}, Ser^{9}-His^{10}, Leu^{15}-Tyr^{16}, and Tyr^{26}-Thr^{27} when incubated with the oxidized B-chain of insulin, and the Leu^{15}-Tyr^{16} bond was cleaved faster than any other bond in the B-chain [39]. When soybean protein was treated with alcalase, the enzyme was able to quickly break the long peptide chain down to small peptide segments with a hydrophobic amino acid at the carboxylic terminus, so peptides degraded by alcalase were expected to be the most bitter [40]. Previous research indicated that the bitterness of soybean protein hydrolysates prepared by alcalase was mainly due to hydrophobic peptides of molecular weight less than 1,000 [41, 42].

Table 1 shows the bitterness values of soybean protein hydrolysates after they were treated with mold extracts. The bitterness values were all significantly lower after treatment with *A. elegans* extract and *A. oryzae* extract at pH 7.0 and pH 4.0, but there were no significant differences among the four treatments, indicating that the two mold extracts had nearly the same ability to decrease the bitter taste under neutral and acidic conditions.

Table 2 displays the changes in free amino acid contents of the soybean protein hydrolysates after they were treated with the mold extracts. The total amounts of free amino acids obviously increased to 295.60 mg/100 mL (pH 7.0) and 58.09 mg/100 mL (pH 4.0) with *A. elegans* extract, and to 329.24 mg/100 mL (pH 7.0) and 356 mg/100 mL (pH 4.0) with *A. oryzae* extract. Interestingly, the total free amino acid content was relatively low upon treatment with *A. elegans* extract at pH 4.0, and mainly consisted of hydrophobic amino acid such as Leu, Phe, and Tyr (the content of these three amino acids reached 42.09 mg/mL and accounted for 73% of the total free amino acids; the ratio of

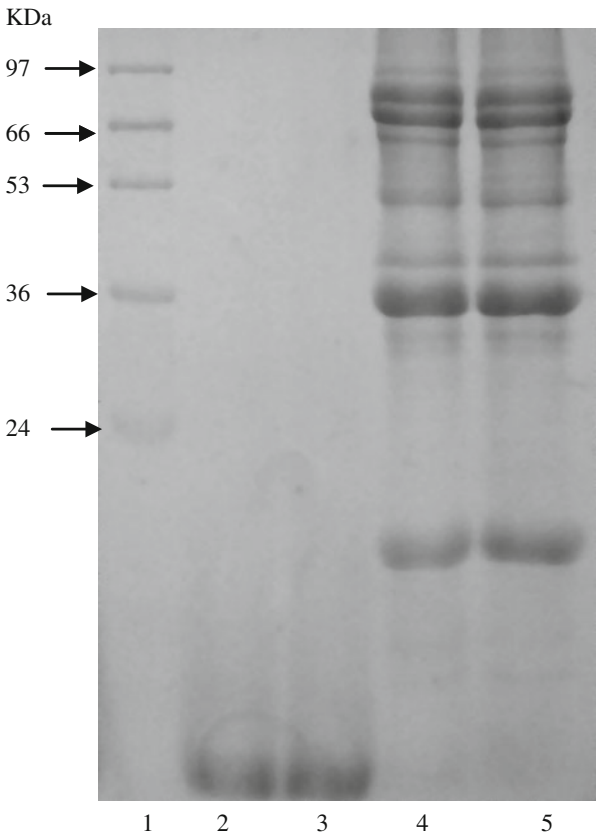

Fig. 3 SDS-PAGE profile of soybean protein hydrolysates. *Lane 1* molecular weight markers; *lanes 2 and 3* soybean protein hydrolysates (bitter peptides); *lanes 4 and 5* soybean protein isolates

hydrophobic/hydrophilic amino acids was 7:1). In contrast, when treated with *A. oryzae* extract at pH 4.0, large quantities of amino acids, especially Glu, Tyr, Phe, Leu, and Arg, were liberated (the ratio of hydrophobic/hydrophilic amino acids was 2:1). This observation provides a clue that *A. elegans* carboxypeptidases are efficient tools for liberating hydrophobic amino acids at the C terminus position, considering there was no aminopeptidase activity detected for either enzyme extract at pH 4.0 (data not shown). As

Table 1 Bitterness values of soybean protein hydrolysates before and after treatment with *A. elegans* extract and *A. oryzae* extract at pH 7.0 and pH 4.0

	pH 7.0	pH 4.0
Before treatment	4.40±0.42 a	4.40±0.42 a
A. elegans extract	0.27±0.10 b, A	0.71±0.24 b, A
A. oryzae extract	039±0.24 b, A	0.67±0.61 b, A

Bitterness values are expressed as average±standard deviation of duplicate scores for each sample. Statistical analysis of the bitterness value was performed to evaluate the analysis of variance, and the statistical significance of the data was determined by Duncan multiple-range test ($P<0.05$)

a, *b* are the significance of the bitterness values both before and after treatment by the mold extracts, *A* is the significance of the bitterness values of soybean peptides treated with two different extracts

Table 2 Changes in the free amino acid content of the soybean protein hydrolysates after treatment with *A. elegans* extract and *A. oryzae* extract at pH 7.0 and pH 4.0 (mg/100 mL)

Free amino acid	*A. elegans* extract		*A. oryzae* extract	
	pH 7.0	pH 4.0	pH 7.0	pH 4.0
Asp	13.75	1.15	4.23	8.23
Glu	20.39	4.28	11.94	57.97
Ser	13.86	1.50	11.55	6.90
Thr	6.04	0.00	13.31	13.94
Gly	2.20	0.00	4.04	3.05
Ala	12.96	0.87	12.58	13.10
Pro	3.75	0.00	9.62	6.46
Tyr	27.66	9.56	18.20	21.75
Val	19.19	2.37	17.26	11.54
Met	4.40	0.00	8.85	10.49
Cys	0.00	0.57	1.05	0.05
Ile	4.36	0.04	15.26	8.61
Leu	50.36	18.42	42.12	42.15
Try	12.12	1.96	5.84	16.43
Phe	44.98	14.11	32.27	25.73
His	8.39	1.32	15.74	14.49
Arg	37.67	1.94	67.39	56.36
Lys	16.99	0.00	37.99	39.20
Total	295.60	58.09	329.24	356.97

to the treatment with *A. elegans* extract at pH 7.0, its debittering effect and the free amino acid composition were close to those observed for the treatment with *A. oryzae* extract. Not only were more hydrophobic amino acids such as Leu, Phe, and Tyr released, which was in agreement with the carboxypeptidase activities shown in Fig. 1, but also large amounts of hydrophilic amino acids, such as Glu, Asp, and Ser, appeared. This indicates that some other exopeptidases, such as aminopeptidases, were active; in fact, several aminopeptidases were detected in *A. elegans* extract at pH 7.0 during our previous research [26].

Conclusion

Carboxypeptidases from *A. elegans* demonstrated a preference for the hydrophobic synthetic substrates Z-Phe-Leu, Z-Phe-Tyr-Leu, and Z-Phe-Tyr, and were efficient tools for decreasing the bitterness of peptides by liberating the fewest free amino acids, which consisted of 73% hydrophobic amino acids, under acidic conditions. Therefore, carboxypeptidases from *A. elegans* display promising prospects for future applications in the protein hydrolysate industry.

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