ISSN 1330-9862
(FTB-2251)

original scientific paper

Purification and Characterization of Antioxidant Peptide from Sunflower Protein Hydrolysate

Jian Ren[1,2]*, *Xi-Qun Zheng*[1,2], *Xiao-Lan Liu*[1,2] *and Huan Liu*[1]

[1]Food and Bioengineering College, Qiqihar University, Qiqihar 161006, Heilongjiang Province, PR China

[2]Key Laboratory of Processing Agricultural Products of Heilongjiang Province, Qiqihar University, Qiqihar 161006, Heilongjiang Province, PR China

Received: March 9, 2009
Accepted: March 29, 2010

Summary

Sunflower proteins were hydrolyzed with Flavourzyme for the production of antioxidant peptide. DEAE-Sepharose Fast Flow, Sephadex G-25 gel filtration chromatography and reversed-phase HPLC were consecutively employed to purify a novel sunflower antioxidant peptide, and the ability to inhibit the autoxidation of pyrogallol was expressed as the antioxidative activity of the peptide. The amino acid sequence was identified as Ala-Cys-Ala-His-Asp-Lys-Val by a Q-Tof2 mass spectrometer. This novel peptide exhibited a high antioxidative activity of 79.42 U/mL, which is expected to protect against oxidative damage in living systems in relation to aging and carcinogenesis. Higher antioxidative activities were presumed mainly due to the presence of hydrophobic amino acids in its sequence.

Key words: antioxidant peptide, sunflower, protein hydrolysate, purification and characterization

Introduction

Lipid oxidation is of great concern for the food industry and consumers because it deteriorates food quality and shortens the shelf life of products. Furthermore, lipid oxidation may lead to diseases following consumption of potentially toxic reaction products (*1*). An antioxidant is defined as any substance that, when present at low concentrations compared to that of an oxidizable substrate, significantly delays or inhibits oxidation of the substrate (*2*). Currently, synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone, and propyl gallate are added to food products to retard lipid oxidation. However, these compounds must be used under strict regulation due to their potential health hazards. Therefore, new interest has been developed as purifying and characterizing safe antioxidants from natural sources (*3*,*4*).

Many investigations have been carried out on the purification and characterization of antioxidant peptides from natural sources, such as bullfrog skin (*1*), lecithin-free egg yolk (*2*), yellowfin sole frame protein (*5*), corn gluten (*6*), skin of sole and squid (*7*) and fermented mussel sauce (*8*). However, little is known about the structure of antioxidant peptides from sunflower.

Sunflower is one of five major oil plants in the world. The defatted sunflower meal has a relatively high content of protein, and a great economic value as food additive. Sunflower production and utilization have increased in many countries, and many investigations have been carried out on the processing, nutrition, and functional properties of sunflower (*9–16*).

*Corresponding author; Phone: ++86 452 274 2712; E-mail: super_renjian1970@163.com

Thus, the objective of the present study is to purify and characterize an antioxidant peptide from a sunflower protein hydrolysate.

Materials and Methods

Materials and chemicals

Sunflower (with high linoleic acid, grown in HeiLongJiang Province, PR China) was obtained from Qiqihar local market. Flavourzyme (500 leucine aminopeptidase units (LAPU)/g) was purchased from Novo Nordisk (Bagsvaerd, Denmark). DEAE-Sepharose Fast Flow and Sephadex G-25 were purchased from Pharmacia (Uppsala, Sweden). C-18 column was purchased from Waters (Milford, MA, USA). The ultrafiltration (UF) membrane system was purchased from Millipore Co. (Bedford, MA, USA). All other chemicals used in the experiments were of analytical grade and purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Preparation of sunflower protein hydrolysate

The factors for the hydrolysis of sunflower protein were optimized in our previous study (unpublished data). Thus, in this work, sunflower proteins were hydrolyzed at 53 °C, pH=7.0, substrate concentration 5.5 % (*m/V*), enzyme to substrate mass ratio 5 %, for 60 min by Flavourzyme. The pH of the mixture was kept constant by continuously adding 1 M NaOH solution to the reaction medium. The enzyme was inactivated by heating in a boiling water bath for 10 min. Then, the sunflower protein hydrolysate was centrifuged at 10 000×*g* for 20 min and the supernatant was used for the determination of antioxidative activity. In the end, the active fractions of molecular mass<6000 Da were separated by ultrafiltration and lyophilized (*7*).

Determination of antioxidative activity of the sunflower protein hydrolysate

The ability to inhibit the autoxidation of pyrogallol was expressed as the antioxidative activity of the sample according to the method of Marklund and Marklund (*17*). A volume of 4.5 mL of Tris–HCl buffer (50 mM, pH=8.2) was kept in a water bath (25 °C) for 20 min. Then, 0.01 mL of 45 mM pyrogallol was added to the above buffer solution. The rate of autoxidation was determined at 325 nm. One unit of antioxidative activity was defined as the amount of sample needed to obtain 50 % inhibition of pyrogallol oxidation (*6*).

Purification of antioxidant peptide

The lyophilized hydrolysate was dissolved in 25 mM Tris–HCl buffer (pH=8.9), loaded onto a column (1.6×20 cm) of DEAE-Sepharose Fast Flow equilibrated with the same buffer, and then eluted with a linear gradient of NaCl from 0 to 1.0 M. Fractions were collected at a flow rate of 1.5 mL/min. A strong antioxidant fraction was lyophilized, and gel filtration chromatography was used as the next step.

The above lyophilized fraction was dissolved in distilled water, centrifuged at 10 000×*g* for 20 min and the supernatant was loaded on a Sephadex G-25 gel filtration column (1.6×100 cm) equilibrated previously with distilled water. The column was then eluted with distilled water and the fractions were collected at a flow rate of 2 mL/min. The fractions showing higher antioxidative activity were pooled and lyophilized for further use.

The above lyophilized fraction was dissolved in acetonitrile at 2 % (by volume) in water with 0.065 % (by volume) of trifluoroacetic acid (TFA) and separated using reversed-phase HPLC on a ZORBAX SB-C18 column with a linear gradient of acetonitrile (2–80 % in 45 mL) in 0.065 % TFA and 1 mL/min flow rate. Absorbance was monitored at 214 nm, and the active peaks were collected and concentrated by lyophilization. The lyophilized active peaks were further chromatographed on a ZORBAX SB-C18 column using a linear gradient of acetonitrile (10–60 % in 45 mL) in 0.065 % TFA at a flow rate of 1 mL/min. The finally purified peptide was analyzed for its amino acid sequence (*1*,*6*,*8*).

Identification of a peptide sequence

The sequence of the antioxidant peptide was determined by Q-Tef2 mass spectrometer (Waters, Milford, MA, USA). The antioxidant peptide was firstly injected into the electrospray source and then dissolved in 50 % methanol solution (by volume). The molecular mass of the purified peptide was measured by a doubly charged $(M+2H)^{+2}$ state. The sequence of the peptide was determined by tandem mass spectrometry analysis (*1*,*6*).

Comparison of peptide sequence

The identified peptide sequence data were compared with a protein database (PIR-NREF) and a bioactive polypeptide database in order to make sure whether the antioxidant peptide was novel (*18*,*19*).

Results and Discussion

Molecular mass profile

Molecular mass of peptides is an important factor in producing protein hydrolysates with desired antioxidative activity. The use of ultrafiltration (UF) membrane system is appropriate to obtain protein hydrolysates with desired molecular mass range (*20*,*21*). Therefore, the sunflower protein hydrolysate was fractionated using three ultrafiltration membranes according to the molecular size, and thus four types of hydrolysates (>20 000, 20 000–10 000, 10 000–6000, <6000) were obtained, and the yields of each fraction were 14.12, 1.33, 56.31 and 28.24 %, respectively. It has been reported that peptides with lower molecular mass are responsible for a range of biological activities including antioxidation (*20*). Higher antioxidative properties of low molecular mass peptides are thought to be due to their low molecular mass as they can easily react with lipid radicals and thereby reduce radical-mediated lipid peroxidation (*22*). Thus, the active fractions of molecular mass<6000 Da were lyophilized and used in the next purification step, and their antioxidative activity was 120.42 U/mL.

Purification of antioxidant peptide

Throughout the purification process, anion exchange chromatography was used as the first step and 50 mL of the sunflower protein hydrolysate (<6000 Da, 2.256 mg/mL) were loaded onto a DEAE-Sepharose Fast Flow. Adsorbed fractions were eluted with a linear gradient of NaCl (0–1 M) (Fig. 1). The fractions of 204–222 mL were pooled and applied onto a Sephadex G-25 gel filtration chromatography column (Fig. 2). The active fractions of 277–301 mL were collected and purified further by reversed-phase HPLC on a ZORBAX SB-C18 column with a linear gradient of acetonitrile/water (2–80 % (by volume) in 45 mL) in 0.065 % TFA and at a flow rate of 1 mL/min. The results showed that the strongest active peak with antioxidative activity of 73.04 U/mL was obtained at a retention time of 33.320 min. This active fraction was finally loaded on a ZORBAX SB-C18 column using a linear gradient of acetonitrile (10–60 % in 45 mL) in 0.065 % TFA at a flow rate of 1 mL/min. As observed in Fig. 3, the strongest active peak with an antioxidative activity of 79.42 U/mL was obtained at a retention time of 35.095 min. In the end, a purified sunflower protein peptide was obtained. Moreover, the antioxidative activity of this purified peptide (79.42 U/mL) was a little higher than that (78.1 U/mL) of the peptide in the reported work (*6*).

Determination of a peptide sequence

The sequence of this sunflower protein peptide was Ala-Cys-Ala-His-Asp-Lys-Val, determined at the National Center of Biomedical Analysis (Beijing, PR China). Compared with the sequences of other known proteins and active peptides (*18*,*19*), it was found that the sequence of this antioxidant peptide was novel. The mass spectrogram of this new antioxidant peptide is presented in Fig. 4.

Many reports have shown that the antioxidant activity of peptides was highly dependent on their sequence and the amino acid composition. Hydrophobic amino acid residues Val or Leu at the N-terminus end and Pro, Asp, His or Tyr in the sequences of antioxidative peptides have been reported to be important in antioxidative activity (*2*,*8*,*23*–*27*). Therefore, the presence of some hydrophobic amino acid residues and their better positioning in the sequence could be attributed to the antioxidative activity of this sunflower protein peptide.

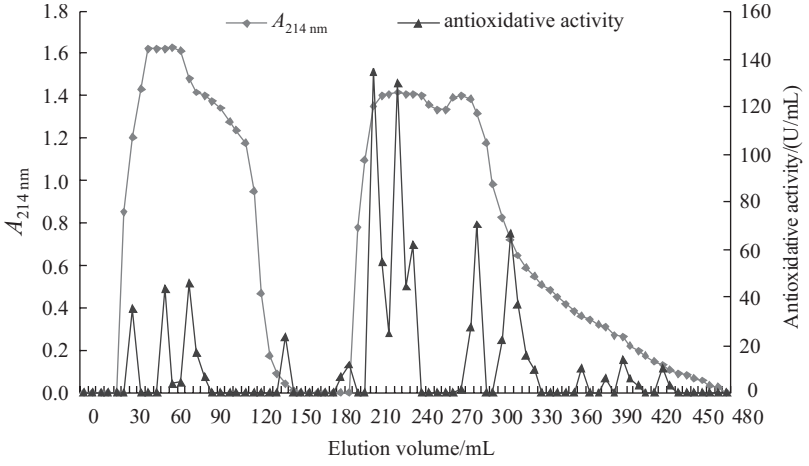

Fig. 1. DEAE-Sepharose Fast Flow ion exchange chromatography elution profile of the sunflower protein hydrolysate

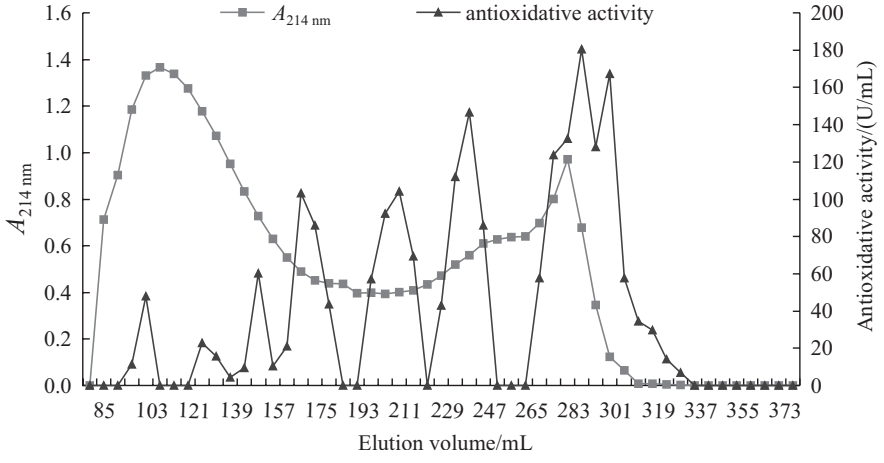

Fig. 2. Sephadex G-25 gel filtration chromatography elution profile of the sunflower protein hydrolysate

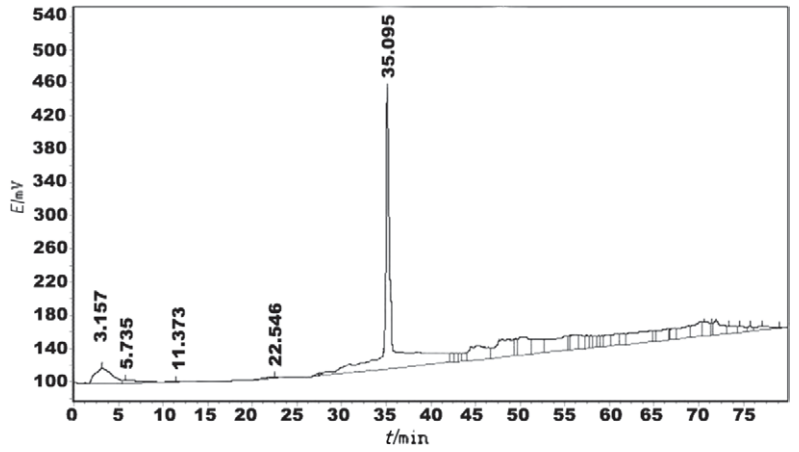

Fig. 3. Reversed-phase HPLC elution profile of an active fraction with the strongest peak on a ZORBAX SB-C18 column

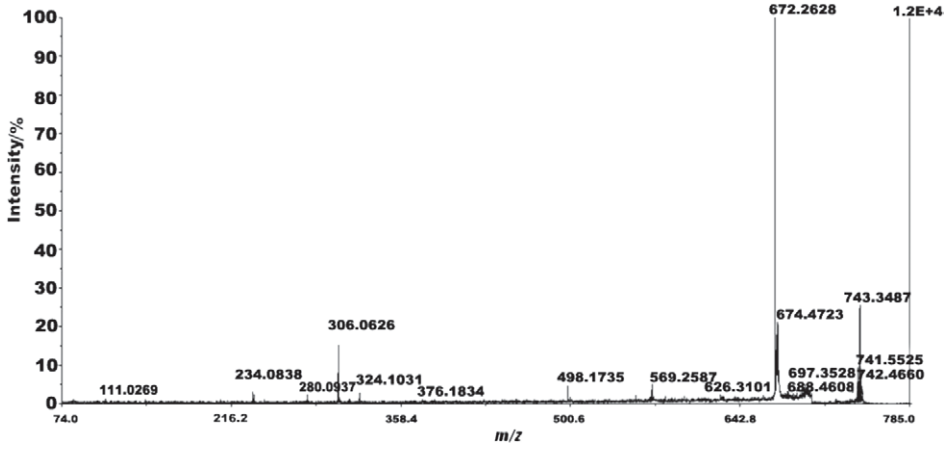

Fig. 4. The mass spectrogram of the new sunflower protein peptide

Conclusions

In the present study, DEAE-Sepharose Fast Flow, Sephadex G-25 gel filtration chromatography and reversed-phase HPLC were combined to purify a novel sunflower protein peptide, and the sequence of this novel peptide was Ala-Cys-Ala-His-Asp-Lys-Val. It exhibited a high antioxidative activity of 79.42 U/mL, which may protect against oxidative damage in living systems in relation to aging and carcinogenesis. The purified sunflower protein peptide has a great potential to be used in food systems as a natural antioxidant. Furthermore, the data presented in this article demonstrate the feasibility of hydrolyzing antioxidant peptide from sunflower protein.

Acknowledgements

This work was supported by Heilongjiang Province Science Foundation for Youths, PR China (QC08C89).

References

1. Z.J. Qian, W.K. Jung, S.K. Kim, Free radical scavenging activity of a novel antioxidative peptide purified from hydrolysate of bullfrog skin, *Rana catesbeiana* Shaw, *Bioresour. Technol. 99* (2008) 1690–1698.
2. P.J. Park, W.K. Jung, K.S. Nam, F. Shahidi, S.K. Kim, Purification and characterization of antioxidative peptides from protein hydrolysate of lecithin-free egg yolk, *J. Am. Oil Chem. Soc. 78* (2001) 651–656.
3. A. Wanita, K. Lorenz, Antioxidant potential of 5-*N*-pentadecylresorcinol, *J. Food Process. Preserv. 20* (1996) 417-429.
4. N.S. Hettiarachchy, K.C. Glenn, R. Gnanasambandam, M.G. Johnson, Natural antioxidant extract from fenugreek (*Trigonella foenum-graecum*) for ground beef patties, *J. Food Sci. 61* (1996) 516–519.
5. S.Y. Jun, P.J. Park, W.K. Jung, S.K. Kim, Purification and characterization of an antioxidative peptide from enzymatic hydrolysate of yellowfin sole (*Limanda aspera*) frame protein, *Eur. Food Res. Technol. 219* (2004) 20–26.
6. X.Q. Zheng, L.T. Li, X.L. Liu, X.J. Wang, J. Lin, D. Li, Production of hydrolysate with antioxidative activity by enzymatic hydrolysis of extruded corn gluten, *Appl. Microbiol. Biotechnol. 73* (2006) 763–770.
7. B. Giménez, A. Alemán, P. Montero, M.C. Gómez-Guillén, Antioxidant and functional properties of gelatin hydrolysates obtained from skin of sole and squid, *Food Chem. 114* (2008) 976–983.
8. N. Rajapakse, E. Mendis, W.Y. Jung, J.Y. Je, S.K. Kim, Purification of a radical scavenging peptide from fermented

mussel sauce and its antioxidant properties, *Food Res. Int. 38* (2005) 175–182.

9. M.A. Sabir, F.W. Sosulski, S.L. Mackenzie, Gel chromatography of sunflower proteins, *J. Agric. Food Chem. 21* (1973) 988–993.
10. E.H. Rahma, M.S. Narasinga Rao, Characterisation of sunflower proteins, *J. Food Sci. 44* (1979) 579–582.
11. E.H. Rahma, M.S. Narasinga Rao, Isolation and characterization of the major protein fraction of sunflower seeds, *J. Agric. Food Chem. 29* (1981) 518–521.
12. M. Kabirullah, R.B.H. Wills, Characterization of sunflower protein, *J. Agric. Food Chem. 31* (1983) 953–956.
13. A. Venktesh, V. Prakash, Functional properties of the total proteins of sunflower (*Helianthus annuus* L.) seed – Effect of physical and chemical treatments, *J. Agric. Food Chem. 41* (1993) 18–23.
14. J. Guéguen, Y. Popineau, I.N. Anisimova, R.J. Fido, P.R. Shewry, A.S. Tatham, Functionality of the 2S albumin seed storage proteins from sunflower (*Helianthus annuus* L.), *J. Agric. Food Chem. 44* (1996) 1184–1189.
15. G.R. Burnett, N.M. Rigby, E.N. Clare Mills, P.S. Belton, R.J. Fido, A.S. Tatham, P.R. Shewry, Characterization of the emulsification properties of 2S albumins from sunflower seed, *J. Colloid Interface Sci. 247* (2002) 177–185.
16. S. Economides, The nutritive value of sunflower meal and its effect on replacing cereal straw in the diets of lactating ewes and goats, *Livest. Prod. Sci. 55* (1998) 89–97.
17. S. Marklund, G. Marklund, Involvement of the superoxide anion radical in the autoxidation of pyrogallol and a convenient assay for superoxide dismutase, *Eur. J. Biochem. 47* (1974) 469–474.
18. The Protein Information Resource (PIR), USA (*http://pir.georgetown.edu*).
19. Institute of Cardiovascular Science, Peking University Health Science Center, Beijing, PR China (*http://biopd.bjmu.edu.cn*).
20. Y.J. Jeon, H.G. Byun, S.K. Kim, Improvement of functional properties of cod frame protein hydrolysates using ultrafiltration membranes, *Process Biochem. 35* (1999) 471–478.
21. W.D. Deeslie, M. Cheryan, Fractionation of soy protein hydrolysates using ultrafiltration membranes, *J. Food Sci. 57* (1991) 411–413.
22. S. Ranathunga, N. Rajapakse, S.K. Kim, Purification and characterization of antioxidative peptide derived from muscle of conger eel (*Conger myriaster*), *Eur. Food Res. Technol. 222* (2006) 310–315.
23. H.M. Chen, K. Muramoto, F. Yamauchi, K. Fujimoto, K. Nokihara, Antioxidative properties of histidine-containing peptides designed from peptide fragments found in the digests of a soybean protein, *J. Agric. Food Chem. 46* (1998) 49–53.
24. H.M. Chen, K. Muramoto, F. Yamauchi, K. Nokihara, Antioxidant activity of designed peptides based on the antioxidative peptide isolated from digests of a soybean protein, *J. Agric. Food Chem. 44* (1996) 2619–2623.
25. J.Y. Je, P.J. Park, S.K. Kim, Antioxidant activity of a peptide isolated from Alaska pollack (*Theragra chalcogramma*) frame protein hydrolysate, *Food Res. Int. 38* (2005) 45–50.
26. H.M. Chen, K. Muramoto, F. Yamauchi, Structural analysis of antioxidative peptides from soybean β-conglycinin, *J. Agric. Food Chem. 43* (1995) 574–578.
27. K. Uchida, S. Kawakishi, Sequence-dependent reactivity of histidine-containing peptides with copper(II)/ascorbate, *J. Agric. Food Chem. 40* (1992) 13–16.