Available online at www.sciencedirect.com

ScienceDirect

journal homepage: www.elsevier.com/locate/jff

Structural characterization of an Indian squid antioxidant peptide and its protective effect against cellular reactive oxygen species

Sekar Sudhakar, Rasool Abdul Nazeer *

Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Chennai, Tamilnadu 603 203, India

ARTICLE INFO

Article history:
Received 10 November 2014
Received in revised form 10 February 2015
Accepted 11 February 2015
Available online

Keywords:
Indian squid
Antioxidant peptide
ROS
FPLC

ABSTRACT

Antioxidant peptides were derived from the edible part of Indian squid, *Loligo duvauceli*, via the use of gastrointestinal enzymes for up to 12 hours. The active α-chymotrypsin hydrolysate exhibited the maximum activity compared to the trypsin and pepsin hydrolysates, as assessed by various radical scavenging and metal chelating assays. Moreover, essential and non-essential amino acids were present in the active hydrolysate. Furthermore, the hydrolysate was purified by ion exchange and gel filtration chromatography using fast protein liquid chromatography (FPLC). The purified peptide exhibited strong free radical scavenging, metal chelation and reducing power abilities that were confirmed with a UV-visible and electron spin resonance (ESR) spectrometer. The peptide also prevented DNA damage and inhibited lipid peroxidation due to its small size (682.5 Da) and content of the high redox potential amino acid sequence Trp-Cys-Thr-Ser-Val-Ser, which was confirmed by ESI-MS/MS. This peptide exhibited no cytotoxic effects on breast cancer cells (MCF7) and scavenged reactive oxygen species at the cellular level under H_2O_2-induced stress. These results suggest that the peptide derived from squid mantle protein acts as a potent antioxidant against oxidative stress and could be used as an efficient and safer adjunct in food processing.

1. Introduction

Antioxidants protect the body from increased levels of reactive oxygen species (ROS) that are elicited by stressful conditions by delaying lipid oxidation in foods. Generally, antioxidants exist in two forms, synthetic and natural. Synthetic antioxidants are used to protect food products, but they can cause health risks (Becker, 1993). Natural antioxidants are often derived from plant sources and are generally considered to be safer for use in food products (Dillard & German, 2000).

α-Tocopherol is widely used as a natural antioxidant in lipid-containing foods, although it has some limitations (Shahidi & Zhong, 2005). Thus, other alternative and safer antioxidants can be isolated from different natural bio-resources and used instead of synthetic compounds. Such antioxidants in proteins are helpful for digestion, absorption and excretion. Antioxidant peptides have lower molecular weights, higher levels of stability and no-risk absorption compared to synthetic antioxidants (Liu, Kong, Xiong, & Xia, 2010). These absorbable short peptides are able to reach peripheral organs and have beneficial effects via transportation through the blood

* *Corresponding author*. Department of Biotechnology, School of Bioengineering, SRM University, Kattankulathur, Chennai 603 203, Tamilnadu, India. Tel.: +091 09444239282; fax: +91-44-2475 2343.
E-mail address: nazeer.ra@ktr.srmuniv.ac.in (R.A. Nazeer).
http://dx.doi.org/10.1016/j.jff.2015.02.028
1756-4646/

(Yust et al., 2003). These peptides inhibit oxidation through different mechanisms, such as free radical scavenging, the chelation of pro-oxidative transition metal ions, the reduction of hydroperoxides and the disabling of reactive oxygen species (ROS) (Hook, Burton, Yasothornsrikul, Hastings, & Deftos, 2001).

The efficacies of bioactive peptides were analyzed based on considerations such as the source of protein, degree of hydrolysis, peptide structure, amino acid composition and proteolytic nature of the enzymes (Memarpoor-Yazdi, Mahaki, & Zare-Zardini, 2013). Enzymatic hydrolysis is an effective method of releasing the antioxidant peptides from edible and non-edible parts of organisms via proteolytic enzymes. According to Dekkers, Raghavan, Kristinsson, and Marshall (2011), in the fish processing industries, more than 60% of the parts of fish are considered waste (e.g., head, skin, fins and visceral mass), and 40% of fish products are edible. This edible portion is rich in protein and is considered to provide essential nutrients that support human health after proteolysis in the gastrointestinal tract.

Recently, antioxidant peptides have been reported to be present in different food protein hydrolysates (e.g., hydrolysates from plants, animals, marine animals and microbes). Cephalopods, such as squid, are abundantly present in the Bay of Bengal and are economically valuable (Quader, 1994). In the present study, the edible part (mantle) of the Indian squid *Loligo duvauceli* was considered to be a source from which to isolate the peptide. This study was designed to resolve the mysteries of the antioxidant peptide after consumption of the mantle of the squid and digestion by gastrointestinal enzymes in the human body, and it can be used as a model for the same mechanism that occurs in our daily lives. For this purpose, the scavenged radicals from oxidative stress were used as an *in vitro* model at the cellular level.

2. Materials and methods

2.1. Materials

Pepsin, α-chymotrypsin, trypsin, α-tocopherol, butylated hydroxytoluene (BHT), sodium phosphate, 2, 2-diphenyl-1 picrylhydrazyl (DPPH), foetal bovine serum (FBS), DMPO (5,5-dimethyl-1-pyrroline N-oxide), penicillin–streptomycin, trypsin–EDTA, Dulbecco's phosphate-buffered saline (DPBS) and 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade.

2.1.1. Sample collection

The Indian squid *L. duvauceli* was collected from the Royapuram (13°6′26″N, 80°17′43″E) coastal area, Chennai, Tamilnadu, India. The squid mantle was dissected and separated and then stored at –20 °C for further use.

2.2. Methods

2.2.1. Proximate analysis

The proximate (i.e., moisture, ash, protein and lipid) composition of the squid *L. duvauceli* mantle was determined on a wet weight basis. The moisture content was determined with 5 g of mantle that were placed in a preheated aluminum dish and then dried in an oven at 100 °C for up to 48 h. The ash content was calculated by burning the pre-dried sample in a crucible at 420 °C for up to 3 h (AOAC, 1995). The crude protein content in the raw sample was determined by the Kjeldal method (AOAC, 1995) and a conversion factor of 6.25 was used. The lipid content was determined by the Bligh and Dyer (1959) method.

2.2.2. Preparation of squid mantle protein hydrolysates by enzymatic hydrolysis

The hydrolysis of the Indian squid mantle was performed according to the method described by Je, Qian, Byun, and Kim (2007). The enzymatic hydrolysis was accomplished according to the physical conditions of the mantle with trypsin, α-chymotrypsin (0.1 M sodium phosphate buffer, pH 8.0 at 37 °C) and pepsin (0.1 M glycine buffer, pH 2.0 at 37 °C) in the enzyme and sample concentration at a ratio of 1:100 (w/w). This mixture was stirred for 12 h at 37 °C and the temperature and pH were checked and maintained every 30 min. The sample was collected every hour up to 12 h (including the 0th h) and then heated to inactivate the enzyme for 10 min at boiling temperature. The hydrolysates were separated by centrifugation at 8000 *g* for 10 min and the supernatants were then lyophilized and stored at –20 °C until use.

2.2.3. Degree of hydrolysis (DH)

The percentages of the cleaved peptide bonds of the protein hydrolysates were determined by the degree of hydrolysis with the ophthalmic aldehyde (OPA) method (Wanasundara, Amarowicz, Pegg, & Shand, 2002) based on the free amino groups that were released upon hydrolysis. The OPA reagent was comprised of 6 mM OPA (first dissolved in 95%, v/v, ethanol) and 5.7 mM DL-dithiothreitol in 0.1 M sodium tetraborate containing 2% (w/v) SDS. The squid mantle (0.4 ml) was mixed with 3 ml of OPA reagent and incubated for 20 min at room temperature before measuring the absorbance at 340 nm using a spectrophotometer. The amounts of the free amino groups in the squid hydrolysates were measured with L-serine as a standard. The total numbers of amino groups in the squid mantle proteins were determined by acid hydrolysis using 6 M HCl at 110 °C for 24 h. It was assumed that complete hydrolysis of all of the peptide bonds in the proteins in the sample occurred during acid hydrolysis.

$$DH\,\% = [((NH_2)_{Tx} - (NH_2)_{T0})/((NH_2)_{Total} - (NH_2)_{T0})] \times 100$$

where,

$(NH_2)_{T0}$ = the amount of free–NH_2 groups at the 0th minute of hydrolysis (mg/ml)

$(NH_2)_{Tx}$ = the amount of free–NH_2 groups in the supernatant after x min of hydrolysis for each experimental point (mg/ml)

$(NH_2)_{Total}$ = the amount of NH_2 groups from acid hydrolysis (mg/ml)