Food Chemistry 141 (2013) 2177–2183

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Biologically active peptides obtained by enzymatic hydrolysis of Adzuki bean seeds

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ARTICLE INFO

Article history:
Received 13 December 2012
Received in revised form 29 April 2013
Accepted 4 May 2013
Available online 14 May 2013

Keywords:
Biologically active peptides
Adzuki bean
Hydrolysates

ABSTRACT

This study investigated the antioxidant and antihypertensive activities of peptides obtained from protein fractions of Adzuki bean seeds. Peptides were obtained by the use of hydrolytic enzymes *in vitro* under gastrointestinal conditions. A determination was made of the activity of the peptide inhibitors of the angiotensin I converting enzyme (ACE), and the antiradical and ion chelating activity of peptides from different protein fractions. The highest peptide levels after the absorption process (<7 kDa) were noted in the albumin fraction (50.69 μg/ml). Furthermore, it was found that peptides from the prolamin fraction were characterised by the highest antiradical activity and ACE inhibitory activity (IC_{50} = 0.17 mg/ml). Peptides obtained from the globulin fraction showed the highest ability to chelate iron ions, and peptides from the glutelin fraction were characterised as being the most effective in the chelation of copper ions.

1. Introduction

In recent years, researchers have focused on the search for natural food components with potential prophylactic and therapeutic effects. In addition to their nutritional value, biologically-active peptides derived from food have a physiological, hormone-like effect on the human body. They are found in milk, eggs, meat and fish of various kinds as well as in many plants. Bioactive peptides are inactive within the sequence of their parent protein and can be released by enzymatic hydrolysis (Hartman & Meisel, 2007). Furthermore, some proteins have a lower functional activity before digestion, than peptides released from them during digestion by the action of hydrolytic enzymes (Korhonen, Pihlanto-Leppälä, Rantamäki, & Tupasela, 1998). Enzymatic hydrolysis is the main process that allows biologically-active peptides to be obtained from food products. It is also known to improve the functional properties of dietary protein without affecting its nutritive value by converting it into peptides with the desired size, charge and surface properties (Moure, Sineiro, Dominguez, & Parajo, 2006). More recently, it has been recognised that many peptides that are released *in vitro* or *in vivo* from animal or plant proteins are bioactive and have regulatory functions in humans beyond normal and adequate nutrition (Hartman & Meisel, 2007; Korhonen, 2009).

Biological peptides with antihypertensive activity via the inhibition of the angiotensin I-converting enzyme (ACE; peptidyldipeptide hydrolase; EC 3.4.15.2) are a well-known group of bioactive compounds from food (Ondetti, Rubin, & Cushman, 1997). Antihypertensive peptides are the most commonly occurring bioactive peptides in foods. They demonstrate their activity by inhibiting the angiotensin-converting enzyme. ACE is a nonspecific dipeptidyl carboxypeptidase associated with the regulation of blood pressure via modulation of the rennin-angiotensin system. This enzyme converts decapeptide angiotensin I into the potent vasoconstricting octapeptide angiotensin II, which leads to an increase in blood pressure. Therefore, inhibition of the ACE results in an antihypertensive effect (Shin et al., 2001).

Furthermore, many peptides have an antiradical activity. Due to the close relationship between oxidative stress and diseases, control of oxidative stress seems to be one of the crucial steps in slowing down the progress of these diseases or preventing complications arising from them. In this regard, a vast number of antioxidants have been isolated and identified from natural sources to control oxidative stress. Besides various well-known natural antioxidants, such as vitamin C, polyphenols, flavonoids, carotenoids or peptides (Wang et al., 2008), transition metal ions, such as Cu^{2+} and Fe^{2+}, can catalyse the generation of reactive oxygen species, resulting in lipid peroxidation and DNA damage. Therefore, the chelation of transition metal ions by antioxidants or antioxidative peptides will retard the oxidation reaction (Stohs & Bagchi, 1995). The antioxidative activity of peptides also results in their ability to chelate metal ions that catalyse the oxidation. The presence of transition metal ions, including Fe^{2+}, plays an important role in the catalysis of oxidative processes leading to the formation of hydroxyl radicals. The main source of hydroxyl radicals in the Fenton reaction is the body, in which the catalysts are iron and copper cations: $H_2O_2 + Fe^{2+} = OH^0 + OH^- + Fe^{3+}$. Therefore, it is important that peptides show a high ability to chelate

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http://dx.doi.org/10.1016/j.foodchem.2013.05.012

transition metal ions in order to prevent the formation of hydroxyl radicals (Gawlik-Dziki & Kowalczyk, 2007).

Pulses (pea, bean, lentil and chickpea) are the seeds of legumes consumed as food in many countries. They are good health-promoting foods and nutritionally good sources of carbohydrates, protein, vitamins and minerals. Additionally, the low fat content and low glycemic index supports their consideration for inclusion in a healthy diet (Świeca, Baraniak, & Gawlik-Dziki, 2013). Regular dietary intake of pulses has been linked with a reduced risk of developing certain diseases, particularly diabetes, cancer and cardiovascular disease (CVD) (Roy, Boye, & Simpson, 2010). One type of bean is Adzuki – a small red bean (*Vigna angularis*) and a popular ingredient in many confectionary products in the orient; moreover, it is becoming increasingly popular in European countries and is used not only in its seed form but also in the form of sprouts. The seeds are also a valuable dietary component, as they contain about 55% of starch, 0.45% of fat and 25% of protein. They are characterised by high levels of lysine and in addition, they are rich in mineral elements. The sprouts are an excellent source of folic acid, vitamin A and vitamin B (Yousif, Deeth, & Caffin, 2002). Studies have shown that Adzuki bean extracts with antioxidant activity attenuate the elevation of blood pressure in an animal model of hypertension (Sato et al., 2008). It has been proved that the seed extract, which is a waste product, also contains active substances. Han et al. (2004) reported that the extract may inhibit the liver damage caused by the use of paracetamol. The extract in 40% ethanol shows an inhibitory effect on the proliferation of gastric cancer. Summing up these facts, both the bean seeds and the water extract of Adzuki bean seeds formed during cooking are valuable sources of active compounds, the properties of which are being systematically studied.

The aim of this study was to evaluate individual protein fractions isolated from the Adzuki bean in terms of their antihypertensive activity, antioxidant activity and their ability to chelate the iron and copper of peptides released during digestion *in vitro*.

2. Materials and methods

2.1. Materials

The experimental material consisted of Adzuki bean (*V. angularis*) dry seeds acquired in Seed Company in Jutrosinie. ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid), DPPH (2,2-diphenyl-1-(2,4,6-triphenyl-hydrazyl), TNBS (picrylosulfonic acid), HHL (N-hippuryl-His-Leu), OPA (o-phtaldialdehyde), ferrozine (sodium salt of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazolidynic acid), pyrocatechol, α-amylase from hog pancreas (50 U/mg), pepsin from porcine gastric mucosa (250 U/mg), pancreatin from porcine pancreas, bile extract, β-mercaptoethanol, PBS (phosphate buffered saline) were purchased from Sigma–Aldrich Company, USA. All other chemicals were of analytical grade.

2.2. Protein sequential fractionation based on solubility criteria

Dry bean seeds were soaked for 12 h in distillate water next boiled for 20 min. The grounded seeds were used to extract of protein with 0.1 M borate buffer, pH 8.3, 1:30 (w/v) for 60 min at room temperature. After this process protein fractions (albumins, globulins, prolamins and glutelins) were isolated on the basis of solubility criterion according to the method Ribeiro, Teixeira, and Ferreira (2004) with slight modification. Albumins, globulins, prolamins and glutelins from Adzuki bean seeds proteins were subsequently sequentially extracted and purified using appropriate extraction solutions. The albumins were extracted in water containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (30 mg/g fresh mass). The insoluble proteins were removed by centrifugation at 9000*g* and 4 °C for 30 min. Obtained supernatant accounted albumin fraction. For globulin extraction, the pellet was resuspended in 100 mM Tris–HCl buffer, pH 7.5, containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), (30 ml/g of dry precipitate mass). The solubilised globulins were obtained by centrifugation at 9000*g* and 4 °C for 30 min. Obtained supernatant accounted globulin fraction. For prolamin extraction, obtained pellet was suspended in methanol, then it was shaken for 1 h. After centrifugation at 9000*g* and 4 °C for 30 min, the prolamin fraction was obtained. The procedure was sequentially repeated to obtain glutelin fraction from the pellet containing the insoluble material. The pellet was resuspended in 50 mM Tris–HCl buffer, pH 10, containing (v/v) β-mercaptoethanol and 1% (w/v) SDS (5 ml/g of dry precipitate mass). The suspension was stirred at room temperature (to keep SDS in soluble form) for 30 min. and centrifuged at 9000*g* for 30 min. All samples were lyophilised and kept at −18 °C until use.

After fractionation samples containing different protein fractions were lyophilised and stored at −18 °C until further use.

2.3. Size-exclusion high performance liquid chromatography

The protein fractions were characterised by SE-HPLC 232 using a Varian ProStar HPLC System separation module (Varian, Palo Alto, USA) equipped with a column (ProSEC 300s Column 7.5 mm ID × 300 mm). The column thermostat was set at 30 °C. The amount of 10 μl of each sample solution was loaded on the column, and protein and peptides were eluted using a 20 mM PBS buffer pH 7.4. The flow rate was 0.4 ml min^{-1}. Ultraviolet detection was performed at a wavelength of 280 nm. The molecular mass of protein fraction was calculated with an external standard (Peptide Molecular Weight Marker, Sigma–Aldrich; SigmaMarker™ Low Range, Sigma–Aldrich) using comparison of retention times.

2.4. In vitro digestion and absorption process

In vitro digestion of protein fractions was carried out according to the method described by Gawlik-Dziki et al. (2012) with slight modification. Briefly, the lentil globulins (4%, w/v) were resuspended in stimulated saliva solution with final concentration 7 mM $NaHCO_3$ and 0.35 mM NaCl, pH 6.75 and stirred for 5 min at 37 °C in darkness. After that, α-amylase (50 U/mg) was added (the ratio of enzyme to substrate was 1:10; w/w) and the mixture was stirred for 10 min at 37 °C in darkness. For the gastric digestion, solution was adjusted to pH 2.5 with 1 M HCl and pepsin (250 U/mg) was added (the ratio of enzyme to substrate was 1:100; w/w). Reaction was carried out for 2 h at 37 °C. Solution was neutralised to pH 7.0 with 1 M NaOH and successively a mixture containing 0.7% solution of pancreatin and 2.5% solution of bile extract (1:2.5, v/v) were added (simulated intestinal digestion). The incubation was carried out for 1 h at 37 °C in darkness and reaction was stopped by heating at 100 °C for 5 min.

Hydrolysates were dialysed with membrane tube (molecular weight cut-off 7 kDa) against phosphate buffered saline (PBS) at the physiological concentration, pH 7.4 (1:4, v/v). Absorption process was carried out without light for 1 h at 37 °C. After this stage, the samples were lyophilised and stored at −18 °C until further use.

2.5. Determination of peptide concentration

The concentration of peptides was determined by the trinitrobenzenesulphonic acid (TNBS) method using L-leucine as the standard (Adler-Nissen, 1979). All assays were performed in triplicate.