Food Chemistry 114 (2009) 38–43

Contents lists available at ScienceDirect

Food Chemistry

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

Antioxidative and ACE inhibitory activities in enzymatic hydrolysates of the cotton leafworm, *Spodoptera littoralis*

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ARTICLE INFO

Article history:
Received 23 June 2008
Received in revised form 27 August 2008
Accepted 2 September 2008

Keywords:
Antioxidant
Bioactive peptides
ACE inhibition
Insect

ABSTRACT

The larvae of the cotton leafworm, *Spodoptera littoralis*, were used as a source of food proteins exerting possible biological activities. A simulated gastrointestinal digestion (IC_{50} = 320 μg/ml) and digestion by mucosal enzymes (IC_{50} = 211 μg/ml) reveals a significantly higher *in vitro* ACE inhibitory activity compared to hydrolysis using thermolysin (IC_{50} = 1392 μg/ml) and alcalase (IC_{50} = 827 μg/ml) as pretreatment. This indicates that the choice of enzymes to generate ACE inhibitory peptides is important. All hydrolysates were also tested for antioxidant activity using two tests: a radical scavenging test using DPPH and the ferric reducing antioxidant power (FRAP) assay, and they showed a similar antioxidant activity which was relatively low compared to the standard antioxidants BHT and vitamin C. As a conclusion, the data obtained suggest that insect protein can be used to generate hydrolysates, exerting both ACE inhibitory and antioxidant activity, which might be incorporated as multifunctional ingredient into functional foods.

1. Introduction

Over the last century, the definition of health has no longer been restricted to the absence of disease, but includes physical fitness as well as mental and physiological well-being. For the development, growth and maintenance of the body, food is required, but food is also recognised to play a key role in the quality of life. Functional foods are those foods that positively affect one or more target functions in the body, beyond the basic nutritional function, in a way that it is relevant to either an improved state of health and well-being and/or reduction of risk of disease (Arvanitoyannis & van Houwelingen-Koukaliaroglou, 2005). One of the components of functional foods may be bioactive peptides, as they exert a physiological effect in the body (Shahidi & Zhong, 2008). These peptides are short chains of amino acids that are inactive within the sequence of parent protein but can be released during gastrointestinal digestion or food processing. A wide range of biological activities has been ascribed to peptides such as antihypertensive (ACE inhibitory), antithrombotic, antioxidative, immunomodulating, opioid, antimicrobial, anticarcinogenic or mineral binding activity (Hartmann & Meisel, 2007; Kitts & Weiler, 2003; Korhonen & Pihlanto, 2003). Moreover, some protein hydrolysates and peptides have an extra advantage of being multifunctional, as they initiate two or more different biological activities (Meisel, 1997). For example, multifunctional hydrolysates exerting both ACE inhibitory and antioxidant activities have been reported in a commercial fermented milk in Europe (Hernandez-Ledesma, Miralles, Amigo, Ramos, & Recio, 2005b), in egg white protein hydrolysates (Davalos, Miguel, Bartolome, & Lopez-Fandino, 2004), and in extracts prepared from chum salmon cartilage and skin (Nagai, Nagashima, Abe, & Suzuki, 2006). In addition, Davalos et al. (2004) identified a multifunctional peptide, namely Tyr-Ala-Glu-Glu-Arg-Tyr-Pro-Ile-Leu, which shows strong ACE inhibitory activity and high radical scavenging activity. Furthermore, the synthetic ACE inhibitor captopril also exerts antioxidant activity (Gurer, Neal, Yang, Oztezcan, & Ercal, 1999). The combination of ACE inhibition and antioxidant activity in one multifunctional preparation could be very useful for the control of cardiovascular diseases.

Angiotensin converting enzyme (ACE) is a key enzyme in the regulation of blood pressure in humans; ACE catalyses the hydrolysis of the inactive decapeptide angiotensin I to the potent vasoconstrictor angiotensin II, an octapeptide (Coates, 2003). Moreover, ACE cleaves bradykinin, a vasodilator, into inactive fragments. The result is an increase in blood pressure (Campbell, 2003). Synthetic ACE inhibitors have been developed as antihypertensive medicine but the use of such inhibitors can cause serious side effects (Antonios & Macgregor, 1995). The search for natural

Abbreviations: ACE, angiotensin converting enzyme; BHT, butylated hydroxytoluene; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FRAP, ferric reducing antioxidant power; HHL, hippuryl-L-histidyl-L-leucine; OPA, *o*-pthaldialdehyde; TPTZ, 2,4,6-tris-(2-pyridyl)-S-triazine.

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doi:10.1016/j.foodchem.2008.09.011

alternatives has resulted in the identification of numerous ACE inhibitory peptides derived from various food sources. Food proteins from vegetable (such as soybean and maize) and animal (such as milk, porcine and beef muscle) origin are reported as good sources of ACE inhibitory peptides (Korhonen & Pihlanto, 2006).

Antioxidants may have a positive effect on human health as they can protect our body against damage by reactive oxygen species (ROS), which attack membrane lipids, protein and DNA and play an important role in many diseases such as cardiovascular diseases, diabetes mellitus, cancer and Alzheimer (Aruoma, 1998; Valko et al., 2007). In addition, lipid peroxidation is a great concern to the food industry as deterioration of food quality has been identified due to oxidation of lipids and formation of secondary lipid peroxidation products. The use of synthetic antioxidants is under strict regulation due to the potential negative health effects caused by such compounds. The substitution of synthetic antioxidants by natural ones is gaining interest due to these health concerns and due to consumers' preferences. Antioxidant activity has been demonstrated in various hydrolysates, e.g. the peptide digests of jumbo squid gelatin (Mendis, Rajapakse, Byun, & Kim, 2005), chum salmon cartilage (Nagai et al., 2006), Alaska pollack skin (Kim et al., 2001), porcine muscle (Saiga, Tanabe, & Nishimura, 2003), soybean (Moure, Dominguez, & Parajo, 2006) and milk (Hernandez-Ledesma, Davalos, Bartolome, & Amigo, 2005a).

In this research, we used the larvae of the cotton leafworm, *Spodoptera littoralis*, as a source of food protein exerting possible biological activities. As insects/invertebrates possess enormous biodiversity and represent a large biomass (95% of the animal kingdom), they offer a tremendous potential as natural resources for new bioactive peptides. Although in the Western world, there is a negative attitude towards the use of insects as food, in many parts of the world insects are part of the daily diet and may be the only source of animal protein. Insects are consumed in Latin America, Africa and Asia (DeFoliart, 1999). Over 2000 different edible insect species have been recorded (Ramos-Elorduy et al., 1997). Furthermore, insect cells have been suggested as alternative for meat production considering the growing population and increase in welfare worldwide (Verkerk, Tramper, van Trijp, & Martens, 2007). We evaluated the effect on the *in vitro* ACE inhibitory activity of a simulated gastrointestinal digestion and digestion by mucosal enzymes of the protein of *S. littoralis*. In addition, the effect on *in vitro* ACE inhibitory activity of a pretreatment of insect protein with two microbial enzymes; thermolysin or alcalase, was studied. Furthermore, the antioxidant capacity of all hydrolysates was evaluated using two techniques: a radical scavenging test using DPPH and the ferric reducing antioxidant power (FRAP).

2. Materials and methods

2.1. Products

Hippuryl-L-histidyl-L-leucine (HHL), ACE (from rabbit lung), *o*-pthaldialdehyde (OPA), sodium dodecyl sulphate (SDS), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,4,6-tris-(2-pyridyl)-S-triazine (TPTZ), pepsin, trypsin, α-chymotrypsin, alcalase, thermolysin and peptidases from porcine intestinal mucosa were purchased from Sigma–Aldrich (Bornem, Belgium). Iodoacetic acid and ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) was purchased from Janssen Chimica (Beerse, Belgium). NaCl was purchased from Chem-Lab (Zedelgem, Belgium).

2.2. Insect breeding

S. littoralis (Lepidoptera) was raised as described previously (Smagghe, Carton, Heirman, & Tirry, 2000). In brief, all stages of a continuous colony of the cotton leafworm *S. littoralis* were maintained under standard conditions of 23 ± 1 °C, 70 ± 5% RH, and a light:dark (16:8) photoperiodic regimen. The larvae were fed an agar-based artificial diet and adults were fed a 20% honey water solution. Sixth instar larvae were collected for our study.

2.3. Protein extraction

Larvae were homogenised in extraction medium (150 mM NaCl; 0.01 mM iodoacetic acid) with an ultra turrax blender. The homogenate was centrifuged at 1500*g*, at 4 °C for 10 min. The supernatant was discarded and the pellet was resuspended in extraction medium followed by centrifugation (1500*g*, at 4 °C for 10 min). This was repeated twice where after the pellet was lyophilized.

2.4. Enzymatic hydrolysis

The pellet obtained after protein extraction was hydrolysed using various enzymes. Alcalase and thermolysin were used as follows: digestion with alcalase (E/S: 48 U/kg) was done for 3 h at 55 °C and pH 8; whilst digestion with thermolysin (E/S: 1/1600) was done for 5 h at 37 °C and pH 8. To simulate the human gastrointestinal digestion process, subsequent hydrolysis with pepsin, trypsin and α-chymotrypsin was conducted. The digestion in the stomach was simulated by lowering pH to 2 with HCl (4 M), adding pepsin (E/S: 1/250) and incubating for 2 h at 37 °C. Trypsin (E/S: 1/250) and α-chymotrypsin (E/S: 1/250) at pH 6.5 (with NaOH, 10 M) and incubation for 2.5 h at 37 °C, simulated the small intestine phase (Vercruysse, Smagghe, Herregods, & Van Camp, 2005a). The porcine mucosal peptidases contain general proteolytic and aminopeptidase activity similar to the enterocytes in the human body. Hydrolysis with these mucosal peptidases was done during 2 h at 37 °C with an enzyme to substrate ratio of 1/500. In this study, different combinations of hydrolysis were used: gastrointestinal digestion, gastrointestinal digestion followed by digestion with mucosal peptidases, digestion with thermolysin, digestion with thermolysin followed by gastrointestinal digestion, digestion with thermolysin followed by gastrointestinal digestion followed by digestion with mucosal peptidases, digestion with alcalase, digestion with alcalase followed by gastrointestinal digestion, digestion with alcalase followed by gastrointestinal digestion followed by digestion with mucosal peptidases.

2.5. Determination of peptide concentration

A spectrophotometric assay using *o*-pthaldialdehyde (OPA) was used for determination of the peptide concentration of the hydrolysates. The OPA reagent (2 ml, 0.8 mg/ml), prepared as described by Church, Swaisgood, Porter, and Catignani (1983), was mixed with 50 μl of sample. After 2 min incubation at room temperature, the absorbance was measured at 340 nm. For calculation of the peptide concentration, a standard curve of casein peptone was used.

2.6. In vitro ACE inhibitory activity

The *in vitro* ACE inhibitory activity was measured using the method of Chang, Chen, Huang, and Chang (2001) with some slight modifications. The method is based on a selective chromogenic reaction for histidyl-leucine with *o*-phaldialdehyde. In brief, the sample, ACE (40 mU/ml) and Hip-His-Leu (15 mM) were mixed and incubated for 2 h at 37 °C. Then, the enzymatic reaction is stopped and the chromogenic reaction is started by adding 2 ml of alkaline OPA reagent. After 20 min of incubation at 25 °C, the absorbance is measured at 390 nm. The IC_{50} value was determined as described previously (Vercruysse et al., 2005a).