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Exploitation of starch industry liquid by-product to produce bioactive peptides from rice hydrolyzed proteins

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ABSTRACT

Small peptides show higher antioxidant capacity than native proteins and may be absorbed in the intestine without further digestion. In our study, a protein by-product from rice starch industry was hydrolyzed with commercial proteolytic enzymes (Alcalase, Neutrase, Flavourzyme) and microbial whole cells of *Bacillus* spp. and the released peptides were tested for antioxidant activity. Among enzymes, Alcalase was the most performing, while microbial proteolytic activity was less efficient. Conversely, the antioxidant activity was higher in the samples obtained by microbial hydrolysis and particularly with *Bacillus pumilus* AG1. The sequences of low molecular weight antioxidant peptides were determined and analyzed for aminoacidic composition. The results obtained so far suggest that the hydrolytic treatment of this industrial by-product, with selected enzymes and microbial systems, can allow its exploitation for the production of functional additives and supplements rich in antioxidant peptides, to be used in new food formulas for human consumption.

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1. Introduction

Rice is the staple diet for about half of the world's population, mostly in Asian countries, providing 35–59% of caloric needs (FAO Report, 2004). It is one of the leading food crops in the world, and in recent years the annual production of milled-rice worldwide is about 460 MMT (Shih, 2012). Accordingly, by-products from the milling of rice, such as bran, broken kernels, bran oil, wax and hulls, are plentiful and readily available. These by-products are valuable sources of food ingredients, but they are currently under-exploited. In addition to starch, which accounts for more than 90% of the kernel mass, other components including proteins, lipids, carbohydrates and small amounts of minerals and vitamins are potentially valuable sources of food ingredients. Although milled-rice is normally sold as premium product, its by-products generally have been underexploited. For instance, rice bran and broken rice are mostly used for animal feed or for the production of alcoholic beverages, starch syrups or powders. In particular broken rice, an unavoidable by-product during milling operations, contains about 80% of starch and 8% of proteins and can be used by starch industry to extract not only powder and crystal starch but also proteins as main by-product (Shih, 2012). Proteins, obtained from rice endosperm by alkaline extraction followed by precipitation at their isoelectric pH (Paraman, Hettiarachchy, Schaefer, & Beck, 2007), are known to have nutritional, hypoallergenic and healthy properties and to be valuable as source for human consumption (Chen Y.J., Chen, Wu, Yu, & Liao, 2010; Fiocchi, Riva, Terracciano, Bernardo, & Riva, 2003).

During the past decades, a great deal of work has been done on protein extraction from rice bran (Guo, Zhang, Ma, & Tian, 2012; Zhang et al., 2009, 2010), but few information is available on protein production from rice by-products deriving from starch and other carbohydrates extraction processes. Currently, these protein residues are normally used as animal feed because of their poor solubility.

Recently, interest has been emerging to identify and characterize bioactive peptides from plant and animal sources. Proteolytic enzyme modification is an efficient approach to improve the functional properties of food proteins. Moreover the greatest advantages of using microorganisms or their enzymes for food processing and for industrial production of bio-based products are their environmental friendliness and consumer acceptance as being a natural process. In particular, antioxidant peptides from foods are considered to be safe and healthy compounds with low molecular

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weight, low cost, high activity and easy absorption. Antioxidant peptides contain 5–16 amino acid residues (Chen, Muramoto, & Yamauchi, 1995). A detailed description of their specific characteristics, enzymatic production, methods to evaluate antioxidant capacity, bioavailability and safety concerns has been reviewed (Bahareh & Sarmadi, 2010). Zhang et al. (2010) reported various studies aimed at investigating the antioxidant peptides derived from the enzymatic hydrolysis of several vegetal and animal protein substrates. Furthermore, Coda, Rizzello, Pinto, and Gobetti (2012) demonstrated the capacity of sourdough lactic acid bacteria to release peptides with antioxidant activity through the proteolysis of native cereal proteins.

Studies dealing with the use of proteolytic enzymes for extraction or modification of proteins from rice materials are limited. Paraman et al. (2007) studied the application of acidic, neutral and alkaline type proteases to hydrolyze rice endosperm proteins. The results showed that the solubility and emulsifying properties of the proteins were enhanced by controlled enzymatic hydrolysis. Zhang et al. (2010) investigated the antioxidant activities of protein hydrolysates from rice endosperm and Neutrase was chosen to be the optimum enzyme to obtain antioxidant peptides. In addition, the alkaline serine proteases, which are active in a pH ranging from neutral to alkaline, are the most important group of enzymes exploited commercially since they are produced in substantial amounts as extracellular enzymes (Gupta, Beg, & Lorenz, 2002).

Generally, alkaline proteases produced from microorganisms are constitutive or partially inducible in nature and, under most culture conditions, *Bacillus* species produce extracellular proteases during post-exponential and stationary phases. The proteolytic system of *Bacillus* contributes to the liberation of bioactive peptides that play a significant role in the enzymatic process involved in many traditionally fermented foods.

In particular, in South East Asian countries such as China, Japan and Korea, microbial fermentation is used widely as the oldest way to preserve food. It is believed that fermentation can increase the nutraceutical value of foods, besides the long storability, possibly due to fragmentation of proteins to bioactive peptides by microbial proteases (Rajapakse, Mendis, Jung, Je, & Kim, 2005). Although the great part of commercial enzymes are obtained by *Bacillus* species and their probiotic characteristics, no many scientifically approached studies are available on nutraceutical effects of their use as whole cells in food hydrolytic processes. On the other hand some traditionally fermented soybean and legumes in Asia and Africa are based on *Bacillus* starter cultures composed by mixed populations of different *Bacillus* species such as *B. subtilis*, *B. laterosporus*, *B. pumilus*, *B. brevis*, *B. macerans*, *B. licheniformis*, *B. polymyxa*, and *B. coagulans* (Ogbadu & Okagbue, 1990). More recently, nutritional characteristics of shrimp hydrolysates by crude protease isolated from *Bacillus* spp. SM98011 were also studied for their content in peptides with molecular mass lower than 3 kDa that have high ACE inhibitory activity and hydroxyl radical scavenger activity (He, Chen, Sun, Zhang, & Gao, 2006). These low molecular mass peptides represented the best candidates for functional properties, including antioxidant properties.

The aim of this work was to demonstrate the potential exploitation of the hydrolyzed proteins of starch industry by-product to produce functional antioxidant peptides both with commercial proteolytic enzymes and whole bacterial cells of *Bacillus* spp.

2. Materials and methods

2.1. Materials

The rice proteins (RP) were obtained as by-product of the starch extraction industrial process from the local company Amideria Il Cervo S.r.l. (Monterenzio, Bologna, Italy).

Reagents were analytical grade from Sigma (St. Louis MO, USA), Merck (Darmstadt, Germany) and Oxoid (Basingstoke, Hampshire, England). Reagents, standards and equipment for electrophoresis were from Bio Rad Laboratories S.R.L. (Segrate, Milano, Italy). The solvents for mass spectrometry analysis were from Carlo Erba (Milano, Italy).

All the enzymes were purchased from Sigma (Sigma Chemical Co., St. Louis MO, USA). Alcalase® 2.4 L is an endopeptidase from *Bacillus licheniformis*, with Subtilisin A as major enzyme component and a specific activity of 2.4 Anson Unit (AU) per gram. Neutrase® 0.8 L is a bacterial protease produced by submerged fermentation of a selected strain of *Bacillus amyloliquefaciens* and a specific activity of 0.8 Anson Unit (AU) per gram. Flavourzyme® 500 U/g is a fungal protease complex produced by submerged fermentation of a selected strain of *Aspergillus oryzae* and contains both endoprotease and exopeptidase activities. One unit hydrolyzes 1 μmol of L-leucine-p-nitroanilide per minute

2.2. Hydrolysis with commercial enzymes and whole cell microbial suspensions

Ten g of RP were dissolved in 100 mL of 0.1% NaOH under stirring conditions for 2 h at room temperature. Final concentration of RP was 1.8 mg/mL. Alcalase 2.4 L, Neutrase 0.8 L and Flavourzyme 500 U/g were used to hydrolyze aliquots of RP above described (1 unit/g of sample). Hydrolysis conditions were: Alcalase, pH 8 and 55 °C; Neutrase, pH 6.5 and 40 °C; Flavourzyme, pH 6 and 60 °C. Reactions were carried out for 2 h and then stopped by boiling in a water bath for 10 min to inactivate the proteases. Samples were maintained at −80 °C until used for further analyses.

The strains of *B. subtilis* (SV27, SV20I), *B. pumilus* (AGI) and *B. licheniformis* (AG2), used for the whole cell suspension hydrolysis, were isolated from different food sources, and belong to the collection of the Department of Agricultural and Food Sciences & Technologies (DISTAL) of Alma Mater University of Bologna. The strains were cultivated in PC broth (5 g/L universal peptone, 2.5 g/L yeast extract, 1 g/L glucose) at 37 °C for 48 h. For enzymatic assays, 5 mL of PC broth were inoculated with a suspension at late exponential phase ($OD_{600} = 0.25$) of the selected microorganism. This was designed as whole-cell suspension (Sanz et al., 1999).

Substrate consisting in 1 mL of the RP and 0.2 mL of the whole-cell suspension, in order to reach an initial cell load of about 10^3, were kept in contact for 72 h. At the end of the period of incubation cell load in the substrate was around 3×10^7. The hydrolysates were then centrifuged at 8000 rpm for 5 min and supernatants maintained at −80 °C until their use for further analyses.

2.3. Freeze-drying of samples

RP-solution and all the other samples obtained after hydrolysis were freeze-dried under vacuum at a temperature of −56 °C, for 48 h, in a Heto Power Dry LL 3000 (Thermo Electron Corporation, Erie, PA, United States), and then maintained at −80 °C until use.

2.4. Determination of the antioxidant activity

The antioxidant activity of all the samples as determined by the ABTS (2,2′-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid) method (Re et al., 1999) optimized for cereal samples as in Ferri, Gianotti, and Tassoni (2013). The addition of antioxidant compounds reduces the ABTS cations, thus causing a reagent decolourization that is measurable spectrophotometrically, depending on the antioxidant type and concentration and on the reaction time (Ferri et al., 2013; Huang, Ou, & Prior, 2005; Prior, Wu, & Schaich, 2005; Re et al., 1999). ABTS stock solution was prepared at the concentration of 7 mM in 2.45 mM $K_2S_2O_8$ and the working solution

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