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Preparation of a rice bran enzymatic extract with potential use as functional food

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Abstract

The production, stabilization, by enzymatic treatment, physicochemical composition, and biological properties (including the anti-proliferative activity), of a water-soluble rice bran enzymatic extract (RBEE) are described. The main component of RBEE is proteins (38.1%) – in the form of peptide and free amino acids – having a 6% content of sulfur amino acids. The second component is fat (30.0%), with oleic and linoleic acids as the major components, and 1.2 mg/g of γ-oryzanol. Carbohydrates (14.2%) are comprised mainly of slowly absorbed carbohydrates. Preliminary studies on the anti-proliferative effect of RBEE on leukemia tumor cell growth in vitro are also reported. This property makes RBEE potentially useful as a functional food for the treatment and prevention of chronic pathological states associated with abnormal proliferation of cells, as is the case with cancer.

Keywords: Rice bran; Rice bran enzymatic extract; Lipase inactivation; Protease; γ-Oryzanol; Antioxidants; Anti-tumor

1. Introduction

The role of dietary plant constituents and their derivatives in the prevention and treatment of a wide variety of diseases has been well known since ancient times (NRCCDH, 1989). Rice bran (RB) is the pericarp and germ of *Oryza sativa* seeds and constitutes about 10% of rough rice grain. RB is a good source of protein, fat, and antioxidants, but is currently under-utilized, in spite of its high potential as a raw material for the preparation of functional foods or nutraceuticals.

The chemical composition of RB shows that its proteins are of high nutritional value (Kennedy & Burlingame, 2003), and may also be hypoallergenic (Tsuji et al., 2001). The main problem limiting the use of these proteins is their high insolubility, due to the aggregation caused by extensive disulfide bonding. RB is also a good source of most of the vitamin E and oryzanol components, although their concentrations may vary substantially, depending on the origin of the RB (Nicolosi, Rogers, Ausmann, & Orthoefer, 1993). RB has shown promising health-related benefits in the prevention of different diseases, including cancer, hyperlipidemia, fatty liver, hypercalciuria, kidney stones, and heart disease (Jariwalla, 2001). RB is a rich source of γ-oryzanol, a mixture of 10 ferulate esters of triterpene alcohols (Xu & Godber, 1999), which is being increasingly focussed upon as an ingredient for drugs, nutraceuticals, functional foods and feeds, as well as cosmetics. Thus, the preparation of products enriched in the antioxidant components of RB could be of great importance for the treatment of disorders associated with an increased generation of oxygen

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active substances and/or free radicals, such as in atherogenesis. Recently, we have described the antioxidant properties of RBEE (Parrado et al., 2003), showing a high capacity for free-radical-scavenging and protection against protein oxidation and lipid peroxidation when cells ex vivo are exposed to free radicals. Here, we describe the production, physicochemical characterization, stabilization, and functionality of RBEE, and its ability to ameliorate or inhibit cell proliferation in cell-culture experiments.

2. Materials and methods

2.1. Analytical methods

Ash and moisture were analyzed according to the standard AOAC methods (AOAC, 1990). The protein content was determined using the Kjeldahl procedure. Mineral nitrogen was determined according to the method described by Bhatty, Sosulski, and Whu (1973). Crude fat was determined gravimetrically after RBEE extraction with hexane for 12 h in a Soxhlet extractor.

Total soluble carbohydrates were determined after extraction with a mixture of ethanol/water (2:3) for 2 h. After centrifugation at 4000*g*, the supernatant was filtered through No. 1 Whatman paper, and total soluble sugars were estimated colorimetrically by the phenol–sulfuric acid method, using a standard curve of glucose (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Individual soluble sugars were determined according to the method described by Perez, Olias, Espada, Olias, and Sanz (1997). Briefly, the supernatant, previously obtained after centrifugation at 4000*g* was evaporated at 60 °C in an air-draft oven to remove ethanol. The dry residue was dissolved in 1 ml of 0.4 M sulfuric acid, and passed through an ExtraSep-C18 column to clarify the solution. Samples were injected into a high-performance liquid chromatography (HPLC) system equipped with a refractometer detector (Waters 410). Separations were performed on a 300×7.8-mm i.d. reversed-phase column (ION-300, Interaction Chromatography Inc., San Jose, CA). The column was maintained at 24 °C by a temperature controller (Waters). A 17 mM sulfuric acid solution was used as solvent for the elution of carbohydrates at a flow rate of 0.4 ml/min. Standards of soluble sugars were used for the identification and quantification of compounds.

Vitamins B_1, B_2, and B_6 were determined by a standard method, using HPLC (Ndaw, Bergaentzle, Aoude-Werner, & Hasselmann, 2000), and vitamin B_{12} was determined using a microbial method (Lichtenstein, Beloian, & Reynolds, 1959). Ca, Mg, and Fe contents were determined by atomic absorption.

2.2. Lipase activity

Lipase activity was estimated using a spectrophotometric assay with *p*-nitrophenyl laurate (*p*-NPL) as substrate. One unit of lipase activity was defined as the amount of enzyme that liberated 1 μmol/min of *p*-nitrophenol under the standard conditions (Hatzinikolaou et al., 1999).

2.3. Protein characterization

2.3.1. Protein solubility

The protein solubilities of RB and RBEE were determined using the method described by Adler-Nissen (1977). Briefly, a 5 ml portion of 2.4 M trichloroacetic acid was added to 10 ml of the sample, the precipitate was removed by centrifugation (8000*g*, 10 min), and the nitrogen concentration of the supernatant was determined.

2.3.2. Amino acid analysis

Amino acid composition was determined by reversed-phase HPLC analysis of 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) derivatives according to the method described by Ward (2000), with γ-aminobutyric acid as internal standard. Briefly, samples were hydrolyzed using 6 N HCl/1% (w/v) phenol vapour at 110 °C for 24 h in vacuo. The amino acids were treated with AQC to form AQC-derivates, which were then analyzed using a Waters HPLC system (Millipore Ltd.) fitted with a reversed-phase C_{18} column. For cysteine estimation, aliquots were first oxidized with performic acid and then analyzed as above.

2.3.3. Molecular weight by size-exclusion HPLC

Molecular mass distribution of peptides in RBEE was determined by size-exclusion chromatography on a Superdex 200HR column (Amershambiotech) using an ÄKTApurifier (Amershambiotech) according to the procedure described by Visser, Slange, and Robben (1992) and Bautista, Hernandez-Pinzón, Alaiz, Parrado, and Millán (1996). The column was equilibrated and eluted with 0.25 M Tris–HCl buffer (pH 7.00) in isocratic mode, at a flow-rate of 0.5 ml/min, and peptides were detected at 215 and 280 nm. A protein standard mixture (aprotinin, 65,000 Da; gastrin-I, 2126 Da; substance-P, 1348 Da; $glycine_6$, 360 Da; $glycine_3$ 189 Da; and glycine, 75 Da) was used to cover the range of 100 Da to 7 kDa.

2.4. Fatty acid analysis

RB samples used for fatty acid composition analysis were extracted with *n*-hexane. The preparation of methyl esters of the fatty acids was carried out according to Pinelli et al. (2003). A gas chromatograph (Perkin–Elmer, mod. 8420) was employed, with a flame ionization detec-

tor (FID), a splitless injector, and capillary column SP-2380 (0.35 i.d., 0.25 mm in thickness, silica phase, from Supelco). The gas chromatographic conditions were as follows: oven temperature programmed: from 160 to 174 °C at 2 °C/min, from 174 to 190 °C at 1 °C/min. FID temperature 250 °C. Helium carrier gas-pressure was 18 psi.

2.5. Oryzanol analysis

Lipids containing γ-oryzanol were extracted from RBEE using hexane. The γ-oryzanol was semipurified using a low-pressure silica column to remove the triglycerides and other lipids. The separation and quantification of γ-oryzanol components were performed by analytical reversed-phase HPLC (Miller, Frenzel, Schmarr, & Engel, 2003).

2.6. Cell culture

HL-60 and MOL-4 cells (acute lymphoid T-cell leukemia) were obtained from Dr. B. Sanchez (Hospital Universitario Virgen del Rocio, Seville, Spain), and cultured in DMEM (Dulbecco's minimal essential medium) supplemented with 10% heat-inactivated fetal calf serum and 0.1% antibiotic. Cells were maintained as monolayer cultures at 37 °C in a humidified atmosphere with 5% CO_2.

Cell growth inhibition assay was performed by a variation of the method described by Mosmann (1983). Samples containing 200 μl of cell suspension (2×10^4 cells/ml) were plated on 96-well flat-bottomed microtitre plates. After adherence of the cells within 24 h of incubation at 37 °C, different RBEE dilutions, on a scale of 1–10 mg/ml, were added separately. After incubation for 72 h at 37 °C in a humidified incubator with 5% CO_2, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide) (dissolved in PBS (phosphate buffered saline) at 5 mg/ml and sterile-filtered) was added to all the wells at a final concentration of 0.5 mg/ml. Following 1 h of incubation, the formazan generated was dissolved with 100 μl DMSO per well. The optical density was measured on an ELISA plate reader (Merck ELISA System MIOS version 3.2) at 550 nm. Absorbance was proportional to the number of cells. The concentrations causing 50% inhibition of cell growth (IC_{50}) were calculated.

3. Results

3.1. Enzymatic process

Some of the fat components of RB are quite unstable, due to the presence of lipase, which decomposes triglycerides, promoting rancidity. To avoid this problem, different approaches have been tested, including thermal treatment and acid treatment, while recently, enzymatic treatment has been proposed (Vercet, Oria, Crelier, & Lopez-Buesa, 2002). In our opinion, enzymatic treatment is the most effective and beneficial, with minimal change to the initial RB composition, thereby minimizing the loss of essential components such, as amino acids, fats, and micronutrients (oryzanols).

RB was processed by enzymatic hydrolysis, using an endoprotease mixture (trypsin- and chymotrypsin-like) as hydrolytic agent, in a bioreactor with controlled temperature (60 °C) and pH (pH 8), using the pH-stat method (Adler-Nissen, 1986). As Fig. 1 shows, the proteins of RB are efficiently hydrolyzed, 20% degree of hydrolysis being reached after 40 min of reaction. The processing of this product follows different steps, including centrifugation, filtration, and concentration, as has been described for similar products (Parrado, Bautista, & Machado, 1991). The final product is a brown syrup completely soluble in water.

One of the main problems of nutritional stability of RB is that the triglycerides break down due to the presence of endogenous lipase, promoting rancidity of the product. We have tested the effect of protease treatments on the lipase activity. Fig. 1 shows the change in lipase activity during the enzymatic hydrolysis process. Initially, there is an increase in lipase activity, due to cell and aggregates degradation by the protease, leading to the liberation and solubilization of the components. After 15–20 min, lipase activity begins to diminish and, after 1 h of reaction, is totally depleted. The inactivation of the endogenous lipases by protease treatment may be the factor responsible for the stabilization of the lipid components of RBEE (results not shown).

3.2. Chemical characterization

The chemical composition of RBEE is shown in Table 1. As these data show, protein is the main component (38.0 ± 2.3%). Due to the use of proteases, which extract, solubilize, and hydrolyze the initial insoluble

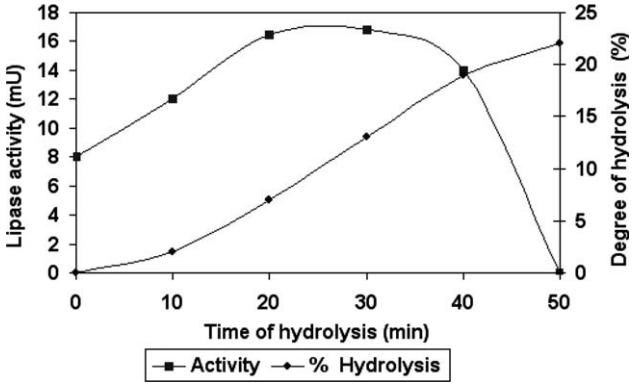

Fig. 1. Enzymatic hydrolysis of rice bran proteins with a mixture of endoproteases (trypsin- and chymotrypsin-like) and inactivation of lipase. (Substrate 15%, Enzyme 0.2 U/g protein, pH 8, 60 °C).

Table 1
Physicochemical characterization of RBEE

	(%, w/w)
Total protein	38.2 ± 2.3
Fat	30.4 ± 0.9
Carbohydrates	14.1 ± 1.1
Ash	6.0 ± 0.2
Ca (mg/kg)	599
Mg (mg/100 g)	787
Fe (mg/100 g)	18
Vitamin B1 (mg/100 g)	<0.1
Vitamin B2 (mg/100 g)	24.1
Vitamin B12 (μg/mg)	0.21
Vitamin B6 (mg/100 g)	1.8

proteins, a specific increase of soluble proteins, peptides, and free amino acids in RBEE is observed. Their physical properties are rather different from those of RB; in RBEE, the proteins are soluble and mainly in the form of peptides and free amino acids. Fig. 2 shows the molecular-weight distribution of the proteinaceous material present in RBEE. The data show that it comprises mainly peptides (<10 kDa) and free amino acids.

This change in the protein structure drastically modifies the water solubility. The protein components of RBEE are totally soluble, independently of the pH, whereas the solubility of RB protein is limited (results not shown). These changes in molecular weight also lead to an increase in the nutritional functionality. The bioabsorption of RBEE protein is better than that of the original RB protein, which is insoluble, due to extensive disulfide bonding and aggregation (Hamada, Spanier, Bland, & Diack, 1998; Matthews, 1977).

With regard to the amino acid composition, RBEE does not differ from RB (Table 2). The most important feature of these data is that RBEE is a good source of sulfur amino acids (cysteine + methionine) which constitute almost 6% of total amino acids.

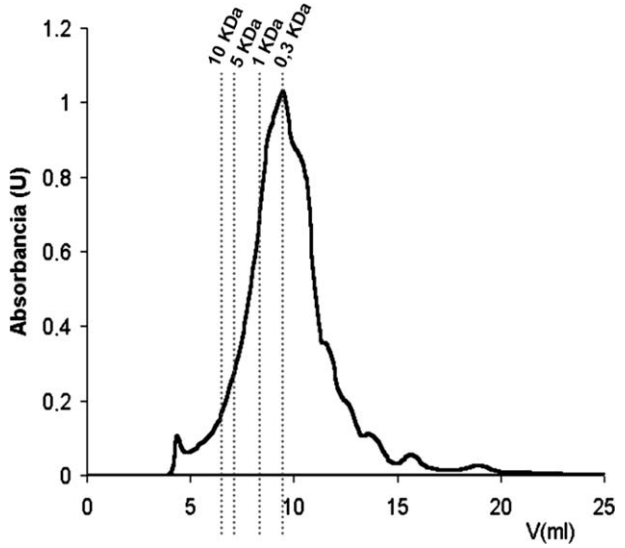

Fig. 2. Size-exclusion chromatography of RBEE.

Table 2
Amino acid composition of RB and RBEE

	RB	RBEE
Asp	7.44	6.48
Ser	5.83	6.28
Glu	11.6	9.57
Gly	6.19	7.25
His	4.03	5.42
Arg	11.6	12.77
Thr	5.19	6.29
Ala	5.37	5.48
Pro	5.01	4.92
Cys	2.74	2.40
Tyr	5.01	2.25
Val	6.41	6.48
Met	2.90	3.74
Lys	4.26	3.46
Ile	4.51	4.92
Leu	6.82	6.62
Phe	5.11	5.62

Results are expressed as grammes per 100 g of proteins.

The RBEE carbohydrates are composed mainly of glucose in the form of polysaccharides, such as starch and dextrins, and free of simple sugars, such as glucose and fructose, and disaccharides, such as maltose, lactose, and sucrose. Table 3 shows the final carbohydrate composition of RBEE non-hydrolysed versus hydrolysed, respectively.

The fat components present in RBEE are mainly soluble, due to protein interactions. The major components are bioactive polyunsaturated fatty acids (see Table 4) and antioxidant phytochemicals, such as oryzanol and vitamin E (see Table 5). As these results show, the oryzanol composition is similar to that found in RB (cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates), but the oryzanol content is 3.4-fold that of the original raw material (RB). This specific enrichment could be due to the enzymatic treatment described above. The vitamin E content is also enriched in the enzymatic extract, almost 3-fold (see Table 5).

3.3. Antiproliferative effect of RBEE

A preliminary study of the anti-proliferative effect of RBEE was carried out by in vitro culture of leukemia

Table 3
Carbohydrate determination of RBEE

Carbohydrates	Non-hydrolyzed RBEE (%) w/w	Hydrolyzed RBEE (%) w/w
Arabinose/rhamnose	0.0026	0.47
Galactose	0.0060	0.33
Glucose	0.029	12.0
Sucrose	0.47	–
Xylose	–	0.31
Mannose	0.0022	0.18
Fructose	–	0.095

Samples were hydrolysed using acidic conditions.

Table 4
Fatty acid composition and content of RBEE and RB

	RB (%)	RBEE (%)
C14:0 Myristic acid	0.21	0.25
C16:0 Palmitic acid	16.4	18.0
C16:1 Palmitoleic acid	0.13	0.14
C18:0 Stearic acid	1.72	1.79
C18:1 Oleic acid	42.4	41.72
C18:2 Linoleic acid	36.4	35.0
C18:3 Linolenic acid	0.80	0.76
C20:1 Arachidic acid	0.60	0.58

Table 5
Oryzanol content and vitamin E content of RBEE and RB

	RBEE (%)	RB (%)
γ-Oryzanol (mg/g)	1.2	0.35
2,4-Methylene cycloartenyl ferulate	22 ± 1.1	25 ± 1.7
Campesteryl ferulate	28 ± 1.3	24 ± 0.9
Cycloartenyl ferulate	21 ± 0.8	23 ± 1.1
Sitosteryl ferulate	14 ± 0.5	13 ± 0.6
Vitamin E (mg/100 g)	<0.02	0.05

cells: HL-60 and MOL-4. The concentration that caused 50% inhibition of cell growth was obtained by determination of formazan dye uptake as explained in Section 2. The concentration was plotted against the percentage of relative cell proliferation after 72 h of treatment, which was calculated assuming that the cell culture in the absence of RBEE had 100% cell proliferation (Fig. 3). The IC_{50} values obtained were: 2.3 mg/ml for HL-60 and 2.8 mg/ml for MOL-4. RBEE does not exhibit cytotoxicity towards peripheral blood lymphocytes.

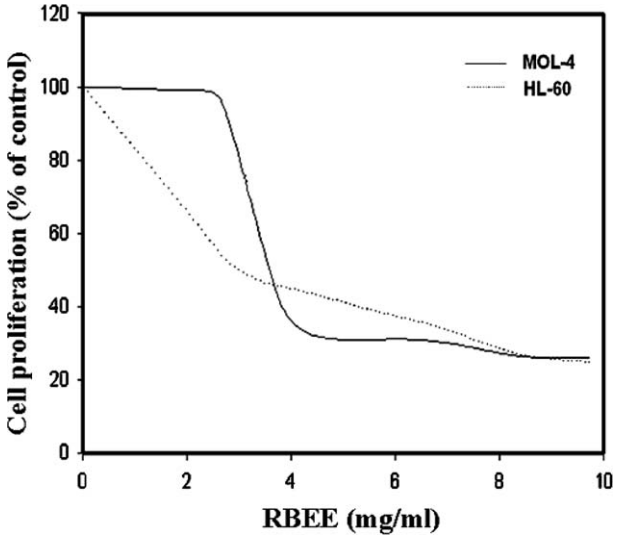

Fig. 3. Leukemia cell (HL-60 and MOL-4) proliferation in response to RBEE treatment. (Cell cultures were treated with increasing doses of RBEE as indicated on the x-axis. The viability and proliferation were determined by formazan formation, and expressed as percentage of untreated control cell proliferation. For details, see Materials and Methods).

4. Discussion

The main aim of our study was the development of a new rice bran derivative and the description of its potential nutritional and health benefits. The chief functional feature of its chemical composition is that all the components are water soluble.

Its essential amino acid profile is comparable to those of the proteins in rice and rice bran, with a relatively high content of sulfur amino acids (6.0%). These amino acids play an important role in strengthening antioxidant defences in processes related to free-radical stress, as they are precursors of two important natural antioxidants: taurine and glutathione (Meister & Anderson, 1983). Taurine is present in animal tissues, particularly those rich in membranes – sources of oxidant generation. There is evidence that a major nutritional role of this amino acid is to protect cell membranes. Glutathione is the most prevalent cellular thiol, and the most abundant low-molecular-weight peptide present in the cells. Glutathione protects cells from toxic effects of reactive oxygen compounds, detoxifying free radicals and peroxides (Horton, 2003). Currently, there is great interest in the potential clinical benefit to be derived from modulating and/or maintaining glutathione levels to protect against oxidative damage, toxic compounds and cell injury due to radiation, physical trauma, and chemotherapy (Pocernich, La Fontaine, & Butterfield, 2000). Thus, the availability of sulfur amino acids may affect antioxidant defences in processes such as burn injury, sepsis and immunological disease, where free-radical-induced tissue damage is patent.

The carbohydrate composition does not produce overloading levels of glucose in plasma, because it is absorbed slowly (results not shown). Because of its slow absorption, it is very suitable for diabetics, elderly people and practitioners of sport.

The fat components present in RBEE are soluble, due to their interactions with proteins and peptides, extracted during the enzymatic treatment. The RBEE oryzanol composition profile is similar to that of RB (cycloartenyl, 24-methylene cycloartenyl, campesteryl, and sitosteryl ferulates) but with two main differences, deriving from its physical properties: (i) the fat components are in a soluble form and (ii) there is a specific enrichment in the γ-oryzanol content, 3.4-fold that in RB.

Different products derived from RB have been reported to possess antioxidant activity (Hiramitsu & Amstrong, 1991;Yasukawa, Akihisa, Kimura, Tamura, & Takido, 1998), and we have recently shown that RBEE has a strong antioxidant capacity, inhibiting lipid peroxidation and protein oxidation induced by cumene hydroperoxide in rat brain homogenate, protection being similar to those of trolox or melatonin (Parrado et al., 2003). The functional anti-hyperlipidemic,

anti-ulcerogenic (Cicero & Gaddi, 2001), and anti-tumoral (Hayashi et al., 1998) properties of RB have also been described. These are a consequence of its antioxidative activity, which is directly correlated with the oryzanol content. The antioxidant capacity of γ-oryzanol has been attributed to its structure, which includes ferulic acid. This constituent is responsible for the strong antioxidant activity (Qureshi, Sami, Salser, & Khan, 2001), which has been demonstrated against cholesterol oxidation and lipid peroxidation in retinal homogenates under oxidative stress (Hiramitsu & Amstrong, 1991). It shows superoxide-dismutase-like activity in that it inhibits pyrogallol autoxidation, which is catalyzed by the superoxide radical. The tocotrienols isolated from RB have also shown in vitro antioxidant activity and suppression of melanoma and adenocarcinoma cell proliferation (Fan, Morioka, & Ito, 2000). Our data show that RBEE, with a high antioxidant capacity, induces the death of leukemia cells, but it is not toxic to peripheral blood lymphocytes. These results provide a comfortable therapeutic window for RBEE application in patients as a supplementary treatment modality with minimal or non-toxic effects.

This new RB-derived product may have potential use in the formulation of solid and liquid food for treatment and prevention of chronic pathological states associated with a high generation of oxygen-active substances and/or free radicals, such as atherosclerosis, neurodegeneration, and cancer, and for elderly persons and practitioners of sports.

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