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Comparison and synergistic effects of intact proteins and their hydrolysates on the functional properties and antioxidant activities in a simultaneous process of enzymatic hydrolysis

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ABSTRACT

Soy protein isolate (SPI), bovine whey protein (BWP) and egg white protein (EWP) were hydrolyzed with the Flavourzyme 500L® protease, and the interactions of these substrates and their mixtures on their functional properties and antioxidant activities were studied using a simplex centroid mixture design. Synergistic effects between the formulations containing binary or ternary mixtures were observed for several parameters, especially the DPPH radical-scavenging activity and emulsion activity index, which exhibited increases of up to 45.0 and 1200.0%, respectively, after enzymatic hydrolysis compared to the isolated substrates. The results suggest that the application of the statistical mixture designs in a simultaneous process of enzymatic hydrolysis using different protein sources is an attractive method for improving enzyme performance and identifying optimum formulations.

Keywords: Enzymatic hydrolysis; Protein hydrolysates; Functional properties; Antioxidant activities; Mixture design

1. Introduction

Processes involving protein hydrolysis have been studied for bioactive peptide production. Bioactive peptides can be defined as specific amino acid sequences that promote beneficial biological activities. Bioactive peptides can be produced by enzymatic hydrolysis using digestive, microbial and plant enzymes. Limited and controlled proteolysis unfolds protein chains, reduces the incidence of allergenic factors and increases the formation of small peptides with biological activities (Korhonen, 2009).

In the last decade, enzymatic hydrolysis of proteins from animal and plant sources for the production of bioactive peptides have attracted much attention, and the antioxidant activities of peptides have been extensively reported in several studies. The action mechanism of peptides with antioxidant properties is related to the inactivation of reactive oxygen species (ROS), scavenging of free radicals, chelation of prooxidative transition metals and reduction of hydroperoxides (Zhou et al., 2012a).

In addition to their antioxidant activities, protein hydrolysates have shown interesting functional properties, such as high solubility, resulting in increases in the concentration of free amino and carboxyl groups. Hydrolysis disrupts the protein tertiary structure and reduces the molecular weight of the protein consequently altering its functional properties (Liu et al., 2010).

According to the literature, different protein sources have been used for enzymatic hydrolysis, such as rice, egg white protein and whey protein (Zhao et al., 2012; Naik et al., 2013; Hoppe et al., 2013). However, these reports investigated enzymatic hydrolysis using separate substrates, and no investigation using statistical mixture designs has been reported.

Mixture designs are a special class of response surface designs where the proportions of the components or factors are considered important rather than their magnitude and are useful in the design of mixtures. The interactions between the components of a mixture can be studied using the mixture design approach aiming to maximize the response. Statistical methods have been applied to different engineering problems

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to improve performance and to find the optimum process variables (Rao and Baral, 2011).

In the present study, a simplex centroid mixture design was used to produce hydrolysates from different protein sources by enzymatic hydrolysis to study the effects of these mixtures on functional properties and antioxidant activities.

2. Materials and methods

2.1. Reagents

Ammonium thiocyanate, ferrous chloride, linoleic acid, trichloroacetic acid (TCA), 2,2′-azobis(2-methylpropionamidine) dihydrochloride (97.0%) (AAPH), fluorescein, (±)-6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma–Aldrich (Steinheim, Germany). All other purchased chemicals were of commercially available grade.

2.2. Preparation of protein hydrolysates

The soy protein isolate (SPI), bovine whey protein (BWP) and egg white protein (EWP) used as the substrates for enzymatic hydrolysis were kindly provided by Bunge Foods S/A (Gaspar, Brazil), Alibra Ingredients Ltd. (Campinas, Brazil) and Cooperovos (Mogi das Cruzes, Brazil), respectively. The commercial protease, Flavourzyme® 500L from *Aspergillus oryzae* (Novozymes Latin America Ltd., Araucária, Brazil) was used for enzymatic hydrolysis. The enzyme concentrations were adjusted to 0 (control) or 50 U per mL of reaction mixture according to the previously determined protease activity. The proteins were suspended in a buffer to a final concentration of 100 mg mL^{-1}, and 50-milliliter aliquots of the mixtures were distributed in 125 mL Erlenmeyer flasks. Hydrolysis was performed at the optimum temperature and pH value of the enzyme (50.0 °C and pH 5.0) for 120 min. After hydrolysis, the samples were incubated in a water bath at 100 °C for 20 min for protease inactivation. The mixtures were centrifuged at 17,000 × *g* at 5 °C for 20 min, and the supernatants containing the peptides were collected and freeze-dried for the determination of their antioxidant activities, functional properties and TCA soluble protein contents.

The protein content in the freeze-dried supernatants was determined using the Biuret method, and the results were expressed in milligrams of protein per grams of freeze-dried sample.

2.3. Statistical mixture design

The experimental mixture design was used to obtain the optimum mixture compositions of the different protein sources for maximum antioxidant activity and to investigate the presence of either synergistic or antagonistic effects in a blend of the components. A three component augmented simplex centroid design was employed in which each component was studied at four levels, namely 0 (0%), 1/3 (33%), 1/2 (50%) and 1 (100%) (Table 1). Quadratic or special cubic regression models were fitted for the variations of all the responses studied as a function of significant ($p < 0.05$) interaction effects between the proportions, thereby obtaining acceptable determination coefficients ($R^2 > 0.70$). Eq. (1) represents these models as follows:

$$Y_i = \sum \beta_i X_i + \sum \beta_{ij} X_i X_j + \sum \beta_{ijk} X_i X_j X_k \quad (1)$$

where Y_i is the predicted response; β_i is the regression coefficient for each linear effect term; and β_{ij} and β_{ijk} are the binary and ternary interaction effect terms, respectively. Statistica® 10.0 software from Statsoft Inc. (Tulsa, Oklahoma, USA) was employed for the experimental design, data analysis and model building.

To confirm the validity of the models, three assays were performed under randomly selected test conditions, and the experimental values were compared with the predicted values by the models within a 95.0% confidence interval.

2.4. Determination of TCA soluble protein content

The TCA soluble protein content of the hydrolysates was determined using a modified version of the method described by Peričin et al. (2009). A 1.0 mL aliquot of the hydrolysate was added to an equal volume of 0.44 mol L^{-1} trichloroacetic acid (TCA). The mixture was incubated for 30 min at room temperature and then centrifuged at 17,000 × *g* for 15 min. A 0.22 mol L^{-1} TCA soluble protein fraction was obtained, and the supernatant of the hydrolysate mixture (without the addition of TCA) was analyzed using the Lowry method (1951), which uses bovine serum albumin as the standard protein to determine the protein content. The results were expressed as a percentage and were calculated as the ratio of the 0.22 mol L^{-1} TCA soluble protein content to the total protein content in the supernatant of the hydrolysate mixture.

2.5. Determination of antioxidant activities

2.5.1. ORAC assay

The antioxidant activity of the hydrolysates was estimated by the ORAC method as developed by Dávalos et al. (2004) and described by Macedo et al. (2011), which uses fluorescein (FL) as the "fluorescent probe". The automated ORAC assay was performed using a Novo Star Microplate reader (BMG LABTECH, Germany) with fluorescence filters for an excitation wavelength of 485 nm and an emission wavelength of 520 nm. The measurements were made in a COSTAR 96 plate. The reaction was performed at 37 °C and was started by the thermal decomposition of AAPH in a 75 mM phosphate buffer (pH 7.4) due to the sensitivity of FL to the pH value. A solution of FL (0.4 μg mL^{-1}) in phosphate buffered saline (PBS) (75 mM; pH 7.4) was prepared daily and stored in complete darkness. The reference standard was a 75 μM Trolox solution prepared daily in distilled water, and the standard was diluted (1500–1.5 μmol L^{-1}) for the preparation of the Trolox standard curve. In each well, 120 μL of the FL solution was mixed with either 20 μL of sample, distilled water (blank) or standard (Trolox solutions) before adding 60 μL of AAPH (108 mg mL^{-1}). The fluorescence was measured immediately after the addition of AAPH, and measurements were taken every minute for 75 min. The ORAC values were calculated from the difference between the area under the FL decay curve and that of the blank (net AUC). Regression equations for the net AUC and antioxidant concentration were calculated for all samples. The ORAC values were expressed as μmol of Trolox equivalent g^{-1} of protein hydrolysate (Trolox EQ μmol g^{-1}).