

WHEY AS A SOURCE OF PEPTIDES WITH HIGH ANTIOXIDANT ACTIVITY: USE OF PANCREATIN AND *ASPERGILLUS SOJAE* PROTEASE

SORO DE LEITE COMO FONTE DE PEPTÍDEOS COM ALTA ATIVIDADE ANTIOXIDANTE: USO DE UMA PANCREATINA E DE UMA PROTEASE DE *ASPERGILLUS SOJAE*

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ABSTRACT

The use of enzymes in the hydrolytic process can release bioactive peptides from food proteins. A pancreatin and an *Aspergillus sojae* proteases were used in this study to obtain whey peptides with high antioxidant activity (AnAc), at enzyme:substrate (E:S) ratios of 0.5:100, 1:100, 2:100, 3:100, 4:100 and 8:100. The DPPH (2-difeny-1-picrylhydrazil), deoxyribose and pyrogallol methods were used to evaluate the AnAc. The highest AnAc values were reached at an E:S ratio of 8:100 by using pancreatin (60.67%) and *A. sojae* protease (59.70%). The possibility of using a low E:S ratio for reducing the costs for scaling-up the process was observed for the *Aspergillus sojae* protease with the deoxyribose and pyrogallol methods since the highest AnAc values were obtained with an E:S ratio of 2:100 (P3: 58.77% and 47.18%, respectively).

Keywords: Milk proteins. Hydrolysis. Antioxidant activity. *Aspergillus sojae* protease. Pancreatin.

RESUMO

O uso de enzimas no processo hidrolítico pode liberar peptídeos bioativos de proteínas alimentares. Uma pancreatina e uma protease de *Aspergillus sojae* foram as proteases usadas neste estudo para obter peptídeos de soro de leite com alta atividade antioxidante (ATAn), nas relações enzima:substrato (E:S) de 0,5:100, 1:100, 2:100, 3:100, 4:100 e 8:100. Os métodos de DPPH (2,2-difenil-1-picril-hidrazila), da desoxirribose e do pirogalol foram utilizados para avaliar a ATAn. As maiores ATAn foram obtidas na relação E:S de 8:100 usando a pancreatina (60,67%) e a protease de *A. sojae* (59,70%). A possibilidade de utilizar uma baixa relação E:S, visando a redução dos custos para o escalonamento do processo, foi observada para a protease de *Aspergillus sojae* com os métodos de desoxirribose e pirogalol, uma vez que as maiores ATAn foram obtidas com uma relação E:S de 2:100 (P3: 58,77% e 47,18%, respectivamente).

Palavras-chave: Proteínas lácteas. Hidrólise. Atividade antioxidante. Protease de *Aspergillus sojae*. Pancreatina.

Introduction

Many milk proteins possess specific biological properties that make these components potential ingredients of health-promoting foods. Increasing attention is being focused on physiologically active peptides derived from milk proteins. These peptides are inactive within the sequence of the parent protein molecule and can be liberated by (1) gastrointestinal digestion of milk, (2) fermentation of milk with proteolytic starter cultures or (3) hydrolysis by proteolytic enzymes (KORHONEN; PIHLANTO, 2006).

There is a growing trend and interest in the use of these bioactive peptides as intervention agents against chronic human diseases and for the maintenance of general well-being. These peptides have exhibited potent biological activities, such as antihypertensive, antioxidant, immunomodulatory, anticancer, antimicrobial, and lipid-lowering activities (UDENIGWE; ALUKO, 2012).

After having previously tested some enzymes, in the present study we used a pancreatin and an *Aspergillus sojae* protease for the production of bioactive peptides from whey proteins. The pancreatin is an enzymatic complex consisting of enzymes secreted by the pancreas and it has proteolytic, amylolytic and lipolytic activities. These proteases are divided into endopeptidases (trypsin, chymotrypsin and elastase) and exopeptidases (carboxypeptidases A and B) (PARK, 2001). The *Aspergillus sojae* protease is activated by zinc and cobalt; it acts as an exopeptidase, and preferentially hydrolyzes leucine bonds, followed by phenylalanine, lysine and arginine derivatives (CHIEN et al., 2002).

Additionally, in the current study we were interested in measuring the antioxidant activity of the whey peptides due to the necessity of finding natural products capable of preventing oxidative reactions. Oxidative stress in the body produces changes in the physical and chemical properties of the membranes, modifying their fluidity and permeability, expanding the intracellular fluid, and increasing the risk of rupture of the cell membranes, resulting in cell death (VASCONCELOS et al., 2007).

Aiming to evaluate the antioxidant activity of the whey peptides, we used four methods based on the reduction of free radicals by electron transfer

(2,2-diphenyl-1-picryl-hidrazyl method, DPPH method), the ability of different compounds to scavenge hydroxyl radicals (deoxyribose method), and superoxide activity (pyrogallol auto-oxidation method).

The objective of this work was the production of bioactive peptides from whey by hydrolytic treatment, using a pancreatin and an *Aspergillus sojae* protease. The antioxidant activity of these peptides was evaluated.

Materials and Methods

Materials

WPC (Kerrylac 750) in powder form was kindly furnished by Kerry of Brazil Ltda. (Três Corações, MG, Brazil). Pancreatin (Corolase PP® from porcine pancreas, activity = 5.97 U mL⁻¹) and a protease from *Aspergillus sojae* (Corolase LAP®, activity = 0.63 U mL⁻¹) were kindly furnished by AB Enzymes (Barueri, SP, Brazil). In this study, one unit of protease (U mL⁻¹) activity was defined as the activity that liberated 1 µg of tyrosine per minute (µg Tyr x mL⁻¹ min⁻¹) under the described conditions (DIAS et al., 2008). The α-deoxyribose (code 121649), pyrogallol (code 16040) and DPPH (code D9132) were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents used in this work were of analytical grade.

Methods

Preparation of whey peptides

The enzymatic hydrolysis of whey protein concentrate (WPC) was used for preparing whey peptides. WPC solutions (10%, w/v) were prepared, and after adjustment of the pH to 7.0 with a 3 mol L⁻¹ NaOH solution, the solutions were heated in an oil bath at 50 °C with continuous stirring (stirrer 752A model from Fisatom, São Paulo, SP, Brazil), and the enzymes were added at the necessary concentration to attain the desired enzyme:substrate ratios. The reaction time was of 5h and the hydrolytic reaction was stopped by heating the samples at 75 °C for 15 sec. The hydrolyzed protein solutions containing the peptides were immediately used for the determination of the degree of hydrolysis and then freeze-dried (Freeze Dry System/FreeZone 4.5, model 77500, Labconco, Kansas City, MO, USA) and stored in

a freezer (-20 °C) until the peptide size evaluation. The pH and temperature values corresponded to the optimal conditions for the enzyme and were provided by the manufacturer.

Antioxidant activity determination

DPPH method

The method described by Li et al. (2006) was used, with modifications. Briefly, 1.5 mL of the hydrolyzed protein solutions (100 mg mL⁻¹), prepared as described above, was mixed with 1.5 mL of a DPPH alcoholic solution (0.1 mmol L⁻¹), and the mixture was allowed to stand at 25 °C for 20 minutes. Then, the mixture was incubated in a water bath until reaching 60 °C, and this temperature was maintained for 20 minutes. Afterwards, the absorbance was measured in a spectrophotometer at 517 nm (SP-2000UV model, Bel Photonics, Osasco, SP, Brazil). A mixture of 1.5 mL of the DPPH solution and 1.5 mL of distilled water was used as control.

The antioxidant activity of the hydrolyzed protein solutions, expressed as the scavenging percentage of the DPPH radicals (% SR), was calculated by the following equation:

$$\text{Antioxidant activity (\%SR)} = (A_C - A_H) / A_C \times 100$$

Where A_C and A_H are the absorbances of the control and hydrolyzed proteins, respectively.

Deoxyribose method

The α-deoxyribose oxidation method proposed by Halliwell et al. (1987), with modifications, was used to evaluate the antioxidant activity of hydrolyzed proteins against hydroxyl radicals (OH[•]). A volume of 0.2 mL of FeSO₄ solution (10 mmol L⁻¹) was mixed with 0.2 mL of hydrolysate solutions (100 mg mL⁻¹) for 5 minutes. Then, 1.0 mL of the α-deoxyribose solution (10 mmol L⁻¹), 2.2 mL of sodium phosphate buffer (0.01 mol L⁻¹, pH 7.4), and 0.4 mL of hydrogen peroxide solution (10 mmol L⁻¹) were added. The mixture was incubated at 37 °C for 60 minutes, and 1.0 mL of trichloroacetic acid solution (2.8 g% w/v) and 1.0 mL of thiobarbituric acid solution (0.8 g w/v) were added. The tubes were heated at 100 °C for 15 minutes, cooled to room temperature, and centrifuged at 4000 rpm for 15 minutes (NT812 centrifuge,

New Technique, Piracicaba, SP, Brazil). Finally, the absorbance of the red pigment in the supernatant was measured in a spectrophotometer at 532 nm (SP-2000UV model, Bel Photonics, São Paulo, SP, Brazil). A mixture prepared without hydrolyzed proteins was used as a control.

The antioxidant activity of the hydrolyzed proteins against hydroxyl radicals was measured as the inhibition percentage of α-deoxyribose oxidation, according to the following formula:

$$\text{Antioxidant activity (\% inhibition)} = (A_C - A_H) / A_C \times 100$$

Where A_C and A_H are the absorbances of the control and hydrolyzed proteins, respectively.

Pyrogallol method

The ability of the hydrolyzed proteins to scavenge superoxide radicals was evaluated by the auto-oxidation of pyrogallol, as proposed by Marklund and Marklund (1974) with modifications proposed by Tang et al. (2010). A mixture containing 2.8 mL of Tris buffer/HCl (50 mmol L⁻¹, pH 8.2), 0.1 mL of hydrolysate solutions (100 mg mL⁻¹), and 0.1 mL of pyrogallol solution (10 mmol L⁻¹) dissolved in HCl (10 mmol L⁻¹) was incubated at 25 °C for 10 minutes. The auto-oxidation of pyrogallol was monitored at 320 nm in a spectrophotometer (model SP-2000UV, Bel Photonics, Osasco, SP, Brazil) at 30 second intervals for 5 minutes. The absorbance curves were plotted versus time, and the slopes were interpreted as the rate of pyrogallol oxidation. A mixture prepared without hydrolyzed proteins was used as a control.

The antioxidant activity of the hydrolyzed proteins against superoxide radicals was expressed as the inhibition percentage of pyrogallol auto-oxidation, according to the following formula:

$$\text{Antioxidant activity (\% inhibition)} = (A_C - A_H) / A_C \times 100$$

Where A_C and A_H are the absorbances of the control and hydrolyzed proteins, respectively.

Statistical analysis

The experiments were conducted in triplicate, and the results were expressed as the mean \pm standard deviation. A randomized design in a factorial analysis scheme (2 enzymes \times 6 enzyme:substrate ratios) was used to verify the presence of significant effects among the different treatments. Analysis of variance was used to determine the effects of the enzyme type and the E:S ratio on the antioxidant activity of the samples. Significant differences ($p < 0.05$) between the means were evaluated using the Tukey test (PIMENTEL-GOMES, 2000). The correlations between the values of antioxidant activity obtained by the different methods were determined using the Pearson correlation coefficient (r), which measures the degree of association between two variables, and p was determined with the Student's t-test and using Bioestat software (AYRES et al., 2007) for the data analysis.

Results

The antioxidant activity of hydrolyzed proteins changed from 15.48% (P1) to 60.67% (P12), as shown in Table 1. The highest antioxidant activity (60.67%) was achieved by using the pancreatin, but the use of the *A. sojae* protease produced the second highest value (59.70%).

There were significant differences among the results obtained by the three methods for the same sample (Table 1) and the highest values were obtained for the pyrogallol method for eight samples (P4 and P5, P7 to P12); the deoxyribose method for two samples (P1 and P3); and the DPPH method for two samples (P2 and P6). Among the eight samples that displayed the highest values using the pyrogallol method, six were obtained with pancreatin and two with *A. sojae* protease.

Table 1 - Antioxidant activity of the hydrolyzed proteins

Samples	E:S ratio	Antioxidant activity		
		DPPH method (radical scavenging %)	Deoxyribose method (Inhibition %)	Pyrogallol method (Inhibition %)
		<i>Aspergillus sojae</i> protease		
P1	0.5:100	34.31 \pm 0.18 ^{Db}	43.50 \pm 1.09 ^{DEa}	15.48 \pm 0.96 ^{Kc}
P2	1.0:100	47.85 \pm 1.11 ^{Ca}	39.92 \pm 3.01 ^{Fb}	34.94 \pm 2.94 ^{lc}
P3	2.0:100	26.10 \pm 1.47 ^{GHc}	58.77 \pm 0.39 ^{Aa}	47.18 \pm 0.97 ^{DEb}
P4	3.0:100	28.15 \pm 1.23 ^{FGb}	49.96 \pm 4.40 ^{BCa}	45.85 \pm 1.98 ^{Efa}
P5	4.0:100	21.98 \pm 1.74 ^{jb}	25.93 \pm 1.96 ^{lb}	49.78 \pm 2.97 ^{BCDEa}
P6	8.0:100	59.70 \pm 0.54 ^{Aa}	52.78 \pm 2.68 ^{Bb}	30.79 \pm 1.85 ^{Jc}
		Pancreatin		
P7	0.5:100	24.41 \pm 2.57 ^{HIc}	33.35 \pm 0.97 ^{Gb}	42.05 \pm 3.65 ^{Fga}
P8	1.0:100	16.89 \pm 0.91 ^{Lc}	23.96 \pm 0.59 ^{lb}	37.81 \pm 3.31 ^{Hla}
P9	2.0:100	18.30 \pm 0.83 ^{KLc}	35.58 \pm 1.17 ^{Gb}	52.83 \pm 2.94 ^{BCa}
P10	3.0:100	19.46 \pm 0.30 ^{Kc}	27.28 \pm 0.80 ^{Hb}	52.46 \pm 1.93 ^{BCa}
P11	4.0:100	22.64 \pm 0.53 ^{Ijb}	35.35 \pm 1.67 ^{Ga}	39.43 \pm 1.99 ^{GHa}
P12	8.0:100	49.44 \pm 0.62 ^{BCb}	47.90 \pm 0.45 ^{CDb}	60.67 \pm 0.41 ^{Aa}

E:S = Enzyme:Substrate; DPPH = 2,2-diphenyl-1-picrylhydrazyl. The values represent the means of triplicate experiments \pm standard deviation. Different capital (column) and small (line) letters represent significantly different ($p < 0.05$) values.

Discussion

The use of different hydrolytic conditions should have released peptides with extensive abilities, chain sizes and chemical compositions, producing this variability of results.

Having both endo- and exoactivities, the pancreatin probably released a significant amount of peptides, which were capable of scavenging superoxide radicals and which reduced the pyrogallol oxidation. For the DPPH and deoxyribose methods,

all the samples that produced the highest values were prepared with the *A. sojae* protease. These enzymes probably released amino acids or peptides, which had a great ability to prevent the degradation of this sugar and which were also able to reduce DPPH.

Considering the reduction of costs for scaling-up the process i.e., the ability to use low E:S ratios, the analysis of the results in Table 1 shows that this beneficial effect was observed in the case of using the *Aspergillus sojae* protease with the deoxyribose and pyrogallol methods because the highest antioxidant activities were obtained with an E:S of 2:100 (P3: 58.77 % and 47.18%, respectively). This beneficial effect was not observed when using the pancreatin with any of the other methods, because the highest antioxidant activities were obtained with an E:S of 8:100.

Conclusion

The pancreatin and the *Aspergillus sojae* protease hydrolyzed whey proteins, producing peptides with high antioxidant activities (59.70% and 60.67%, respectively). The hydrolytic condition that produced the highest result (60.67%) was that using the pancreatin at an E:S ratio of 8:100. The use of the *Aspergillus sojae* protease was more advantageous than pancreatin in terms of the reduction of costs for scaling-up the process because it was possible to obtain higher antioxidant activity with an E:S of 2:100.

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