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Simultaneous hydrolysis of proteins from different sources to enhance their antibacterial properties through the synergistic action of bioactive peptides

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ABSTRACT

Antimicrobial peptides have been identified as possible substitutes for chemicals used as drugs or food preservatives, owing to their high specificity, easy degradability in the environment, and low toxicity. In the current study, three different protein sources (viz., soy protein isolate [SPI], bovine whey protein [BWP], and egg white protein [EWP]) were hydrolyzed using Flavourzyme® 500L, individually or in binary/ternary mixtures, to verify the antibacterial activities of the hydrolysates obtained. The most adequate formulations to inhibit the tested bacterial strains were 1) the ternary mixture of SPI, BWP, and EWP in equal proportions for *Escherichia coli* ATCC 11229, 2) all binary mixtures of the protein hydrolysates for *Salmonella enterica* serotype Choleraesuis ATCC 14028, and 3) the binary mixture of SPI and BWP for *Staphylococcus aureus* ATCC 6538, which decreased the bacterial growth by ≥19%, ≥15%, and ≥48%, respectively. The enzymatic process proposed in our study proved to be a promising alternative for producing protein hydrolysates with high potential bioactivity, by simultaneously hydrolyzing mixtures of different protein sources to obtain peptides that could act synergistically to inhibit pathogenic bacteria.

1. Introduction

Bioactive peptides from dietary proteins have been extensively studied over the last decade to determine their potential uses and their effects on human health. These bioactive molecules are short peptides with only 2–20 amino acid residues, and may present antimicrobial, antioxidant, and antihypertensive activities, among others. Therefore, they can potentially be used as functional foods or nutraceuticals, where their bioactivities can aid in the prevention and control of diseases (de Castro and Sato, 2015).

Bacterial resistance to conventional antibiotic compounds has drawn interest towards some new natural substances, such as antimicrobial peptides, which will not induce antibiotic resistance (Bamdad et al., 2015). Antimicrobial peptides are distributed widely in nature and have great diversity in their primary structures. Most antimicrobial peptides are similar, especially with regard to the composition, net charge, and hydrophobicity of their amino acids (Akalin, 2014). Cationic peptides are the most extensively studied owing to their antimicrobial activities against gram-positive and gram-negative bacteria (Ntwasa, 2012; Bamdad et al., 2015). In addition, some studies have shown that anionic peptides can contribute to the amphiphilic nature of the molecule, which is necessary for interaction with the lipid membrane of cells (Jiang et al., 2008; Benkerroum, 2010; Demers-Mathieu et al., 2013).

The aim of this work was to study the simultaneous hydrolysis of animal and plant proteins (viz., soy protein isolate [SPI], bovine whey protein [BWP], and egg white protein [EWP]) as a strategy to enhance their antibacterial properties against two gram-negative bacteria (*Escherichia coli* ATCC 11229 and *Salmonella enterica* serotype Choleraesuis ATCC 14028) and one gram-positive bacterium (*Staphylococcus aureus* ATCC 6538).

2. Materials and methods

2.1. Reagents

Flavourzyme® 500L and nutrient culture broth were purchased from Sigma-Aldrich (São Paulo, Brazil). All other chemicals were purchased in the grade commercially available.

2.2. Preparation of protein hydrolysates

The SPI, BWP, and EWP used as 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 Flavourzyme® 500L concentration was ad-

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justed to 50 U per milliliter of reaction, according to the protease activity, as previously determined (Charney and Tomarelli, 1947). Control samples were prepared with the enzyme previously inactivated in a water bath at 100 °C for 20 min. The proteins were suspended in 100 mM acetate buffer (pH 5.0) to a final concentration of 100 mg mL^{-1}. Fifty-milliliter aliquots of the mixtures were distributed into 125 mL Erlenmeyer flasks and the hydrolysis was carried out at the optimum temperature and pH of the enzyme (50 °C, 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 peptides were collected and freeze-dried for the determination of antibacterial activities.

2.3. Statistical mixture design

A statistical mixture design was used to obtain the optimum mixture composition of the different protein sources for maximum inhibition of the bacterial strains and to investigate the presence of either a synergistic or an antagonistic effect in a blend of components. A three-component augmented simplex-centroid design was employed, in which each component was studied in 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 variations of all studied responses as a function of significant ($p < 0.05$) interaction effects between the proportions, with acceptable determination coefficients ($R^2 > 0.80$). Eq. (1) represents these models:

$$Yi = \sum_{i=1}^{q} \beta iXi + \sum \sum_{i<j}^{q} \beta ijXiXj + \sum \sum_{i<j<k}^{q} \sum \beta ijkXiXjXk \quad (1)$$

where Y_i is the predicted response (bacterial growth); q represents the number of components in the system; X_i, X_j, and X_k are the coded independent variables; 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 software from Statsoft Inc. (Tulsa, Oklahoma, USA) was employed for the experimental design, data analysis, and model building.

2.4. Antibacterial activity of the protein hydrolysates

2.4.1. Microorganisms and culture conditions

E. coli ATCC 11229, *S.* Choleraesuis ATCC 14028, and *S. aureus* ATCC 6538 were used throughout this work. To obtain working cultures, stock microorganisms were initially inoculated into 125 mL Erlenmeyer flasks containing 50 mL of nutrient broth. The flasks were incubated in a shaker with stirring at 100 rpm for 24 h at 37 °C under aerobic conditions to activate growth. After incubation, the cultures were subcultured by transferring a 1 mL aliquot of the reactivated microorganisms into 125 mL Erlenmeyer flasks with 50 mL of nutrient broth and incubating at 37 °C and 100 rpm for 8 h. The culture suspensions were diluted in the same medium to obtain the final optical density required for inoculation of the microtiter plate wells.

2.4.2. Antibacterial activity of the protein hydrolysates

Antibacterial activity was assayed using a Novo Star microplate reader (BMG Labtech, Ortenberg, Germany). The measurements were made in a 96-well microtiter plate. First, the protein solutions were filtered through a Millex® membrane filter with pore size of 0.45 mm (Millipore, Sao Paulo State, Brazil). Then, 100 µL of the culture suspension (diluted in nutrient broth to an OD_{600} of 0.2) and an equal volume of protein solution (0.5 mg mL^{-1} dissolved in the same medium) were added to each well of a microtiter plate. The plate was incubated for 24 h at 37 °C, after which the OD_{600} was measured. As a control experiment, 100 µL of each culture suspension and 100 µL of nutrient broth supplemented with the corresponding non-hydrolyzed proteins (0.5 mg mL^{-1}) were applied to the wells. All assays were performed in six replicates. The growth, expressed as a percentage, was calculated as the ratio of OD_{600} of the samples to OD_{600} of the control. Antibacterial activity was calculated as a decrease in the percentage growth.

2.5. Calculations and statistics

The statistical analyzes were performed using the Minitab® 16.1.1 software package from Minitab Inc. (State College, Pennsylvania, USA). Values are expressed as the arithmetic mean. The Tukey test was used to determine significant differences between the groups analyzed. The differences were considered significant at $p < 0.05$.

3. Results and discussion

3.1. Bacterial growth and antibacterial activities of the protein hydrolysates

The results for relative bacterial growth in the presence of the protein hydrolysates are presented in Table 1. Some assays showed percentage growth up to 100%, which characterized a promotion of the bacterial growth by the protein hydrolysates compared with their respective non-hydrolyzed counterparts. The most important decreases in antibacterial activity were detected for the samples in run 2 (BWP), run 5 (binary mixture of SPI and EWP), and run 7 (ternary mixture of SPI, BWP, and EWP), which resulted in increases of *S. aureus* ATCC 6538 growth ranging from 11.98% to 49.59% compared with their respective controls. In these assays, the enzymatic hydrolysis probably resulted in peptides that were easily assimilated by the microorganisms as nitrogen sources, which stimulated the bacterial growth.

The bacterial growth of the tested microorganisms in the presence of EWP (run 3) showed values at around 100%, but there was no significant difference ($p < 0.05$) in relation to the control samples. However, for most of the samples, the enzymatic hydrolysis exhibited a

Table 1
Matrix of the simple centroid mixture design and results for bacterial growth in media supplemented with different protein hydrolysates.

Runs	Independent variables			Bacterial growth (%)		
	x_1	x_2	x_3	***E. coli* ATCC 11229**	***S.* Choleraesuis ATCC 14028**	***S. aureus* ATCC 6538**
1	1	0	0	79.70 ± 6.81^{d}	$86.84 \pm 1.22^{b, c}$	68.69 ± 1.87^{a}
2	0	1	0	$91.19 \pm 2.27^{a, b, c}$	91.61 ± 4.28^{b}	136.14 ± 1.11^{b}
3	0	0	1	101.03 ± 5.06^{a}	100.50 ± 0.08^{a}	96.68 ± 3.08^{c}
4	1/2	1/2	0	$88.58 \pm 2.11^{b, c, d}$	$86.47 \pm 0.62^{b, c}$	55.93 ± 2.35^{d}
5	1/2	0	1/2	99.02 ± 0.43^{a}	$89.15 \pm 2.50^{b, c}$	149.59 ± 7.17^{e}
6	0	1/2	1/2	$93.56 \pm 1.13^{a, b}$	$88.07 \pm 2.01^{b, c}$	81.34 ± 0.86^{f}
7	1/3	1/3	1/3	$81.60 \pm 2.23^{c, d}$	84.19 ± 1.35^{c}	111.98 ± 4.25^{g}

$^{a, b, c...}$The results are presented as the mean (n=3) ± SD, and those with different letters are significantly different, with $p < 0.05$. x_1 – soy protein isolate (SPI); x_2 – bovine whey protein (BWP); x_3 – egg white protein (EWP).