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ORIGINAL ARTICLE

Antimicrobial and radical scavenging properties of bovine collagen hydrolysates produced by *Penicillium aurantiogriseum* URM 4622 collagenase

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Abstract A 2^3 full factorial design was used to identify the main effects and interactions of pH, collagen concentration and temperature on the degree of collagen hydrolysis (DH) by collagenase from *Penicillium aurantiogriseum* URM 4622. Increases in both pH and collagen concentration improved DH, and a positive interaction effect was observed for these variables. On the other hand, temperature had a negative main effect on DH. The maximum value of DH (4.65 μg/mL) was achieved at 7.5 mg/mL collagen concentration, pH 8.0 and 25 °C. The peptide profile showed several peptides with molecular weights lower than 2 kDa and exhibited antibacterial activity against *Escherichia coli*, *Bacillus subtilis and Staphylococcus aureus*. An antioxidant activity of 84.7± 0.24 % towards the radical ABTS•+was obtained with 50 mg/mL hydrolysates. This study demonstrated that collagen hydrolysed by *P. aurantiogriseum* URM 4622 collagenase possesses interesting antibacterial and antioxidant activities.

Keywords Collagen peptides · Bioactive peptides · Antioxidant activity · Antibacterial activity · Collagenase · *Penicillium aurantiogriseum*

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Introduction

Collagen is a cheap and resourceful meat byproduct whose main product is a gelatin that is used extensively as a food additive to increase the texture, water-holding capacity and stability of several food products. In addition, collagen is a source of biologically active peptides with promising applications (Gómez-Guillén et al. 2011).

Collagen peptides are considered to be ideal ingredients in the field of functional foods because of their high antioxidant and antihypertensive activity, along with their low antigenicity (Jian-xin and Zheng 2009). Therefore, the interest of food, pharmaceutical, and cosmetic industries in the application of collagen hydrolysates has grown during the last two decades (Korhonen and Pihlanto 2006). Collagen hydrolysates can be used as seasonings, non-allergic preservatives for drugs, ingredients for dietary materials and parentally fed products (Nakayama et al. 2000), and antioxidant and antimicrobial agents (Gómez-Guillén et al. 2011) and for treating diseases such as collagen-induced arthritis (Zhu et al. 2007).

Collagen peptides were reported to affect lipid absorption and metabolism in rats (Saito et al. 2009), to reduce proinflammatory cytokine production in mice (Zhuang et al. 2009

and to improve bone mineral density in osteoporotic rats (Beynen et al. 2010) and joint disease in dogs (Watanabe-Kamiyama et al. 2010). Recently, some collagen peptides were reported to act protectively against ultraviolet radiation-induced damage on mice skin (Zhuang et al. 2009).

Some studies were performed to confirm the in vivo biological activity of collagen peptides, and some convincing data were obtained in animal models (Zhu et al. 2007) and humans (McAlindon et al. 2011). Because of this ability, collagen hydrolysates, mainly obtained from mammalian sources, have long been used in pharmaceutical and dietary supplements (Gómez-Guillén et al. 2011).

The most common way to generate bioactive peptides is through enzymatic hydrolysis of whole protein molecules. The bioactivity of such hydrolysis-generated peptides appears to be inherent to size and specific amino acid sequences (Korhonen 2009). Proteolytic enzymes from various sources (animal, plant, and microbes) have been successfully employed in the production of bioactive peptides (Korhonen and Pihlanto 2006).

Since microorganisms can be cultured through well established methods to produce high amounts of enzymes, microbial proteases are attractive biocatalysts for commercial production of protein hydrolysates and bioactive peptides. In this sense, investigation of novel proteases is essential to obtain protein hydrolysates with the desired properties. The collagenolytic fungus *Penicillium aurantiogriseum* URM 4622 was shown to produce a biotechnologically relevant extracellular serine collagenase (Lima et al. 2011a, b). In the current study, a collagenase preparation from this strain was employed to hydrolyse collagen type I. The molecular weight distribution of the collagen peptides obtained under optimal conditions of hydrolysis was identified, and the biological (antioxidant and antimicrobial) activities of the protein hydrolysates were assessed to evaluate their potential for food applications.

Material and methods

Microorganism and culture medium

P. aurantiogriseum (URM 4622) strain was obtained from the Culture Collection of the Department of Mycology of the Federal University of Pernambuco (Micoteca). The strain was maintained at 4 °C in malt extract agar medium, consisting of 0.5 % (w/v) malt extract, 0.1 % (w/v) peptone, 0.5 % (w/v) glucose, and 1.5 % (w/v) agar.

The soybean flour medium described by Lima et al. (2011b), composed of 1.6 % (w/v) filtered soybean flour (SF), 0.1 % (w/v) NH_4Cl, 0.06 % (w/v) $MgSO_4 \cdot 7H_2O$, 0.435 % (w/v) K_2HPO_4, 0.01 % (w/v) glucose, and 1.0 % (v/v) mineral solution, was used for collagenase production. The mineral solution was prepared by adding, per 100 mL of distilled water, 100 mg $FeSO_4 \cdot 7H_2O$, 100 mg $MnCl_2 \cdot 4H_2O$, 100 mg $ZnSO_4 \cdot H_2O$, and 100 mg $CaCl_2 \cdot H_2O$. This fermentation medium was sterilized in an autoclave at 121 °C for 20 min.

Collagenase production

Inoculum spores were produced in agar plates containing a cell culture grown for 5 days at 28 °C, and then suspended in 3 mL of a 0.9 % (w/v) NaCl and 0.01 % (v/v) Tween 80 solution previously sterilized at 121 °C for 20 min. After inoculation of 300 μL spore suspension (10^6 spores/mL), fermentation was carried out at 24 °C and 200 rpm in 250-mL Erlenmeyer flasks containing 50 mL of the soybean flour medium (pH 7.21). The broth obtained at the end of fermentation (72 h) was vacuum filtered through 0.45-μm nitrocellulose membranes to remove the mycelium. As the target collagenase is extracellular, the filtrate was analyzed to determine the final protein concentration and collagenase activity, and then used as a source of the enzyme (referred to as crude extract). The crude extract was used for collagenase purification.

Collagenase partial purification by aqueous two-phase system (ATPS)

The experiments of collagenase purification were performed according to Lima et al. (2013). A 40 % (w/w) phosphate solution was prepared at room temperature (25±1 °C) by mixing the amounts of dibasic and monobasic sodium phosphates needed to reach pH 6.0. The required amounts of this solution were mixed with 60 % (w/w) 1,500 g/mol PEG solution. The resulting solutions were then added to 15-mL graduated tubes. The crude extract (120 U) corresponding to 20 % (w/w) of the total mass was subsequently added, along with enough water to give a 10-g system. After 1.0 min of vortex shaking, the two phases were left to separate by settling for 120 min. Since a previous work demonstrated that the crude extract contained a collagenase (Lima et al. 2013), the PEG-rich phase obtained by ATPS (60 U) was used to hydrolyse collagen.

Azocoll assay for collagenase activity determination

The Azo dye-impregnated collagen (Azocoll; Sigma Chemical Co., St Louis, MO) assay was carried out according to a modified version of the method developed by Chavira et al. (1984). Azocoll was washed and suspended in 0.05 M Tris–HCl buffer (pH 7.2) containing 1 mM $CaCl_2$ up to a final concentration of 0.5 % (w/v). Subsequently, 150 μL of cell-free filtrate and 150 μL of buffer were mixed with 270 μL of azocoll suspension in a 2.0-mL reaction tube. The reaction

tubes were incubated at 37 °C in a water bath under agitation. After 3 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000×*g* and 4 °C for 20 min (model KR-20000 T; Kubota Seisakusho, Tokyo, Japan). The absorbance of the supernatant was measured at 520 nm by a UV–vis spectrophotometer (model B582; Micronal, São Paulo, Brazil). One unit of enzyme activity (U) was defined as the amount of enzyme, per millilitre, that led, after 3 h of incubation, to an absorbance increase of 0.1 at 520 nm, because of the formation of azo dye-linked soluble peptides.

Protein determination

Protein concentration was determined according to Smith et al. (1985), using bovine serum albumin as a standard.

Assay for collagen hydrolysis

The experiments were performed according to Endo et al. (1987). The standard reaction mixture, containing different concentrations (2.5, 5.0, and 7.0 mg/mL) of collagen (type 1, bovine Achilles tendon; Sigma Chemical Co., St Louis, MO) in 5 mL of different buffers (0.05 M citrate (pH 6), 0.05 M Tris–HCl (pH 7 and 8)), was incubated with 1 mL of PEG-rich phase obtained by ATPS (containing 60 U of collagenase) at different temperatures (25, 37, and 49 °C). After 18 h of incubation, the samples were chilled in ice for 5 min to stop the reaction and centrifuged at 10,000×*g* and 4 °C for 20 min (model KR-20000 T; Kubota Seisakusho, Tokyo, Japan). The degree of collagen hydrolysis (DH) was quantified by assessing the hydroxyproline content (μg/mL) in the supernatant by the method of Creemers et al. (1997). The supernatant (peptide fraction) was further collected and concentrated. Its peptide profile was determined, and its antimicrobial and antioxidant activities were tested.

Effect of process parameters on the DH

A 2^3 full factorial design was used to study their effects and interactions on the DH. The levels of these variables in the full factorial design were chosen based on previous results on the collagenolytic serine proteinase characterization conducted by our research-group (Lima et al. 2011a). As far as the time is concerned, a hydrolysis time of 18 h was fixed, because it was previously selected as the optimum time for collagen hydrolysis by this enzyme (Lima et al. 2013). Eight assays, with all possible combinations of the factor levels, were performed. The real and coded values selected for the three factors are given in Table 1.

Table 1 Matrix of the 2^3 full factorial design combinations (actual levels), observed response values and statistically main effects on the degree of hydrolysis (DH) and interactions. Best result is in boldface

Assay[a]	pH	T (ºC)	S_o (mg/ml)	DH (μg/ml)
1	6	25	2.5	0.562
2	8	25	2.5	2.096
3	6	49	2.5	0.23
4	8	49	2.5	1.963
5	6	25	7.5	0.938
6	8	25	7.5	4.655
7	6	49	7.5	0.857
8	8	49	7.5	3.785
9	7	37	5.0	2.749
10	7	37	5.0	2.896
11	7	37	5.0	3.057
12	7	37	5.0	2.915
Factor				Effect on DH
pH				2.47
T				–0.35
S_o				1.34
pH*T				–0.14b
pH*So				0.84
T*So				0.12b

[a] Results refer to 18-h of incubation

[b] Not significant values. S_o=substrate (collagen) concentration; T=hydrolysis temperature

For model fitting, the actual factor levels were converted into coded, dimensionless values according to the equation:

$$X_1 = \frac{X_1 - X_0}{\Delta X_i / 2} \quad (1)$$

where x_i are the coded value corresponding to the actual values X_i, X_0 is the average of the two extreme levels, and ΔX_i is the range of variation of the i^{th} factor.

The full model for a 2^3 design includes a constant term, three main effects, three two-factor interactions, and one three-factor interaction. The following linear regression model was employed to predict the response:

$$y = b_0 + \sum b_i x_i + \sum b_y x_i x_j \quad (2)$$

where $\hat{y}$ is the predicted response, b_0 is the constant term, b_i are the linear coefficients, b_{ij} are the interaction coefficients, and x_i and x_j are the coded values of the independent variables. The relative significance of the main and interaction coefficients was assessed from the error estimate based on the third-order term, at the 95 % confidence level. All statistical and graphical analyses were carried out using the Statistica 8.0 software (StatSoft Inc., Tulsa, OK).

Peptide profile by mass spectrometry and amino acid sequence (MALDI TOF-MS/MS)

Peptide profiles were analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) using an Autoflex III mass spectrometer (Bruker Daltonics, Bremen, Germany), equipped with a 355 nm Nd:YAG laser. The sample was mixed with the matrix solution consisting of 10 mg/mL α-cyano-4-hydroxycinnamic acid in 50 % acetonitrile with 0.3 % trifluoroacetic acid. Then, 0.5 μL of this mixture were spotted onto a MALDI target plate (MTP 384 ground steel, Bruker Daltonics, Bremen, Germany) and left to dry at room temperature. Mass spectra were acquired in positive reflectron mode with an acceleration voltage of 19 kV and a laser frequency of 100 Hz. The ion detection range was m/z 700–4480. After that, the selected parent ions were fragmented using LIFT mode. External calibration was performed using a standard mixture of peptides (Bruker Daltonics). Data were acquired by the Flex Control software, and spectra processed by the Flex Analysis one (Version 3.0, Bruker Daltonics). The obtained amino acid sequences were compared with sequences in the protein database using the NCBI BLAST program (www.ncbi.nlm.nih.gov).

Determination of the antimicrobial activity of hydrolysates

The antimicrobial activity of potential bioactive peptides contained in type I collagen hydrolysates generated by the *P. aurantiogriseum* collagenase was tested by the minimum inhibitory concentration (MIC) method as described by the Standard Clinical and Laboratory Standards Institute (CLSI. Performance standards for antimicrobial susceptibility testing. CLSI approved standard M100-S15. Wayne and PA 2003). The microorganisms used were *Enterococcus faecalis* ATCC 6057, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Klebsiella pneumoniae* ATCC 29665 and *Staphylococcus aureus* ATCC 6538. These strains were obtained from the Culture Collection of the Department of Antibiotics of the Federal University of Pernambuco. The amount of hydrolysate tested against these microorganisms ranged from 0.5 to 5 mg/mL. The negative control was performed by adding the peptides to the culture medium, whereas the positive one without any peptide in the medium containing the inoculated bacteria.

Determination of the antioxidant activity of hydrolysates

Experiments were carried out using the ABTS assay (Re et al. 1999) that implies the formation of the 2,2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical chromophore by oxidation of ABTS with potassium persulphate. The ABTS radical cation was produced by reaction of 7 mmol/L ABTS stock solution with 140 mmol/L potassium persulphate (final concentration), and allowing the mixture to stand in the dark for at least 16 h at room temperature before use. For the assay, the ABTS•+solution was diluted with ethanol to an absorbance of 0.7±0.02 at 734 nm. A 30-μL sample (containing 5–50 mg/mL of hydrolysate) was mixed with 1 mL of diluted ABTS•+solution. The absorbance (734 nm) reading was taken after 6 min. Trolox (6-hydroxy-2,5,7,8-tetrametichroman-2-carboxylic acid) was also used as a reference standard. The negative control was done by adding buffer instead of the peptides. The percentage inhibition of absorbance at 734 nm was calculated and plotted as a function of the concentration of the reference antioxidant (Trolox).

Results and discussion

Effect of process parameters on the DH

The purpose of the first step of this work was to identify the influence of pH, substrate (collagen) concentration, and hydrolysis temperature on the DH by *P. aurantiogriseum* collagenase, according to the 2^3 factorial design.

Table 1 lists the matrix and the observed response values of the experiments, which were performed for an incubation time of 18 h, according to previous findings (results not shown). The highest DH (4.655 μg/mL) was obtained in the assay 6, which was carried out at pH 8.0 and 25 °C using a collagen concentration of 7.5 mg/mL.

A full factorial model was fitted to the DH data, which included three main effects, three two-factor interactions and a three-factor interaction. The statistically significant estimates of the effects (at the 95 % confidence level) are listed in Table 1. A simplified model based only on the effects of Table 1 showed a small lack of fit (2.88). A determination coefficient (R^2) of 0.86 was obtained for the DH, indicating that the statistical model can explain 86 % of variability in response, and that the statistical model could significantly represent the actual relationships between the parameters chosen. The values of the significant effects indicate that, on an average, DH was the highest when factors 1 (pH) and 3 (collagen concentration) were at their highest levels and factor (2) (hydrolysis temperature) was at its lowest level (Table 1). As shown in Table 1, pH exerted the highest significant effect (2.47) followed by collagen concentration (1.34), while temperature exerted the lowest effect (−0.35), all of them within a 95 % confidence interval ($p<0.05$). In addition, a significant positive interaction was also observed between pH (1) and collagen concentration (3) (Table 1), which means that an increase in pH with a simultaneous increase in collagen concentration led to an increase in DH.

These results are similar to those reported by Jian-xin and Zheng (2009), who observed that the major-minor order of

factors affecting the degree of collagen hydrolysis was pH, collagen concentration, and temperature. Similarly, Zhuang et al. (2012) observed that pH exerted the highest significant effect on the enzymatic hydrolysis of collagen. Moreover, Kong et al. (2011) observed that the degree of collagen hydrolysis is dependent on the collagen concentration and temperature of hydrolysis, and that the highest degree of collagen hydrolysis occurs at the highest collagen concentration and the lowest temperature; on the other hand, the pH did not exert any significant influence.

Since the correlation between DH and bioactivity may occur or not, depending on the properties of the hydrolysed protein, only the most favourable condition (assay 6) for collagen hydrolysis was set aside for further study. A confirmation experiment was run in triplicate under these conditions, yielding similar DH (4.63±0.09 μg/mL) (Table 1). Such a low standard deviation confirms the high reproducibility of this result. All subsequent studies (peptide profile, antimicrobial and antioxidant activities) were carried out using the triplicates.

Peptide profile by mass spectrometry and amino acid sequence (MALDI-TOF MS/MS)

The molecular weight ranges of digested collagen type I were analyzed with MALDI-TOF MS (Fig. 1a, b). Peptides from collagen I have molecular weights less than 11 kDa, with several peptides less than 2 kDa. This result demonstrates that the collagenase produced by *P. aurantiogriseum* effectively hydrolysed collagen and produced several small collagen peptides. Thus, its biological activity should be further investigated.

To identify the cleavage sites within the collagen triple helix, the amino acid sequences of three peptides (919.41 kDa, 1264.60 kDa, and 1676.97 kDa) were determined by MALDI-TOF MS/MS. The experimentally obtained sequences (Fig. 1a) were compared with the sequences for the α_1 and α_2 chains of bovine type I collagen. The sequences Gly-Asp-Hyl-Gly-Glu-Thr-Gly-Glu-Gln-Gly-Asp-Arg and Phe-Leu-Pro-Gln-Pro-Pro-Gln-Glu-Lys-Ala-His-Asp-Gly-Gly-Arg were identical to sequences in the α_1 chain commencing at Gly1093 and Phe1198, respectively. The sequence Phe-Asp-Gly-Asp-Phe-Tyr-Arg was identical to a sequence in the α_2 chain commencing at Phe1109. The cleavage pattern of the collagenase produced by *P. aurantiogriseum* differs from that of other known collagenases. For example, the cleavage sites of the *Clostridium histolyticum* collagenase were identified at amino acid residues 397 and 406 in both the α_1 and α_2 chains (French et al. 1992), while those of collagenase from ginger at amino acid residues 286, 499 and 712 in both the α_1 and α_2 chains (Kim et al. 2007).

Antimicrobial activity of collagen hydrolysates

Table 2 lists the results on the ability of collagen hydrolysates to inhibit the growth of many bacteria. Collagen hydrolysed for 18 h by the *P. aurantiogriseum* collagenase preparation (carried out in triplicate) showed antibacterial activity against one Gram-positive bacterium (*S. aureus* - MIC 0.55 mg/mL) and two Gram-negative bacteria (*E. coli* - MIC 0.625 mg/mL and *B. subtilis* - MIC 5 mg/mL). This experiment was performed in triplicate, and all the three runs showed the same behavior.

Corrêa et al. (2011) also used a microbial protease to obtain peptides with antimicrobial activity; however, those peptides were only able to inhibit the growth of two Gram-positive bacterial strains (*Bacillus cereus* ATCC 9634 and *Corynebacterium fimi* NCTC 7547) using a much higher concentration (250 mg/mL) than that used in the present work. Similarly, Arruda et al. (2012) observed that a concentration of 50 mg/ml was needed to inhibit the growth of *S. aureus* and a concentration of 62.5 mg/mL to inhibit those of *E. coli* and *B. subtilis*. These values were about 100 times higher than those used in the present study to inhibit the growth of *S. aureus* and *E. coli* and 12 times higher than that used for *B. subtilis*.

As is well known, the cell envelope of Gram-negative bacteria is both structurally and functionally more complex than that of Gram-positive ones. Since in the present study both Gram-positive and Gram-negative bacteria were inhibited, these differences in bacterial membrane composition suggest that the mode of action of our collagen peptides probably involved the cytoplasmic membrane or specific targets within the cell. According to Floris et al. (2003), differences in membrane composition have implications for the mode of action and the specificity of antibacterial compounds.

Published information on the antimicrobial properties of hydrolysates or peptides from collagen or gelatin is very scarce. As previously described in Section 3.2, the molecular weights of the peptides obtained from the hydrolysis of collagen type I by *P. aurantiogriseum* collagenase were <11 kDa, and several peptides were <2 kDa. Gómez-Guillén et al. (2011) reported antimicrobial activity in peptide fractions from tuna and squid skin gelatins within a range of 1–10 kDa and <1 kDa. The hydrolysates were tested using the agar diffusion assay against 18 strains of bacteria (both Gram-positive and Gram-negative), among which *Lactobacillus acidophilus*, *Bifidobacterium animalis* subsp. *lactis*, *Shewanella putrefaciens* and *Photobacterium phosphoreum* were the most susceptible species.

According to Gómez-Guillén et al. (2011), the reduced molecular weight of the peptide fractions (which was related to the elimination of aggregates), better exposure of the amino acid residues and their charges, and structure acquisition may facilitate the interaction with bacterial membranes.

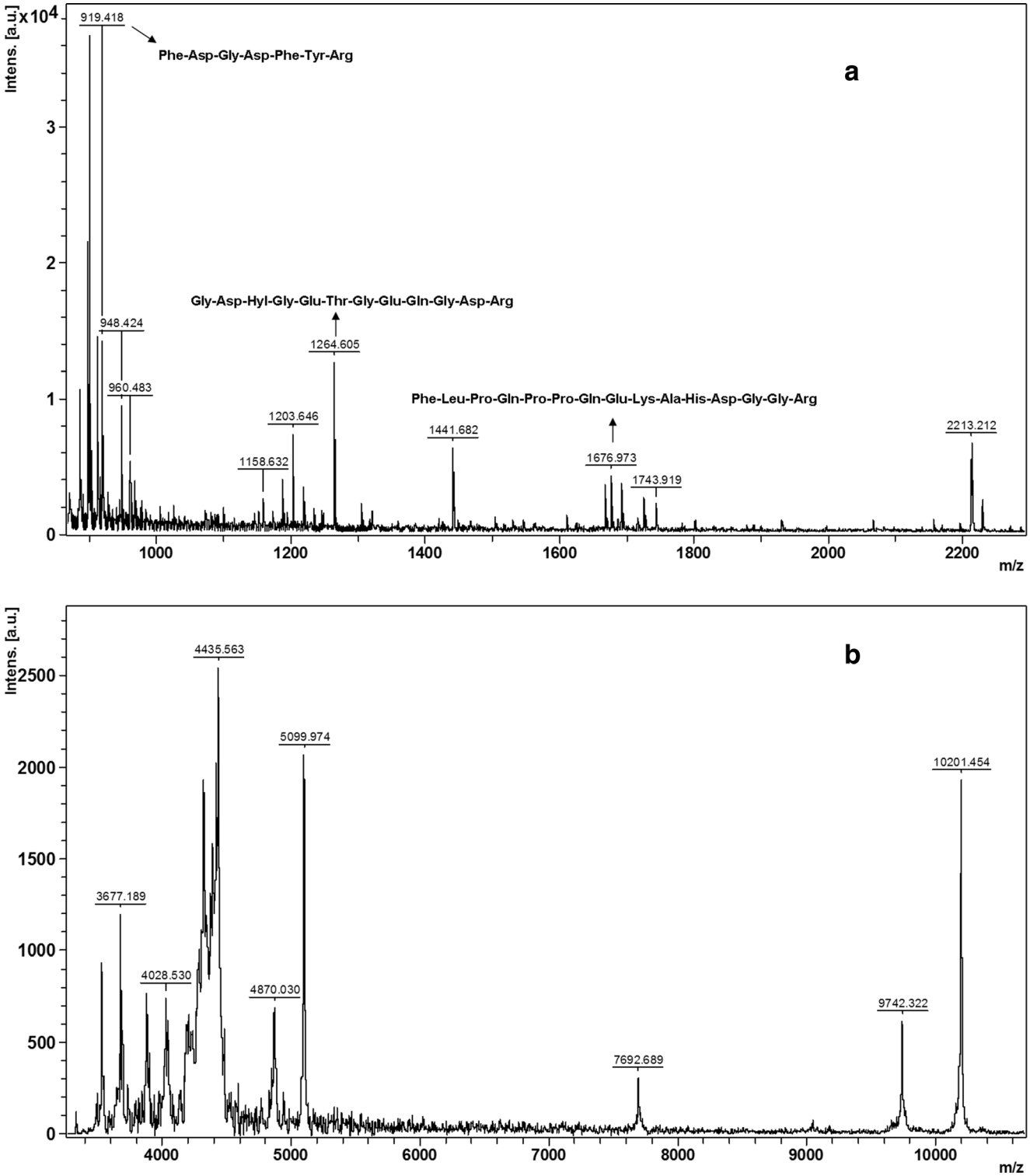

Fig. 1 Peptide profile by mass spectrometry and amino acid sequence (MALDI TOF- MS/MS). **a** Mass spectrum in the range of 0–3 kDa. **b** Mass spectrum in the range of 3–20 kDa

The three peptides sequenced in the present study (GDKGETGEQGDR, FLPQPPQEKAHDGGR, and FGGDFYR) had hydrophobic amino acids in their sequences, which would have allowed peptides to enter the bacterial membrane. The relationship between peptide characteristics and antimicrobial activity has not yet been clearly understood. Several factors such as amino acid composition, sequence, molecular weight and type of bacteria need to be considered.

Table 2 Determination of the minimal inhibitory concentration (MIC) of collagen peptides against *Enterococcus faecalis*, *Bacillus subtilis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae* and *Staphylococcus aureus*

Peptides concentration (mg/ml) Microorganisms	5.0	2.5	1.6	1.25	1.0	0.83	0.71	0.625	0.55	0.5
Enterococcus faecalis (ATCC 6057)	–	–	–	–	–	–	–	–	–	–
Escherichia coli (ATCC 25922)	+	+	+	+	+	+	+	+	–	–
Pseudomonas aeruginosa (ATCC 27853)	–	–	–	–	–	–	–	–	–	–
Bacillus subtilis (ATCC 6633)	+	–	–	–	–	–	–	–	–	–
Klebsiella pneumonae (ATCC 29665)	–	–	–	–	–	–	–	–	–	–
Staphylococcus aureus (ATCC 6538)	+	+	+	+	+	+	+	+	+	–

(+) presence of growth inhibition; (–) absence of growth inhibition

Thus, both the sequence and concentration of the peptide and the composition of the bacterial membrane would influence the mode of interaction (Di Bernardini et al. 2011).

Antioxidant activity of collagen hydrolysates

The antioxidant activity of the collagen hydrolysates (carried out in triplicate) determined using the radical ABTS at seven different concentrations is shown in Table 3. It can be seen that the antioxidant activity significantly increased with increasing the peptides concentrations in the collagen hydrolysate (Table 3) and reached a maximum value of 84.7 %±0.24 at a concentration of 50.0 mg/ml. Jia et al. (2010) applied various microbial proteases to produce collagen peptides with antioxidant activity, and the highest antioxidant activity (44.09 %±1.37) was obtained with protease produced by *Bacillus licheniformis*.

Peptides and protein hydrolysates obtained from the proteolysis of various food proteins are reported to possess antioxidant activities. Antioxidant mechanisms include radical-scavenging (both hydrogen-donating capability and free radical quenching) activity, inhibition of lipid peroxidation, metal ion chelation, or a combination of these properties (Sarmadi and Ismail 2010). Antioxidant activities might protect biological systems against damage related to oxidative stress in human diseases. These antioxidant peptides and hydrolysates might also be employed to prevent oxidation reactions (such as lipid peroxidation) that lead to the deterioration of foods and foodstuffs (Hogan et al. 2009).

Table 3 Antioxidant activity of collagen peptides

Peptides concentration (mg/ml)	ABTS radical scavenging activity (%)
50.0	84.7±0.24
40.0	82.7±0.91
20.0	73.0±0.49
10.0	45.4±1.49
8.0	43.0±0.18
6.7	40.5±0.25
5.0	37.7±0.78

The results of this study suggest that the use of microbial enzymes obtained by fermentation could be a good alternative method for producing collagen protein hydrolysates and collagen-derived peptides with antioxidant activity (Gómez-Guillén et al. 2011). Functional foods with such natural antioxidants may in fact be potentially employed without the toxic side effects associated with the use of synthetic equivalents (Gómez-Ruiz et al. 2008).

Conclusion

This study describes a simplified strategy to hydrolyse collagen type I by using collagenase produced by *P. aurantiogriseum* and purified by ATPS. Compared to the crude extract, the use of ATPS allowed obtaining a more concentrated and more purified enzyme preparation, using only one purification step. Furthermore, the obtained enzyme preparation could be applied directly to the hydrolysis of collagen in the presence of PEG, making unnecessary the application of a further extraction step. The most significant variables for collagen hydrolysis were singled out, along with their interactions. Collagen hydrolysis was improved when the collagen concentration and pH were raised, and the temperature was lowered. The molecular weight distribution of the collagen peptides obtained under the most favourable hydrolysis conditions demonstrated the presence of a large proportion of low-molecular-weight peptides. The peptides with antioxidant and antimicrobial activities were produced through hydrolysis with a novel microbial collagenase preparation. The bioactivities of the protein hydrolysates could have resulted from the synergistic effect of different peptides within the mixture. The physicochemical characterization and properties of collagen hydrolysates are under investigation

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