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Effects of enzymatic hydrolysis (Flavourzyme®) assisted by ultrasound in the structural and functional properties of hydrolyzates from different bovine collagens

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Abstract

This study aimed at evaluating the functional (antioxidant and antimicrobial activity) and structural properties of different bovine collagen hydrolysates obtained through ultrasound assisted enzymatic hydrolysis (Flavourzyme®). The use of previous or simultaneous ultrasound strengthened the enzymatic hydrolysis of bovine collagen samples, generating higher number of bands in wave number (cm^{-1}). The best treatment was the enzymatic hydrolysis, while for the powder fiber and hydrolysate, it was the ultrasound hydrolysis. The sample that showed the highest inhibiting action to the gram-negative bacteria *Salmonella choleraesuis* growth and gram-positive bacteria *Staphylococcus aureus* hydrolyzed with enzyme and simultaneous ultrasound. Therefore, the enzymatic hydrolysis (Flavourzyme®) was concluded to enable the hydrolysis of samples, providing structural rupture and better functionality to the different bovine collagen hydrolysates.

Keywords: antioxidant activity; antimicrobial activity; FTIR; hydrolysis degree; ultrasound.

Practical Application: The hydrolysis providing better functionality to the different bovine collagen hydrolysates.

1 Introduction

The enzymatic hydrolysis through animal or vegetable proteases applied to bovine slaughtering by products makes it possible to obtain collagen originated peptides, aggregating value to these residues (Gómez-Guillén et al., 2011; Khan et al., 2011; Di Bernardini et al., 2011; Selvakumar et al., 2012; Zhang et al., 2013). The enzyme Flavourzyme® has been widely used to obtain protein hydrolysates with functional properties (Kristinsson & Rasco, 2000; Yust et al., 2013; Castro & Sato, 2015; Ambigaipalan et al., 2015). It can act under neutral or slightly acid conditions of hydrolysis, and it is a complex fungal protease which originates from the submerged fermentation of a specific *Aspergillus oryzae* strain without genetic modification (Slizyte et al., 2005; Yust et al., 2013). Ultrasound has been used to change the structure and functional properties of food proteins (Chandrapala et al., 2011; Arzeni et al., 2012a), mainly proteins of animal origin (O'Sullivan et al., 2015). The use of ultrasound as a previous treatment or simultaneous to the hydrolysis process provokes the rupture of the proteins' tertiary and quaternary structures; this is provoked by the effects of cavitation in the medium, consequently these structural changes make it easier for the enzyme to access the protein structure, provoking increase in the hydrolysis and the bioactivity (Ozuna et al., 2015). The objective of this study was to evaluate the effect of ultrasound assisted enzymatic hydrolysis (Flavourzyme®) on the functional and structural properties of different bovine collagen hydrolysates.

2 Materials and methods

Different bovine collagens were used to obtain the different protein hydrolysates, which are shown in Table 1. The Flavourzyme 1000L® (Novozymes®) was supplied by the company Tovanni Benzaquen Ingredients (São Paulo, SP, Brazil) and the remaining reagents of analytical degree (PA) were purchased from the company Sigma Aldrich Basil Ltda. (São Paulo, SP, Brazil).

2.1 Hydrolysis treatments

The enzymatic reactions were performed following the methodology employed by Schmidt & Salas-Mellado (2009) with 8% enzyme was used. The following equipment was used for the hydrolysis reactions: ultra thermostatic bath (Model SL152, 2000 W power, SOLAB, Piracicaba, SP, Brazil), ultrasonic bath (Model ECO-SONICS – q3.8/40a, 88w power and 40 KHz frequency, ULTRONIQUE, SERVYLAB, São Leopoldo, RS, Brazil) and centrifuge COLEMAN (Model 90 – 1, Santo André, SP, Brazil). The hydrolysates were lyophilized (lyophilizer TERRONI, Model LS 3000, São Carlos, SP, Brazil). For the EHU treatment the hydrolysis process total time was 2 hours, while the UEH treatment took 4 hours (Table 2).

2.2 Hydrolysates structural characterization

A Fourier-transform infrared spectrophotometer (FTIR) Shimadzu IR Prestige-21 (Shimadzu Corporation, Kyoto, Japan) was used and the spectra collected in the range 4000-400 cm^{-1} according to the methodology described by Demiate et al. (2000).

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Table 1. Different types of bovine collagen used to obtain protein hydrolysates.

Types of collagen	Natural fiber	Powder fiber	Gelatin 1	Gelatin 2	Hydrolysate 1	Hydrolysate 2
Name/abbrev.	Fibra/FB	Fibra pó/FP	Gelita®/GEL	Rousselot®/ROU	Peptiplus®/PTL	Peptan B®/PEP
Manufacturer	Novaprom Food Ingredients Ltda (Lins – SP - Brazil)	Novaprom Food Ingredients Ltda (Lins – SP- Brazil)	Gelita do Brasil Ltda (Cotia – SP- Brazil)	Rousselot Gelatinas do Brasil Ltda (Amparo – SP-Brazil)	Gelita do Brasil Ltda (Cotia – SP-Brazil)	Rousselot Gelatinas do Brasil Ltda (Amparo – SP-Brazil)
Description	Particle size between 1.80 and 1.92 mm, raw material: bovine skin, alkaline treatment, pH between 7 and 9.5	Particle size between 0.45 and 0.57 mm, raw material: bovine skin, alkaline treatment, pH between 7 and 9.5	Raw material: leather or other sources, partial alkaline hydrolysis (severe treatment), 236 g Bloom; 40 mP viscosity; 11% humidity; pH 5.6; <= 2% ash	Raw material: leather or other sources, partial alkaline hydrolysis (severe treatment), 250g Bloom; 3.8 mP viscosity; 12.4% humidity; pH 5.5; <=2% ash	Raw material: bovine leather/ swine skin, chemical + enzymatic hydrolysis, 39 mP viscosity; 5.9% humidity; 92.1% protein; pH 5.7; <=2% ash	Raw material: bovine leather/ swine skin, chemical + enzymatic hydrolysis, 4 mP viscosity; 7.2% humidity; 92.15% protein; pH 6; 0.65% ash

Source: Rousselot (2014); Gelita (2014).

Table 2. Treatments of hydrolysis used to obtain protein hydrolysates from different bovine collagens.

Abbr.	Description of treatments	Enzyme	Ultrasound	T °C	Time (h)	pH
CC	Crude collagens	No	No	50	2	7.0
HU	Ultrasound hydrolysis	No	Yes	50	2	7.0
EH	Enzymatic hydrolysis	Yes	No	50	2	7.0
EHU	Enzymatic Hydrolysis with simultaneous ultrasound	Yes	Yes	50	2	7.0
UEH	Previous ultrasound followed by enzymatic hydrolysis	Yes	Yes	50	4	7.0

2.3 Determination of the hydrolysates' hydrolysis degree and the antioxidant activity of hydrolysates

The method by Lowry et al. (1951) was used to determine the hydrolysis degree. The bovine albumin (Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil) was used as a standard for the method. The method DPPH by Brand-Williams et al. (1995) modified by Sánchez-Moreno et al. (1998) was used to evaluate the antioxidant activity. The spectrophotometer UV (Model UV-M51, BEL Photonics, SERVYLAB, São Leopoldo, RS, Brazil) was used for the analysis.

2.4 Determination of the antimicrobial activity of hydrolysates

The Minimal Inhibitory Concentration (MIC) was determined through the indirect method of bacterial growth in liquid medium (Pierozan et al., 2009) on Gram-negative (*Salmonella choleraesuis* – ATTC 10708) and Gram-positive (*Staphylococcus aureus* – ATTC 6538). MIC values were determined as triplicate runs.

2.5 Statistical evaluation

After obtaining the spectra through FTIR, each spectrum was treated with the absorbance normalized between 0 and 1, smoothed (15 points), baseline corrected and CO_2 zone removed using the Shimadzu IR solution 1.40 software. A chemometric approach composed of principal component analysis (PCA) was implemented in the Pirouette v. 4.0 (Infometrix®, Bothell, WA, USA). The analysis was performed in the spectrum region ranging from 500 to 1722 cm^{-1}, and the dataset was mean-centered and submitted to 1st derivative. PCA is a data reduction technique that was applied to verify the difference and to extract important information from FTIR spectra between the samples, and a 3D-dimensional scatter plot was built to project the samples (Zielinski et al., 2014). Experiment 1 evaluated which ultrasound application, previous or simultaneous, was better to the enzymatic treatment. The experiment analysis was performed in split plot with a completely randomized design, and the different collagens were distributed in the plots with ultrasound application (previous vs. simultaneous) and the interactions distributed randomly in the subplots within each plot, according to the statistical model (Equation 1):

$$Y_{ijk} = \mu + \alpha_i + (\alpha\gamma)_{ik} + \beta_j + (\alpha\beta)_{ij} + \varepsilon_{ijk} \quad (1)$$

where, Y_{ijk} = value observed in the i-th whole plot, k-th repetition, and j-th subplot; μ = overall mean of the response variable; α_i = fixed effect of i-th collagen; $(\alpha\gamma)_{ik}$ = residual effect of whole plot (error A); β_j = fixed effect of j-th ultrasound application; $(\alpha\beta)_{ij}$ = fixed effect of interaction between i-th collagen and j-th ultrasound application; ε_{ijk} = residual effect of the subplots (error B) or random effect associated to ijk-th observation, supposed $\varepsilon_{ijk} \overset{iid}{\sim} N(0, \sigma^2)$.

Experiment 2 evaluated the effect of the collagen, enzymatic concentration and the application or not of simultaneous ultrasound. The experiment analysis was carried out in split plot with completely randomized design, when the different collagens were distributed in plots and the enzyme concentrations (0 and 8%) and the use of ultrasound or not (US free vs. simultaneous US) and the interactions distributed randomly in the subplots

within each plot, according to the statistical model described in Equation 2:

$$Y_{ijkl} = \mu + \alpha_i + (\alpha\delta)_{il} + \beta_j + \gamma_k + (\beta\gamma)_{jk} + (\alpha\beta)_{ij} + (\alpha\gamma)_{ik} + (\alpha\beta\gamma)_{ijk} + \varepsilon_{ijkl} \quad (2)$$

where, Y_{ijkl} = value observed in i-th whole plot, l-th repetition and jk-th subplot; μ = overall mean of the response variable; α_i = fixed effect of i-th collagen; $(\alpha\delta)_{il}$ = residual effect of whole plot (error A); β_j = fixed effect of j-th enzymatic concentration; γ_k = fixed effect of k-th ultrasound application; $(\beta\gamma)_{jk}$ = fixed effect of the interaction between j-th enzymatic concentration and k-th ultrasound application; $(\alpha\beta)_{ij}$ = fixed effect of the interaction between i-th collagen and j-th enzymatic concentration; $(\alpha\gamma)_{ik}$ = fixed effect of the interaction between i-th collagen and k-th ultrasound application; $(\alpha\beta\gamma)_{ijk}$ = fixed effect of the interaction between i-th collagen, j-th enzymatic concentration and k-th ultrasound application; ε_{ijkl} = residual effect of the subplots (error B) or random effect associated to $ijkl$-th observation, supposed $\varepsilon_{ijkl} \overset{iid}{\sim} N(0,\sigma^2)$.

Data was submitted to the outliers investigation from the studentized residuals. Later on, they were submitted to the univariate variance analysis (ANOVA) through the GLM procedure, with averages adjusted through the ordinary least squares method with the command LSMEANS and compared by the minimum significant difference (test t) at 5% significance level. In addition, Spearman's correlation analysis between the variables under study was carried out. Statistical analyses were carried out in the application SAS®System for Windows™ version 9.4 (SAS Institute Inc., Cary, NC - USA).

3 Results and discussion

3.1 Structural characterization of hydrolysates

According to the results of the principal component analysis (PCA) the samples were separated into three distinct groups, considering the similarity of bands (Figure 1), explaining 92.41% of data variance. The enzymatic hydrolysis with simultaneous ultrasound provided this sample with the highest structural rupture, generating smaller peptides. The use of ultrasound only in the hydrolysis, which tends to attack the protein quaternary and tertiary structures, and also for the fact that the sample was a commercial hydrolysate. The partial rupture of secondary structures of the hydrolysates, which presented lower integral absorbance (area of bands) when compared to the origin of their proteins, suggesting that the collagen was hydrolyzed in polypeptides (Li et al., 2013). The use of previous or simultaneous ultrasound generating higher number of bands in wave number (cm^{-1}), however, it was not possible to confirm that peptides with antioxidant ability have been generated, since the ability of the peptide generated in capturing free radicals is what determines its antioxidant potential.

3.2 Effect of previous or simultaneous ultrasound on the enzymatic hydrolysis process

The ultrasound did not increase considerably the functionality do hydrolysates and the effect of the interaction collagen x ultrasound was significant ($p < 0.05$) for the variables protein and hydrolysis degree and not significant ($p > 0.05$) for

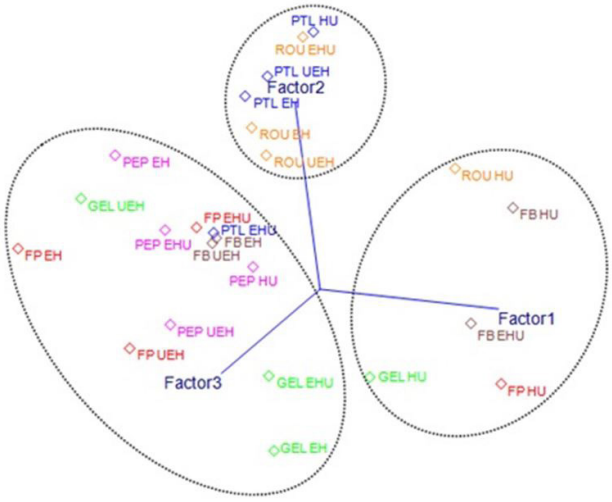

Figure 1. 3D-scatter plot obtained through PCA based on the 1722 to 500 cm^{-1} region. FB = collagen natural fiber; FP = powder collagen fiber; PEP = hydrolyzed 1 collagen; PTL = hydrolyzed 2 collagen; GEL = gelatin 1; ROU = gelatin 2; HU = ultrasound hydrolysis; EH = enzymatic hydrolysis; EHU = ultrasound assisted enzymatic hydrolysis; UEH = previously treated with ultrasound followed by enzymatic hydrolysis.

the antioxidant activity (results not shown). This might mean that the simultaneous ultrasound with the enzymatic hydrolysis did not favor the increase in the high functionality peptide production because during the hydrolysis process, the enzyme action might be hampered by the ultrasound effect. As it had been expected, there was a negative correlation ($p < 0.001$) between the values of protein (ptn) and hydrolysis degree (DH) ($r = -0.80$). The hydrolysis degree is directly linked to the enzyme action during the hydrolysis process, which in turn can be affected either positively or negatively by the conditions imposed to the medium, such as amount of substrate, ultrasound use and even the enzyme concentration. There was a negative correlation ($p < 0.05$) between the protein and antioxidant activity values ($r = -0.37$).

3.3 Total average of protein (ptn), Hydrolysis Degree (DH) and Antioxidant Activity (AA) from the evaluation of the previous and simultaneous ultrasound effect on the enzymatic hydrolysis

The use of simultaneous ultrasound in the enzymatic hydrolysis process generated lower protein content when compared to the use of previous ultrasound, consequently the hydrolysis degree value for the simultaneous ultrasound was higher than that for the previous ultrasound (Table 3), however, some caution in the hydrolysis process is important, since high hydrolysis degree values can positively or negatively affect the functional properties of hydrolysates (Kristinsson & Rasco, 2000; Chen et al., 2012).

The simultaneous ultrasound favored the enzymatic action in the hydrolysis presented lower residual protein content generating peptides with lower molecular mass, however, this does not mean that peptides with higher functionality have been

Table 3. Protein, hydrolysis degree and antioxidant activity total average for the samples of collagen hydrolysates, evaluating the ultrasound effect on the enzymatic hydrolysis.

	Ultrasound	
	Previous	Simultaneous
Protein, mg/mL	7.89[a]	4.33[b]
Hydrolysis Degree, %	0.00[b]	40.80[a]
Antioxidant activity, %	33.63[a]	38.01[a]

Averages on the same line with small different letters indicate significant difference between ultrasound with "t" test ($p < 0.05$).

generated. The enzyme access to the structure and the alteration in the conformation of proteins were made easier by the use of simultaneous ultrasound in the hydrolysis, causing an increase in the hydrolysis degree and the bioactivity of hydrolysates; however, this alteration depends on the nature of this protein and its denaturation and aggregation degrees (Arzeni et al., 2012a). Low values of residual protein were expected and indicated that the enzyme acted upon the protein structures generating smaller peptide fractions. The highest antioxidant activity value (38.0%) was found for the hydrolyzed simultaneous ultrasound. A different result was found by Knezevic-Jugovic et al. (2012) who compared the ultrasound and the high pressure processing with carbon dioxide effects on the proteolytic hydrolysis of egg white protein and the antioxidant activity of the hydrolysates obtained, the results found in that study, revealed that the combination of ultrasound as pre-treatment (1 hour, 30 kHz, pH 8.3) followed by the enzymatic hydrolysis with alcalase (50 °C and pH 8.0) increased the antioxidant activity of the hydrolysates obtained. The use of previous or simultaneous ultrasound to the enzymatic hydrolysis process of the samples gave similar the final antioxidant activity of the hydrolysates. The simultaneous ultrasound with the enzymatic hydrolysis provided the hydrolysates with higher protein hydrolysis, generating higher hydrolysis degree and higher antioxidant activity.

3.4 Effect of ultrasound, enzyme and different types of collagen for protein, hydrolysis degree and antioxidant activity

The effect of the interaction collagen × enzyme × ultrasound was significant ($p < 0.0001$). The variations of their average values are directly linked to the type of collagen, percentage of enzyme and use or not of the ultrasound in the hydrolysis process, and these might affect directly the functionality of peptides generated in the hydrolysis (Table 4).

There was negative correlation ($p < 0.001$) between the values of protein and hydrolysis degree ($r = -0.81$). Higher protein content resulted in lower hydrolysis degree, showing lower enzyme and ultrasound action in relation to the substrate (results not shown). Protein and antioxidant activity values presented negative correlation ($p < 0.0001$) ($r = -0.62$), indicating that higher or lower protein values are related to the variation in the antioxidant activity, this is due to the generation of high functionality peptides during the hydrolysis. The hydrolysis degree and antioxidant activity values presented positive correlation ($p < 0.01$) ($r = 0.41$), in which the functionality of the hydrolysates generated was increased by the higher rupture of the protein structure caused by the enzymatic hydrolysis simultaneous or not to the ultrasound.

Table 4. Probabilistic values to evaluate the effect of ultrasound (simultaneous), enzyme (0 and 8%) and the different types of collagen on the variables protein (mg/mL), hydrolysis degree (%) and antioxidant activity (%).

Effects	Ptn	DH	AA
Collagen	0.0001	0.0001	0.0001
Enzyme	0.0001	0.0001	0.0001
Ultrasound	0.0001	0.0001	0.4639
Enzyme × Ultrasound	0.0001	0.0001	0.0014
Collagen × Enzyme	0.0001	0.0001	0.0001
Collagen × Ultrasound	0.0120	0.0007	0.0063
Collagen × Enzyme × Ultrasound	0.0001	0.0001	0.0670
Average	6.19	16.43	32.68
VC	2.14	1.32	22.94

Ptn = protein; DH = hydrolysis degree; AA = antioxidant activity; VC (%) = variation coefficient.

3.5 Averages of protein, hydrolysis degree and antioxidant activity and the effect of the interaction ultrasound × enzyme × collagen

The crude FB sample presented protein average value equal 7.49 mg/mL, but when it was treated with 8% enzyme and simultaneous ultrasound its protein content was 4.17 mg/mL, representing a hydrolysis degree of 44.5%. The reduction in the protein value was provoked by the enzyme action along with the ultrasound, the latter provoked medium cavitation, facilitating the protein structural rupture and enzyme action (enzyme/substrate), consequently smaller protein fractions were generated. However, despite the enzyme and simultaneous ultrasound having provided this sample with higher hydrolysis degree, the antioxidant activity value found for this treatment it was similarly to the treatment using enzyme only (Table 5). This might be explained by the maximum generation, in the short term, of peptides with the elimination of DPPH radicals, which were then hydrolyzed in inactive sequences (García-Moreno et al., 2014). Probably the use of simultaneous ultrasound and enzymatic hydrolysis deviated the enzyme action upon the substrate, leading its action to bonds different from the usual ones, without rupture of the protein structure. The antioxidant activity of peptides is associated to the antioxidant power of amino acids present in the sequence. Its functionality is ascribed to the chelating ability and imprisonment of the lipid radical of the imidazole ring (Je et al., 2005; Chi et al., 2015). Peptides were also found in the antioxidant peptide sequences, such as alanine, leucine and glycine (Hsu, 2010). Despite the enzymatic hydrolysis treatment with simultaneous ultrasound having provided the sample with higher hydrolysis degree it did not result in higher antioxidant activity, showing that a protein hydrolysis that is too high might affect negatively the functionality of the hydrolysates, this is due to the generation of peptides which are not functional or present low function.

3.6 Antimicrobial potential of hydrolysates

To inhibit the gram-negative bacteria *Salmonella choleraesuis*, the sample that presented the best result was the FB EHU, with sample concentration below 10%, and for the gram-positive bacteria *Staphylococcus aureus,* the samples were PEP EHU, PEP UEH, ROU EHU, FB UEH, FB EH, FP EHU and FP EH, with sample concentration below 10% (results not shown). In a study carried out

Table 5. Protein, hydrolysis degree and antioxidant activity averages for the different samples of collagen hydrolysates.

Collagen**	Enzyme, %	Sim.US*		Enzyme average	Collagen average	Sim.US*		Enzyme average	Collagen average	Sim.US*		Enzyme average	Collagen average
		without	with			without	with			without	with		
		Protein, mg/mL				Hydrolysis degree, %				Antioxidant activity, %			
FB	**0**	7.49^{aA}	7.54^{aA}	7.52	6.22^{B}	0.00^{aB}	0.00^{aB}	0.00	17.3^{A}	5.32^{bB}	20.0^{aB}	12.7	28.6^{B}
	8	5.66^{aB}	4.17^{bB}	4.92		24.6^{bA}	44.5^{aA}	34.5		43.9^{aA}	45.3^{aA}	44.6	
Ultrasound average		6.58	5.86			12.3	22.2			24.6	32.7		
FP	**0**	8.64^{aA}	8.22^{bA}	8.43	7.37^{A}	0.00^{bB}	5.68^{aB}	2.84	17.0^{AB}	15.8^{bA}	28.7^{aA}	22.2	20.9^{B}
	8	6.75^{aB}	5.88^{bB}	6.32		25.4^{bA}	37.0^{aA}	31.2		24.7^{aA}	14.3^{aB}	19.5	
Ultrasound average		7.69	7.05			12.7	21.3			20.2	21.5		
PEP	**0**	6.84^{aA}	6.72^{aA}	6.78	5.65^{C}	0.00^{aB}	1.98^{aB}	0.99	16.0^{B}	35.8^{aA}	45.2^{aA}	40.5	42.0^{A}
	8	5.05^{aB}	4.00^{bB}	4.53		23.9^{bA}	38.0^{aA}	30.9		40.2^{aA}	46.8^{aA}	43.5	
Ultrasound average		5.95	5.36			11.9	20.0			38.0	46.0		
PTL	**0**	6.41^{aA}	6.44^{aA}	6.42	5.39^{D}	0.00^{aB}	0.00^{aB}	0.03	13.7^{C}	43.1^{aA}	30.0^{bB}	36.5	43.6^{A}
	8	4.80^{aB}	3.93^{bB}	4.36		21.6^{bA}	33.3^{aA}	27.4		52.2^{aA}	47.8^{aA}	50.0	
Ultrasound average		5.61	5.18			10.8	16.7			47.6	38.9		
GEL	**0**	7.46^{aA}	7.60^{aA}	7.53	6.16^{B}	0.00^{aB}	0.00^{aB}	0.00	17.9^{A}	21.4^{aB}	19.8^{aA}	20.6	29.4^{B}
	8	5.70^{aB}	3.89^{bB}	4.79		23.6^{bA}	47.9^{aA}	35.8		47.2^{aA}	29.2^{bA}	38.2	
Ultrasound average		6.58	5.75			11.8	23.9			34.3	24.5		
ROU	**0**	7.54^{aA}	7.49^{aA}	7.52	6.24^{B}	0.00^{aB}	0.61^{aB}	0.31	17.4^{A}	2.8^{bB}	25.6^{aB}	14.2	29.0^{B}
	8	5.69^{aB}	4.25^{bB}	4.97		24.8^{bA}	44.1^{aA}	34.5		46.0^{aA}	41.6^{aA}	43.8	
Ultrasound average		6.61	5.87			12.4	22.4			24.4	33.6		
Ultrasound general average		6.50	5.84			12.0	21.1			31.5	32.9		

**FB = collagen fiber; FP = powder collagen fiber; PEP = hydrolyzed collagen 2; PTL = hydrolyzed collagen 1; GEL = gelatin 1; ROU = gelatin 2; Sim.US* = simultaneous ultrasound. Averages on the same line with small different letters indicate significant difference between ultrasound with "t" test ($p < 0.05$).

by Soares (2013) using soybean cake protein hydrolysate obtained at pH 7.0 with the use of papain, against the gram-positive bacteria *Staphylococcus aureus*, a 3.753 mg/mL hydrolysate concentration was able to inhibit the bacterial growth. The use of simultaneous ultrasound in the enzymatic hydrolysis process favored the antimicrobial activity, which might be explained by the better peptide bond with the bacterial cellular membrane, inhibiting the growth of microorganisms.

4 Conclusion

The use of previous or simultaneous ultrasound strengthened the enzymatic hydrolysis of bovine collagen samples, generating higher number of bands in wave number (cm^{-1}). The best treatment was the enzymatic hydrolysis, while for the powder fiber and hydrolysate 2, it was the ultrasound hydrolysis. The sample that showed the highest inhibiting action to the gram-negative bacteria *Salmonella choleraesuis* growth and gram-positive bacteria *Staphylococcus aureus* hydrolyzed with enzyme and simultaneous ultrasound. Therefore, the enzymatic (Flavourzyme®) hydrolysis was the best treatment in the hydrolysis of samples, providing structural rupture and better functionality to the different bovine collagen hydrolysates.

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