Ciência Rural, Santa Maria, v.48: 03, e20170649, 2018
http://dx.doi.org/10.1590/0103-8478cr20170649
ISSNe 1678-4596
FOOD TECHNOLOGY

Enzymatic hydrolysis (pepsin) assisted by ultrasound in the functional properties of hydrolyzates from different collagens

Alessandra Roseline Vidal[1] Carine da Fonseca Cechin[1] Rogério Luis Cansian[2] Renius de Oliveira Mello[1] Michele Mantelli Schmidt[1] Ivo Mottin Demiate[3] Aniela Pinto Kempka[4] Rosa Cristina Prestes Dornelles[1*]

[1]Departamento de Tecnologia e Ciências dos Alimentos, Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil. E-mail: rosacrisprestesdornelles@outlook.com. *Corresponding author.
[2]Departamento de Engenharia de Alimentos, Universidade Regional do Alto Uruguai e das Missões (URI), Erechim, RS, Brasil.
[3]Departamento de Engenharia de Alimentos, Universidade Estadual de Ponta Grossa UEPG), Ponta Grossa, PR, Brasil.
[4]Departamento de Engenharia de Alimentos, Universidade Estadual de Santa Catarina (UDESC), Pinhalzinho, SC, Brasil.

ABSTRACT: *Enzymatic hydrolysis (pepsin) assisted with or without ultrasound in the functional properties of hydrolyzates from different collagens were analyzed. Degree of hydrolysis, antioxidant activity (DPPH) and antimicrobial activity (MIC) were assessed. The treatment that resulted in greater antioxidant activity for the fiber sample was with the use of 4% of enzyme and concomitant ultrasound (40.7%), leading to a degree of hydrolysis of 21.7%. For the powdered fiber sample the hydrolysis treatment with use of 4% of enzyme resulted in lower protein content (6.97mg/mL), higher degree of hydrolysis (19.9%) and greater antioxidant activity (38.6%). The hydrolyzates showed inhibitory capacity against gram-negative bacteria Salmonella choleraesuis and gram-positive bacteria Staphylococcus aureus. It can be concluded that enzymatic hydrolysis concomitant or not with the use of ultrasound increased the functionality of the fiber and powdered fiber samples, for the other samples its use as supplementary treatment was not productive, due to the worse results of antioxidant activity (DPPH) reported. However, it provided greater hydrolysis degree.*
Key words: *pepsin, degree of hydrolysis, antioxidant activity, antimicrobial activity, ultrasound.*

Hidrólise enzimática (pepsina) assistida por ultrassom nas propriedades funcionais de hidrolisados de diferentes colágenos

RESUMO: *Foram avaliados os efeitos da hidrólise enzimática (pepsina) assistida com ou sem ultrassom nas propriedades funcionais de hidrolisados de diferentes colágenos. Foi analisado o grau de hidrólise, a atividade antioxidante (DPPH) e a atividade antimicrobiana (MIC). O tratamento que possibilitou maior atividade antioxidante para a amostra fibra foi com a utilização de 4% de enzima e ultrassom concomitante (40,7%), levando a um grau de hidrólise de 21,7%. Para a amostra fibra pó o tratamento de hidrólise com uso de 4% de enzima resultou em menor teor de proteína (6,97mg/mL), maior grau de hidrólise (19,9%) e maior atividade antioxidante (38,6%). Os hidrolisados mostraram capacidade inibitória contra a bactéria gram-negativa Salmonella choleraesuis e gram-positiva Staphylococcus aureus. Pode-se concluir que a hidrólise enzimática concomitante, ou não, ao uso do ultrassom apresentou aumento da funcionalidade das amostras fibra e fibra pó. Para as demais amostras, sua utilização como tratamento complementar, a hidrólise não foi interessante, devido aos piores resultados de atividade antioxidante (DPPH) encontrados. Porém, proporcionou maior grau de hidrólise.*
Palavras-chave: *pepsina, grau de hidrólise, atividade antioxidante, atividade antimicrobiana, ultrassom.*

INTRODUCTION

Collagen can be found in various forms in tissues of multicellular organisms, being the dominant protein in connective tissue (LIU et al., 2010). The growing interest in the process of obtaining, fractioning and characterizing collagens and their derivatives (DECKER & PARK, 2010) is due to the functional properties of these compounds and to the possibility of using them as substituents of synthetic agents in various industrial processes (GOMÉZ-GUILLÉN et al., 2011). Enzymatic hydrolysis of collagen, obtained from by-products of the slaughter of bovines, with animal, plant and microbial proteases (GOMÉZ-GUILLÉN et al., 2011; KHAN et al., 2011) enables the obtainment of bioactive peptides, adding value to

Received 09.13.17 Approved 12.19.17 Returned by the author 03.01.18
CR-2017-0649.R1

these residues (BERNARDINI et al., 2011). Bioactive peptides execute certain biological activities, including antimicrobial, antioxidant and antihypertensive activities (LI et al., 2007; HERREGODS et al., 2011).

Types of bioactive peptides generated from a particular protein depend on two factors: the primary protein sequence of the substrate and the specificity of the enzyme used to generate such peptides (HARNEDY & FITZGERALD, 2012). Proteolytic enzymes are classified as endo and exopeptidases. Studies have shown that ultrasound can modify the functional and structural properties of food protein (CHANDRAPALA et al., 2011). However, this structural modification depends on the protein nature, of the degree of denaturation and of aggregation (ARZENI et al., 2012). Differences between the various types of by-products of collagen and the assessment of the effects of enzymatic hydrolysis (pepsin) assisted by ultrasound in the functional properties of protein hydrolyzates have not been fully exploited yet, which provides a large field for study. The objective of this study was to assess the effects of enzymatic hydrolysis (pepsin) assisted with or without ultrasound in the functional properties (antioxidant activity (DPPH) and antimicrobial activity (MIC)) of hydrolyzates of various collagens.

MATERIALS AND METHODS

Different commercial bovine/porcine collagen products were used to obtain the protein hydrolyzates, presented in table 1. Enzyme Pepsin 1:2.500 (powder, ≥400 units/mg protein, P7125, SIGMA®) and the other analytical grade reagents (PA) were acquired from Sigma-Aldrich Brasil Ltda company (São Paulo, SP, Brazil).

The different treatments used for to obtain of protein hydrolyzates from different collagen are evidenced in table 2. For the conducting of enzymatic reactions the methodology described by LIN et al. (2012) was used with modifications, in which 4% (p/p) of pepsin enzymes were tested. For the hydrolysis reactions the following equipment were used: ultra-thermostatic bath (SL152 model, 2000 W power, SOLAB, Piracicaba, SP, Brazil), ultrasonic bath (ECO-SONICS-Q – 3.8/40A model, 88W power and frequency of 40KHz, ULTRONIQUE, SERVYLAB, São Leopoldo, RS, Brazil) and COLEMAN centrifuge (90-1 model, Santo André, SP, Brazil). The hydrolyzates were lyophilized (lyophilizer TERRONI, LS3000 model, São Carlos, SP, Brazil). For the EHU (enzymatic hydrolysis with concomitant ultrasound) treatment the total time of the hydrolysis process was 3 hours, while for the UEH (previously treated with ultrasound and subsequent enzymatic hydrolysis) treatment was of 5 hours.

The methodology described by LOWRY et al. (1951) was used to determine the degree of hydrolysis of the hydrolyzates. The bovine albumin (Sigma-Aldrich Brasil Ltda, São Paulo, SP, Brazil) was used as the standard for the method. Protein of raw collagens (untreated) was used as basis for

Table 1 - Different collagens used to obtain the protein hydrolyzates.

Collagen type	Description	Manufacturer	Name/Acronym
Natural fiber	Particle size between 1.80 and 1.92mm; Extracted from bovine skin through alkaline treatment, pH between 7 and 9.5	Novaprom Food Ingredients Ltda (Lins – SP - Brazil)	Fiber/FB
Powdered fiber	Particle size between 0.45 and 0.57mm; Extracted from bovine skin through alkaline treatment, pH between 7 and 9.5	Novaprom Food Ingredients Ltda (Lins – SP- Brazil)	Powdered fiber/FP
Gelatin 1	Extracted from leather or from various raw materials through alkaline partial hydrolysis (drastic treatment); 236 g Bloom; 40mP viscosity; 11% humidity; 5.6 pH; < = 2% ashes	Gelita do Brasil Ltda (Cotia – SP- Brazil)	Gelita®/GEL
Gelatin 2	Extracted from leather or from various raw materials through alkaline partial hydrolysis (drastic treatment); 250g Bloom; 3.8mP viscosity; 12.4% humidity; 5.5 pH; < = 2% ashes	Rousselot Gelatinas do Brasil Ltda (Amparo – SP- Brazil)	Rousselot®/ROU
Hydrolyzate 1	Extracted from bovine leather/porcine skin through chemical + enzymatic hydrolysis; 39mP viscosity; 5.9% humidity; 92.1% protein; 5.7 pH; < = 2% ashes	Gelita do Brasil Ltda (Cotia – SP- Brazil)	Peptiplus®/PTL
Hydrolyzate 2	Extracted from bovine leather/porcine skin through chemical + enzymatic hydrolysis; 4mP viscosity; 7.2% humidity; 92.15% protein; 6 pH; < = 0.65% ashes	Rousselot Gelatinas do Brasil Ltda (Amparo – SP- Brazil)	Peptan B®/PEP

Source: (PRESTES et al., 2013; ROUSSELOT, 2014; GELITA, 2014).

Table 2 - Different treatments used to obtain the protein hydrolyzates from collagens.

Acronym	Description of the treatments	Enzyme	Ultrasound	T°C
CC	Crude collagens	No	No	45
HU	Hydrolysis through ultrasound	No	Yes	52 ± 2.5
EH	Enzymatic hydrolysis	Yes	No	45
EHU	Enzymatic hydrolysis with concomitant ultrasound	Yes	Yes	52 ± 2.5
UEH	Previously treated with ultrasound and subsequent enzymatic hydrolysis	Yes	Yes	52 ± 2.5

calculating the degree of hydrolysis. To evaluate the antioxidant activity of the hydrolyzates the DPPH method of Brand-WILLIAMS et al. (1995) modified by SÁNCHEZ-MORENO et al. (1998) was used. An UV spectrophotometer was used to perform the analysis (UV-M51 model, BEL Photonics, SERVYLAB, São Leopoldo, RS, Brazil).

The Minimal Inhibitory Concentration (MIC) was determined through the indirect method of bacterial growth in liquid medium (GAIO et al., 2015) on Gram-negative (*Salmonella choleraesuis* - ATTC 10708) and Gram-positive (*Staphylococcus aureus* - ATCC 6538) bacteria. The MIC values were determined in triplicate (tested concentrations: <10; 10; 12.5; 15; 17.5; 20 and 22.5%).

To evaluate the results of protein (Lowry), degree of hydrolysis (Lowry) and antioxidant activity (DPPH) two experiments were carried out:

Experiment 1 evaluated which application of ultrasound, prior or concomitantly, was better for the enzymatic treatment of the different collagen products (greater bioactivity and degree of hydrolysis). Split-plot randomized design was performed, with the various collagens distributed in the plots and the application of ultrasound (prior vs. concomitantly) and its interactions distributed at random in the subplots of each plot, according to the statistical model (Equation 1):

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

In which, Y_{ijk} = value observed in the i-th installment, k-th repetition and j-th sub-plot; μ = general mean of variable response; α_i = fixed effect of i -th collagen; $(\alpha\gamma)_{ik}$ = residual effect of plots (error A); β_j = fixed effect of j -th application of ultrasound; $(\alpha\beta)_{ij}$ = fixed effect of the interaction between the i-th collagen and the j-th application of ultrasound; ε_{ijk} = residual effect of the subplots (error B) or random effect associated with the ijk-th observation, supposing $\varepsilon_{ijk\sim}{}^{iidN}(0, \sigma^2)$.

Experiment 2 assessed the effect of collagen product, of enzyme concentration and of the application or not of concomitant ultrasound. The split-plot randomized design was performed, with the various collagens distributed in the plots and the enzyme concentrations (0 or 4%) and the application or not of ultrasound (without US vs. concomitant US) and its interactions distributed at random in the subplots of each plot, according to the statistical model described in equation 2:

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

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

The data were subjected to outlier investigation based on the standardized residues. Subsequently, they were subjected to univariate analysis of variance (ANOVA) through the GLM procedure, their means adjusted through the method of ordinary least squares using LSMEANS statement and compared through minimum significant difference (t test) at 5% level of significance. In addition, Spearman correlation analysis was conducted between the studied variables. The statistical analyses were performed in the SAS®System for Windows™ application version 9.4 (SAS Institute Inc., Cary, - NC, USA).

RESULTS AND DISCUSSION

Effects of the use of prior or concomitant ultrasound in enzymatic hydrolysis

The effect of collagen was significant ($P<0.0001$) for protein (ptn), hydrolysis degree (DH) and antioxidant activity (AA) variables (results not shown), showing that results obtained for these analyses depended not only on the hydrolysis process applied, but also on the type of sample tested. Ultrasound and the collagen x ultrasound interaction were not significant ($P>0.05$) in the process of enzymatic hydrolysis, that is, the use of prior or concomitant ultrasound did not result in significant difference over the studied variables. There was positive correlation ($P<0.05$) between hydrolysis degree and antioxidant activity ($r = 0.41$) (results not shown), which showed that they are dependent on each other; higher hydrolysis degree values may positively or negatively affect the antioxidant activity of the hydrolyzate, depending on the process and sample tested. The protein variable showed no correlation ($P>0.05$) with the other variables.

The use of ultrasound prior to or concomitantly with the enzymatic hydrolysis process provided similar results for the analyzed variables (results not shown). It can be concluded that the ultrasound did not inhibit enzyme activity when used concomitantly with hydrolysis (within the tested conditions). It is possible to say that this is a particularity of pepsin which can be activated through the use of ultrasound, due to the increase of combinations between substrate and enzyme (YU et al., 2013; YU et al., 2014). A similar result was reported by YU et al. (2014), who verified that the application of ultrasound (278.8 ± 7.4W, 40kHz) for 30-60 minutes, under controlled temperature of 0 to 4°C, activated pepsin enzyme activity. This effect is attributed to changes in its secondary and tertiary structure. To evaluate the effect of different types of collagen, of the enzyme and of ultrasound over the different variables analyzed, the process with the use of concomitant ultrasound was chosen. Enzymatic hydrolysis with concomitant ultrasound is more feasible and economical compared to hydrolysis with prior ultrasound and subsequent addition of the enzyme.

Effects of (concomitant) ultrasound, of the enzyme (0 and 4%) and of the various types on the collagen over protein content, degree of hydrolysis and antioxidant activity

The effect of collagen, of the enzyme and of the collagen x enzyme interaction was significant ($P<0.001$) for protein (ptn), hydrolysis degree (DH) and antioxidant activity (AA) variables (results not shown), showing that the results depend on the type of collagen and on the enzyme concentration used in the enzymatic hydrolysis process. The effect of the enzyme x ultrasound, collagen x ultrasound and collagen x enzyme x ultrasound interactions was significant ($P<0.05$) only for the antioxidant activity variable, that is, these three factor determined the results obtained for this analysis. There was negative correlation ($P<0.001$) between the residual protein content and hydrolysis degree ($r= -0.41$) (results not shown), higher protein levels resulted in lower hydrolysis degree, which is due to less break downs during the hydrolysis process.

Protein values were lower and degree of hydrolysis higher for FB samples treated with 4% of pepsin enzyme and 4% of enzyme and concomitant ultrasound (compared to other treatments) due to the greater rupture of the protein structure of the collagen (Table 3). Protein residual content of hydrolysates (collagen average) obtained by different enzyme concentrations ranged from 4.81 to 7.96 mg/ml and the degree of hydrolysis of from 5.29 to 10.3%. The best treatment for obtaining greater antioxidant activity for sample FB was with the use of 4% of enzyme and concomitant ultrasound (Table 4), showing that the mutual action of the enzyme and ultrasound caused greater protein rupture, which favored the increase in the functionality of the hydrolyzate obtained from this sample.

The collagen fiber is obtained from bovine hide, treated with calcium hydroxide, and goes through the process of degreasing and subsequent drying, resulting in long chains of polypeptides. The pepsin enzyme acts over peptide bonds of very large amino acids (MAXIMO & CUNHA, 2010) and the ultrasound acts over hydrogen bonds and hydrophobic interactions, breaking tertiary and quaternary structures of proteins due to the effects of cavitation. These structural changes allowed the access of the enzyme to the structure, causing an increase in the degree of hydrolysis (OZUNA et al. 2015). The use only of enzymatic hydrolysis or ultrasound did not generate functional peptides (with antioxidant activity) as to acid hydrolysis and with use of temperature (without enzyme and ultrasound) (Table 4). GARCÍA-MORENO et al. (2014) reported that antioxidant activity depends on the enzyme treatment and sample used. Hydrolyzates samples with low antioxidant activity and considerable degree of hydrolysis are derived from maximum generation, in a short period, of peptides with activity of elimination of DPPH radicals, these are

Table 3 - Means of protein and degree of hydrolysis values for the different samples of collagen hydrolyzates.

Collagen[*]	Enzyme, %		Mean collagen	Enzyme, %		Mean collagen
	0	4		0	4	
	Residual Protein, mg/mL			Degree of hydrolysis, %		
FB	7.00^{aB}	5.62^{bA}	6.31^{B}	0.60^{b}	19.8^{aA}	10.2^{A}
FP	8.70^{aA}	7.22^{bB}	7.96^{A}	1.15^{b}	18.9^{aA}	10.1^{A}
PEP	5.62^{aD}	4.96^{bB}	5.29^{D}	0.00^{b}	10.6^{aC}	5.29^{C}
PTL	5.12^{aE}	4.51^{bB}	4.81^{E}	2.06^{b}	13.7^{aB}	7.88^{B}
GEL	6.58^{aC}	5.34^{bC}	5.96^{C}	0.58^{b}	18.9^{aA}	9.75^{AB}
ROU	6.77^{aBC}	5.37^{bD}	6.07^{BC}	0.23^{b}	20.4^{aA}	10.3^{A}

[**]FB – natural collagen fiber; FP – powdered collagen fiber; PEP – hydrolyzed collagen 2; PTL – hydrolyzed collagen 1; GEL – gelatin 1; ROU – gelatin 2. Means in the same row with different lowercase superscript indicate significant difference between treatments by ultrasound according to "t" test (P<0.05). Means in the same column with overwritten uppercase and different letters indicate significant difference between treatments by collagen according to "t" test (P<0.05).

then hydrolyzed in inactive sequences (GARCÍA-MORENO et al., 2014).

The hydrolysates of samples PTL and PEP showed similar antioxidant activity following enzymatic hydrolysis with or without ultrasound treatment. Despite low value of hydrolysis degree, PEP and PTL samples treated with ultrasound had higher antioxidant activity. The same occurred in the study by KLOMPONG et al. (2007), who reported that the capturing of DPPH radicals by hydrolyzates of muscle from Trevally (*Selaroides leptolepis*) using flavourzyme and alcalase enzymes increased when the degree of hydrolysis decreased.

The greatest antioxidant activity reported for GEL was of 43.9% and for ROU of 53.7%, concerning hydrolysis treatment with use of ultrasound only. When the ROU sample was treated with enzyme it showed higher values of hydrolysis degree; however, antioxidant activity was inferior to treatment with enzyme, when compared to the other treatments. This showed that a higher degree of hydrolysis will not always result in increased antioxidative activity, since what determines the greater functionality of the hydrolyzate is the formation of peptides with of high free radicals capture power.

Enzymatic hydrolysis treatment with concomitant ultrasound and enzymatic hydrolysis were beneficial, evaluating antioxidant activity (DPPH), for samples FB and FP only, respectively, which are the structurally larger samples. For samples with smaller chains of amino acids, hydrolysis with the pepsin enzyme was not productive, even when being assisted by ultrasound, probably for having broken the structure of these samples in unsuitable places for the release of peptides of high functionality, having possibly generated very small peptides or even free or inactive amino acids. Hydrolysis treatment using ultrasound only provided for the commercial samples which were already hydrolyzed (PEP and PTL) and partially hydrolyzed (GEL and ROU) higher values of antioxidant activity. The pepsin enzyme is normally used in the process of collagen extraction, because it extracts with more ease the collagenous fraction of the substrate, it is an enzyme that is aggressive to protein.

Results of antimicrobial activity

Virtually all hydrolyzates showed antimicrobial activity suited to inhibiting the growth of Gram-negative bacteria *Salmonella choleraesuis*, a sample concentration lower than 10% being necessary, with the exception of samples FB HU, FP HU, GEL HU (did not solubilize) and PTL EHU (17.5%), PTL UEH (15%), PTL EH (20%) (Table 5). For inhibition of Gram-positive bacteria *Staphylococcus aureus* the samples which required percentage greater than 10% were FB HU, FP HU, GEL HU (did not solubilize) and PEP UEH (22.5%), the others required concentration lower than 10% to inhibit the growth of the bacteria. The performance of the hydrolyzates was probably favored by the better connection of the peptide with the bacterial cell membrane, causing inhibition of microorganisms.

GIULIANI et al. (2007) reported that peptides with antimicrobial activity are small, with molecular mass below 10kDa (KIM & WIJESEKARA, 2010). They have positive charge and are composed of amphiphile molecule (having hydrophobic and hydrophilic regions) (GIULIANI et al., 2007). Hydrolyzates had antimicrobial activity, showing potential for application in food products for this purpose.

Table 4 - Means of antioxidant activity values for the different samples of collagen hydrolyzates.

Collagen**	Enzyme, %	--------------------Conc US*--------------------		Mean enzyme	Mean collagen
		Without	With		
-------------------------Antioxidant activity, %-------------------------					
FB	0	33.4	16.5^{B}	24.9	29.1^{C}
	4	25.9^{b}	40.7^{aA}	33.3	
---------Mean ultrasound---------		29.6	28.6		
FP	0	17.7^{B}	14.6	16.2	21.5^{D}
	4	38.6^{aA}	15.0^{b}	26.8	
---------Mean ultrasound---------		28.2	14.8		
PEP	0	38.8^{b}	49.8^{aA}	44.3	31.5^{BC}
	4	17.4	20.0^{B}	18.7	
---------Mean ultrasound---------		28.1	34.9		
PTL	0	23.5^{b}	41.1^{a}	32.3	30.7^{BC}
	4	27.6	30.6	29.1	
---------Mean ultrasound---------		25.5	35.8		
GEL	0	36.8	43.9^{A}	40.4	36.7^{AB}
	4	38.7^{a}	27.5^{bB}	33.1	
---------Mean ultrasound---------		37.7	35.7		
ROU	0	42.6^{b}	53.7^{aA}	48.2	38.1^{A}
	4	28.1	28.0^{B}	28.1	
---------Mean ultrasound---------		35.4	40.8		
------Overall mean ultrasound------		30.7	31.8		

**FB – natural collagen fiber; FP – powdered collagen fiber; PEP – hydrolyzed collagen 2; PTL – hydrolyzed collagen 1; GEL – gelatin 1; ROU – gelatin 2.*Conc US – concomitant ultrasound. Means in the same row with different lowercase superscript indicate significant difference between treatments by ultrasound according to "t" test (P<0.05). Means in the same column with overwritten uppercase and different letters indicate significant difference between treatments by enzyme and collagen according to "t" test (P<0.05).

CONCLUSION

The use of ultrasound prior to or concomitant with enzymatic hydrolysis optimized the structural rupture of fiber samples, gelatin 1 and gelatin 2. Enzymatic hydrolysis treatments with concomitant ultrasound and enzymatic hydrolysis provided for the fiber and powdered fiber samples, respectively, lower protein content, higher degree of hydrolysis and higher antioxidant activity. Hydrolysis treatment with ultrasound only resulted in greater antioxidant activity for the commercial samples of hydrolyzate 1, hydrolyzate 2, gelatin 1 and gelatin 2, even though it resulted in low degree of hydrolysis. It is thus possible to infer that a higher degree of hydrolysis not always results in increased antioxidant activity.

The sample that showed the highest antioxidant activity was gelatin 2. The hydrolyzates showed inhibition against Gram-negative bacteria *Salmonella choleraesuis* and Gram-positive bacteria *Staphylococcus aureus*. The performance of the hydrolyzates was favored by the better connection of the peptide with the bacterial cell membrane, causing inhibition of microorganisms, which allows them to be used in the development of food products for this purpose.

It can be concluded that enzymatic hydrolysis concomitant or not with the use of ultrasound was only favorable for the functionality of the fiber and powdered fiber samples. However, it provided for all samples greater structural disruption. For future researches, in which the goal is to obtain peptides for parenteral diet, enrichment of medium or food supplementation, or even obtainment of amino acids, the use of pepsine enzyme and concomitant ultrasound as hydrolysis process would be interesting;

Table 5 - Minimum inhibitory concentration (MIC) of different collagen hydrolyzates, required for inhibition of Gram-negative (*Salmonella choleraesuis* - ATTC 10708) and Gram-positive (*Staphylococcus aureus* - ATCC 6538) bacteria.

Collagen hydrolyzates**	---Bacteria---	
	Salmonella choleraesuis	*Staphylococcus aureus*
FB EHU	<10,0%	<10,0%
FB HU	-	-
FB UEH	<10,0%	<10,0%
FB EH	<10,0%	<10,0%
FP EHU	<10,0%	<10,0%
FP HU	-	-
FP UEH	<10,0%	<10,0%
FP EH	<10,0%	<10,0%
PEP EHU	<10,0%	<10,0%
PEP HU	<10,0%	<10,0%
PEP UEH	<10,0%	22.5%
PEP EH	<10,0%	<10,0%
PTL EHU	17.5%	<10,0%
PTL HU	<10,0%	<10,0%
PTL UEH	15.0%	<10,0%
PTL EH	20.0%	<10,0%
GEL EHU	<10,0%	<10,0%
GEL HU	-	-
GEL UEH	<10,0%	<10,0%
GEL EH	<10,0%	<10,0%
ROU EHU	<10,0%	<10,0%
ROU HU	<10,0%	<10,0%
ROU UEH	<10,0%	<10,0%
ROU EH	<10,0%	<10,0%

**FB – natural collagen fiber; FP – powdered collagen fiber; PEP –hydrolyzed collagen 2; PTL – hydrolyzed collagen 1; GEL – gelatin 1; ROU – gelatin 2.HU – hydrolysis through ultrasound; EH – enzymatic hydrolysis; EHU–ultrasound-assisted enzymatic hydrolysis; UEH – previously treated with ultrasound and subsequent enzymatic hydrolysis. *Samples with "-" in their results did not solubilize for analysis performance.

however, for this study, in which the goal was to increase the functionality of hydrolyzates, their concomitant use was not productive.

ACKNOWLEDGEMENTS

To Fundação de Amparo à Pesquisa do Estado do Rio Grande do Sul (FAPERGS), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Novaprom and Rousselot companies. To the Universidade Estadual de Ponta Grossa (UEPG) and Universidade Regional Integrada do Alto Uruguai e das Missões (URI), to CAPES Statute No. 27/2010 – Pro-Equipments Institutional, the Universidade Federal de Santa Maria (UFSM), the coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and to the Post-Graduate Program in Food Science and Technology.

REFERENCES

ARZENI, C. et al. Comparative study of high intensity ultrasound effects on food proteins functionality. **Journal of Food Engineering**, v.108, p.463-472, 2012. Available from: <https://doi.org/10.1016/j.jfoodeng.2011.08.018>. Accessed: Mar. 12, 2016. doi: 10.1016/j.jfoodeng.2011.08.018.

BERNARDINI, R. D. et al. Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. **Food Chemistry**, v.134, p.1296-1307, 2011. Available from: <https://

doi.org/10.1016/j.foodchem.2010.07.004>. Accessed: Mar. 03, 2016. doi: 10.1016/j.foodchem.2010.07.004.

BRAND-WILLIAMS, W. et al. Use of a free radical method to evaluate antioxidant activity. **Food Science and Technology**, v.28, p.25-30, 1995. Available from: <https://doi.org/10.1016/S0023-6438(95)80008-5>. Accessed: Mar. 13, 2016. doi: 10.1016/S0023-6438(95)80008-5.

CHANDRAPALA, J. et al. Effects of ultrasound on the thermal and structural characteristics of proteins in reconstituted whey protein concentrate. **Ultrasonics Sonochemistry**, v.18, p.951-957, 2011. Available from: <https://doi.org/10.1016/j.ultsonch.2010.12.016>. Accessed: Mar. 02, 2016. doi: 10.1016/j.ultsonch.2010.12.016.

DECKER, E. A. & PARK, Y. Healthier meat products as functional foods. **Meat Science**, v.86, p.49-55, 2010. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/20580991>. Accessed: Mar. 14, 2016. doi: 10.1016/j.meatsci.2010.04.021.

GAIO, I. et al. Antibacterial activity of basil essential oil (***Ocimum basilicum L.***) in Italian-type sausage. **Journal für Verbraucherschutz und Lebensmittelsicherheit**, v.10, p.323-329, 2015. Available from: <https://link.springer.com/article/10.1007/s00003-015-0936-x>. Accessed: Mar, 18, 2016. doi: 10.1007/s00003-015-0936-x.

GARCÍA-MORENO, P. J. et al. Antioxidant activity of protein hydrolysates obtained from discarded Mediterranean fish species. **Food Research International**, v.65 (Part C), p.469-476, 2014. Available from: <https://doi.org/10.1016/j.foodres.2014.03.061>. Accessed: Mar, 20, 2016. doi: 10.1016/j.foodres.2014.03.061.

GELITA. **Analysis certificate**. Gelita do Brasil Ltda (Cotia–SP-Brazil), 2014.

GIULIANI, A. et al. Antimicrobial peptides: an overview of a promising class of therapeutics. **Central European Journal of Biology**, v.2, p.1-33, 2007. Available from: <https://link.springer.com/article/10.2478/s11535-007-0010-5>. Accessed: Mar. 01, 2016. doi: 10.2478/s11535-007-0010-5.

GOMÉZ-GUILLÉN, M. C. et al. Functional and bioactive properties of collagen and gelatin from alternative sources: A review. **Food Hydrocolloids**, v.25, p.1813-1827, 2011. Available from: <https://doi.org/10.1016/j.foodhyd.2011.02.007>. Accessed: Mar. 02, 2016. doi: 10.1016/j.foodhyd.2011.02.007.

HARNEDY, P. A. & FITZGERALD, R. J. Bioactive peptides from marine processing waste and shellfish: A review. **Journal of Functional Foods**, v.4, p.6-24, 2012. Available from: <https://doi.org/10.1016/j.jff.2011.09.001>. Accessed: Mar. 05, 2016. doi: 10.1016/j.jff.2011.09.001.

HERREGODS, G. et al. Angiotensi I-converting enzyme inhibitory activity of gelatin hidrolysates and identification of bioactives peptides. **Journal of Agricultural and Food Chemistry**, v.59, p.552-558, 2011. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/21174470>. Accessed: Mar. 13, 2016. doi: 10.1021/jf1037823.

KHAN, M. I. et al. Meat as a functional food with special reference to probiotic sausages. **Food Research International**, v.44, p.3125-3133, 2011. Available from: <https://doi.org/10.1016/j.foodres.2011.07.033>. Accessed: Mar. 14, 2016. doi: 10.1016/j.foodres.2011.07.033

KIM, S. & WIJESEKARA, I. Development and biological activities of marine-derived bioactive peptides: A review. **Journal of Functional Foods**, v.2, p.1-9, 2010. Available from: <https://doi.org/10.1016/j.jff.2010.01.003>. Accessed: Mar. 11, 2016. doi: 10.1016/j.jff.2010.01.003.

KLOMPONG, V. et al. Antioxidative activity and functional properties of protein hydrolysate of yellow stripe trevally (Selaroides leptolepis) as influenced by the degree of hydrolysis and enzyme type. **Food Chemistry**, v.102, p.1317-1327, 2007. Available from: <https://doi.org/10.1016/j.foodchem.2006.07.016>. Accessed: Mar. 11, 2016. doi: 10.1016/j.foodchem.2006.07.016.

LI, B. et al. Isolation and identification of antioxidative peptides from porcine collagen hydrolysate by consecutive chromatography and eletrospray ionization-mass spectrometry. **Food Chemistry**, v.102, p.1135-1143, 2007. Available from: <https://doi.org/10.1016/j.foodchem.2006.07.002>. Accessed: Mar. 11, 2016. doi: 10.1016/j.foodchem.2006.07.002.

LIN, L. et al. Angiotensin-I-converting enzyme (ACE)-inhibitory and antihypertensive properties od squid skin gelatin hydrolysates. **Food Chemistry**, v.131, p.225-230, 2012. Available from: <https://doi.org/10.1016/j.foodchem.2011.08.064>. Accessed: Mar. 10, 2016. doi: 10.1016/j.foodchem.2011.08.064.

LIU, Z. Y. et al. Purification and characterization of pepsin-solubilized collagen from skin and connective tissue of giant red sea cucumber (*Parastichopus californicus*). **Journal of Agriculture and Food Chemistry**, v.58, p.1270–1274, 2010. Available from: <https://www.ncbi.nlm.nih.gov/pubmed/20085374>. Accessed: Mar. 10, 2016. doi: 10.1021/jf9032415.

LOWRY, O. H. et al. Protein measurement with the folin phenol reagent. **The Journal of Biological Chemistry**, v.193, p.265-275, 1951. Available from: <http://www.jbc.org/content/193/1/265.full.pdf>. Accessed: Mar. 11, 2016.

MAXIMO G. J. & CUNHA R. L. Mechanical properties of collagen fiber and powder gels. **Journal of Textures Studies**, v.41, p.842-862, 2010. Available from: < http://onlinelibrary.wiley.com/wol1/doi/10.1111/j.1745-4603.2010.00258.x/full>. Accessed: Mar. 12, 2016. doi: 10.1111/j.1745-4603.2010.00258.x.

OZUNA, C., et al. Innovative applications of high-intensity ultrasound in the development of functional food ingredients: Production of protein hydrolysates and bioactive peptides. **Food Research International**, v.77, p.685-696, 2015. Available from:<https://doi.org/10.1016/j.foodres.2015.10.015>. Accessed: Mar. 12, 2016. doi: 10.1016/j.foodres.2015.10.015.

PRESTES, R. C. et al. Effects of the addition of collagen and degree of comminution in the quality of chicken ham. **Poultry Science Association**, v.22, p.885–903, 2013. Available from: <http://dx.doi.org/ 10.3382/japr.2013-00809>. Accessed: Mar. 13, 2016. doi: 10.3382/japr.2013-00809.

ROUSSELOT. **Analysis certificate**. Rousselot Gelatinas do Brasil Ltda (Amparo–SP-Brazil), 2014.

SANCHEZ-MORENO, C. et al. A procedure to measure the antiradical efficiency of polyphenols. **Journal of the Science of Food and Agriculture**, v.76, p.270-276, 1998. Available from: <https://www.researchgate.net/publication/229455469_A_procedure_to_measure_the_antiradical_efficiency_of_polyphenols>. Accessed: Mar. 14, 2016. doi: 10.1002/(SICI)1097-0010(199802)76:2<270::AID-JSFA945>3.0.CO;2-9.

YU, Z. L. et al. Effect of ultrasound on the activity and conformation of a-amylase, papain and pepsin. **Ultrasonics Sonochemistry**, v.21, p.930-936, 2014. Available from: <https://doi.org/10.1016/j.ultsonch.2013.11.002>. Accessed: Mar. 15, 2016. doi: 10.1016/j.ultsonch.2013.11.002.

YU, Z. et al. Influence of ultrasound to the activity of tyrosinase. **Ultrasonics Sonochemistry**, v.20, p.805-809, 2013. Available from: <https://doi.org/10.1016/j.ultsonch.2012.11.006>. Accessed: Mar. 15, 2016. doi: 10.1016/j.ultsonch.2012.11.006.