

Optimization of enzymatic hydrolysis process of mechanically separated chicken meat**Otimização do processo de hidrólise enzimática de carne de frango mecanicamente separada**

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ABSTRACT

The main of this work was to evaluate the influence of the temperature, pH and enzyme concentration in the hydrolysis of mechanically separated chicken meat (MSCM) with and without previous heat treatment. The influence of pH (8.08 to 8.92), temperature (50.75°C to 59.25°C), enzyme concentration (0.38 to 4.62%) and reaction time (0.17 to 5.83 h) on the degree of hydrolysis and protein concentration were evaluated. The conditions that promoted the best degree of hydrolysis where 58°C, pH 8.5, enzyme concentration of 4.62 and reaction time of 3 h without previous heat treatment. The hydrolysed presented IC₅₀ of 4 mg/mL. The MSCM hydrolyzed in these conditions is alternative of product with antioxidant properties with potential of application in foods.

Key words: alcalase, heat treatment, protein, antioxidant activity.

RESUMO

O objetivo deste trabalho foi avaliar a influência da temperatura, pH e concentração de enzima na hidrólise de carne de frango separada mecanicamente (CFMS) com e sem tratamento térmico prévio. Foram avaliadas a influência do pH (8,08 a 8,92), temperatura (50,75°C a 59,25°C), concentração enzimática (0,38 a 4,62%) e tempo de reação (0,17 a 5,83 h) no grau de hidrólise e concentração de proteínas. As condições que promoveram o melhor grau de hidrólise foram: 58°C, pH 8,5, concentração enzimática de 4,62 e tempo de reação de 3h sem tratamento térmico prévio. O hidrolisado apresentou IC₅₀ de 4 mg/mL. O MSCM hidrolisado nestas condições é alternativa de produto com propriedades antioxidantes com potencial de aplicação em alimentos.

Palavras-chave: alcalase, tratamento térmico, proteínas, atividade antioxidante.

1 INTRODUCTION

Mechanically separated chicken meat (MSCM) obtained from chicken carcass is one of the co-products generated in poultry industrialization of cutting as in the preparation of breast meat and thigh parts such as the back, neck, wings and other skeletal parts from the processing, are intended for mechanical separation. In this mechanical separation, the meat undergoes a grinding process, separating the bone residue from MSCM. It is wrapped in plastic and frozen into blocks for subsequent use. Among the uses of MSCM, is the use in the production of cooked sausages such as sausages and bologna (Auriema *et al.*; Famenin *et al.*, 2019; 2019; Júnior *et al.*, 2019). The MSCM has a minimum of 12% proteins and maximum of 30% lipids (BRASIL, 2000).

The MSCM can be used in the production of hydrolysates in order to obtain bioactive peptides (Mora *et al.*, 2014), which can be applied for the nutritional enrichment of foods. These

peptides have functional properties as antioxidant, antimicrobials, emulsifying capacity and others (Mora *et al.*, 2014; Nwachukwu & Aluko, 2018; Karami & Akbari-Adergani, 2019).

The use of protein hydrolysates depends on the content of amino acids, the molecular weight of the peptide fraction and the presence of dipeptides, tripeptides and oligopeptides (Neklyudov *et al.*, 2000) The reduced size of the peptides also influences their antioxidant activity, the smaller the peptide obtained in the hydrolysate, the greater its antioxidant capacity for direct applications (Wu *et al.*, 2005; Nie; *et al.*, 2017).

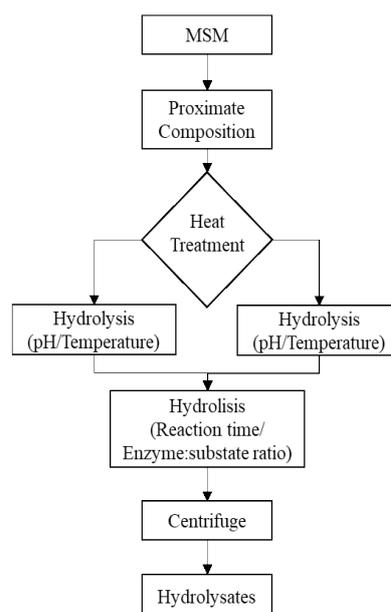
Meat products and by-products as a source of functional and biological components. The recovery of animal co-products will become appreciated as an important issue in the food industry (Bechaux *et al.*, 2019; Borges *et al.*, 2019).

Protein hydrolysis from chicken meat or co-products has been studied with the application of several proteolytic enzymes (Nie *et al.*, 2017; Kurozawa *et al.*, 2018), depending on the enzyme used, different degrees of hydrolysis are obtained, which are related to pH and temperature optimum of the actuation of the enzyme (Kurozawa *et al.*, 2009; Rossi *et al.*, 2009).

In this sense, the objective of the work was to evaluate the influence of the temperature, pH, enzyme concentration and time in the enzymatic hydrolysis of MSCM with and without previous heat treatment to obtain the maximum protein content, and from the best condition were analyzed the antioxidant potential of the hydrolysate.

2 MATERIAL AND METHODS

Figure 1 presents a simplified diagram showing the steps taken in the present study



The MSCM was provided by a company in the northern region of the state of Rio Grande do Sul, Brazil. In order to eliminate possible interferences related to changes in the raw material, the MSCM was transported frozen in a thermal box to the University of Passo Fundo, where it was thawed at 4°C, homogenized manually and vacuum packed in low density polyethylene containers in portions between 750-950 g and stored at -18°C. The MSCM was characterized according to AOAC (1997) in relation to protein, lipids, ash and moisture.

The enzyme used was Alcalase 2.4L (Novozymes, Bagsvaerd, Denmark) obtained from *Bacillus licheniformis* with activity of 2.4 U/g.

The heat-treatment of MSCM procedure was carried out at 80°C for 15 min and cooled to room temperature (Rossi *et al.*, 2009). The thermal treatment of the MSCM was carried out in order to promote the denaturation of the muscle protein, because according to Rossi *et al.* (2009), it facilitates the enzymatic action in the hydrolysis of the MSCM.

The enzymatic hydrolysis process to obtain the MSCM protein hydrolysate was studied with and without previous heat treatment. A central rotational composite design (DCCR) 2² was performed to evaluate the influence of pH (8.08 to 8.92) and temperature (50.75°C to 59.25°C) on the degree of hydrolysis and protein concentration. The fixed independent variables were reaction time (3h) and enzyme concentration (2.5%), based on studies reported in the literature. The pH of the samples was the adjusted using NaOH 1N solution.

Based on the reaction condition (pH and temperature) that presented the highest degree of hydrolysis (Table 1), a new complete DCCR 2² was performed, evaluating the influence of the enzyme concentration (0.38 to 4.62%) and reaction time (0.17 h to 5.83 h) in relation to the degree of hydrolysis and protein concentration (Table 2). The levels were defined based on the study of Kurozawa *et al.* (2009).

The degree of hydrolysis was assessed according to the methodology described in AOAC (2005). Initially, 5 mL of the hydrolysate sample (10% w/v solution) were added, in a erlemeyer of 125 mL, with the pH adjusted to 7.0 with 0.2 mol/L sodium hydroxide (NaOH) (Vetec) or with 0.2 mol/L hydrochloric acid (HCl) (Synth), and 5 mL of the formaldehyde-phenolphthalein solution (formaldehyde - Kinetic and phenolphthalein - Nuclear), also adjusted to pH 7.0 with the same solutions (NaOH and HCl both 0.2 mol/L) and 3 drops of 1% phenolphthalein.

The erlemeyer contents were titrated with 0.2 mol/L NaOH until the color turns to light pink and the volume (V₁) recorded. The same volume used in the first titration was then added as an excess, making the pink color intense and the volume (V₂) registered. The solution was again

titrated, but with 0.2 mol/L HCl until the pink color disappears (back- titration) and the volume (V_3) registered.

The amount of NaOH spent on the titration (X) and the amount of HCl spent on the back-titration, were obtained using Eq. 1 and 2:

$$X = (FcNaOH) \quad (1)$$

$$Y = \frac{(FcHCl)(V_3)}{1000 mL} \quad (2)$$

Where: V_1 = volume used in first titrate with NaOH 0.2 mol/L (mL); V_2 = same volume of first titrate (mL); V_3 = volume used in retro-titrate with HCl 0.2 mol/L (mL); Fc = titrate solution factor concentration; X= mols of NaOH used in titration; Y= mols of HCl used in retro-titrate.

The α -amino nitrogen (K) was determined considering the volume of NaOH used in titrate (X) minus volume of HCl used in retro-titrate (Y). Degree of hydrolysis was obtained by Eq. 3.

$$DH (\%) = \frac{K}{N_{total}} \quad (3)$$

Where: DH= degree of hydrolysis; N_{total} = nitrogen percentage determined by Kjeldahl method.

The determination of total protein of hydrolyzed MSCM was done according to the methodology described by AOAC (1997) and AOAC (2005).

The determination of antioxidant activity was carried out with the hydrolyzed sample that presented the more protein content, by the free radical capture method 2,2-Diphenyl-1-picrylhydrazyl (DPPH), described by Brand-Williams et al. [1995]. 0.5 mL of 0.1 mM DPPH ethanolic solution (Êxodo Científica) and 0.5 mL of aqueous solutions containing 1.5; 2.5; 5; 10; 15; 25% of the hydrolysates were added in test tubes, which were incubated for 30 min in the absence of light, then reading on a UV-1600 spectrophotometer (Pro-analysis) at 515 nm. Analyzes were performed in triplicate and the antioxidant activity was obtained by linear regression analysis and expressed in IC_{50} values.

Electrophoresis was performed with MSCM (crude) and the hydrolysate with more protein content, following the methodology of Laemmli (1970), using sodium dodecyl sulfate and polyacrylamide gel 15% (SDS-PAGE) and molar mass standard 5 to 250 kDa (PageRuler, 4-20% Trisglycine SDS-PAGE). The samples were subjected to a constant current of 300 mA and a

voltage of 250 V for approximately 60 min. The bands of 34 proteins present were visualized using a bright blue dye solution (Blue R-250).

The results were treated statistically with software *Statistica* version 5.0, at 95% confidence level.

3 RESULTS AND DISCUSSION

The crude MSCM shows values of proteins 12.81 ± 0.22 g/100 g, lipids 28.67 ± 0.51 g/100 g, ash 0.75 ± 0.11 g/100 g and moisture 54.73 ± 0.87 g/100 g. All results found are in accordance with Brazil current legislation (BRASIL, 2000), mainly protein >12% and fats <30%.

Table 1 presents the results of protein concentration and degree of hydrolysis obtained in the enzymatic hydrolysis of the MSCM with and without thermal treatment in relation to pH and temperature evaluated.

Heat treated MSCM obtained the highest levels of protein and degree of hydrolysis in the central point (runs 9, 10 and 11), at 55 °C and pH 8.5, and run 6 (59.25°C and pH 8.5). While for the tests did not heat-treated the major protein content and consequent degree of hydrolysis were obtained in runs 4, 6 and 8, represented by the higher values of temperature (55-59.25) and pH (8.5-8.92). This behavior is consistent with the performance characteristics of the enzyme. Kurozawa et al. (2009) who obtained results with the same trend and similar to those found in this study.

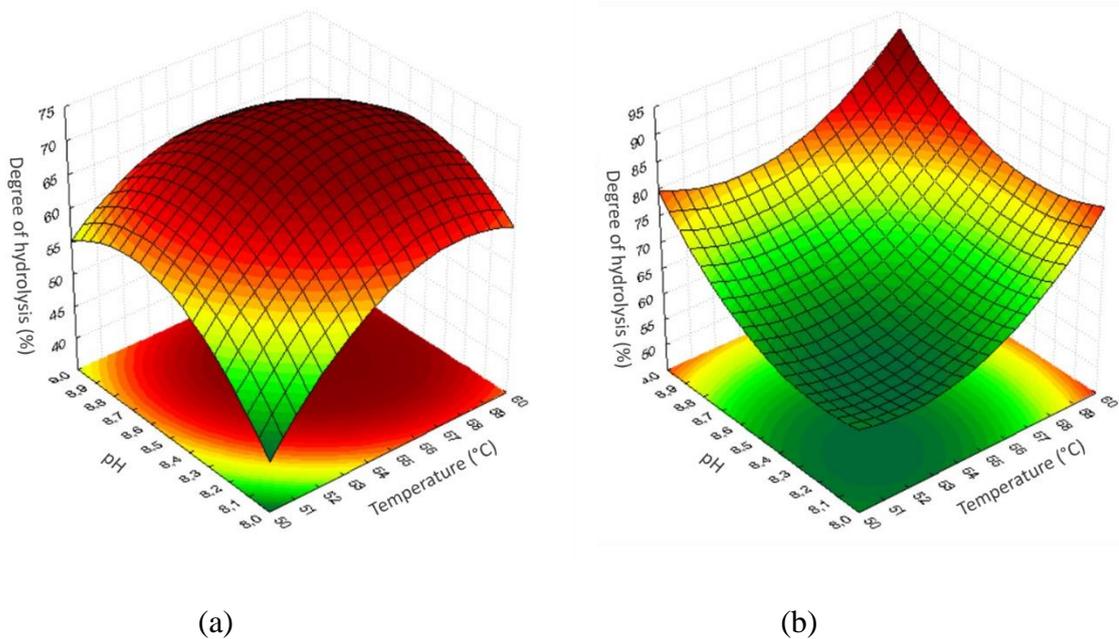
Table 1. Protein content and degree of hydrolysis (DH) of chicken MSM treated (HT) and not heat-treated (NHT) in relation to temperature and pH.

Runs	T (°C)	pH	HT		NHT	
			Protein (mg/mL)	DH (%)	Protein (mg/mL)	DH (%)
1	-1 (52)	-1 (8.2)	34.922 ± 0.146	61.97	31.821 ± 0.016	59.38
2	-1 (52)	1 (8.8)	32.914 ± 0.148	66.72	35.768 ± 0.463	68.72
3	1 (58)	-1 (8.2)	35.004 ± 0.363	66.92	35.771 ± 0.369	68.73
4	1 (58)	1 (8.8)	34.653 ± 0.158	66.09	38.574 ± 0.417	75.37
5	-1.41 (50.75)	0 (8.5)	31.195 ± 0.418	57.90	32.277 ± 0.125	60.46
6	1.41 (59.25)	0 (8.5)	36.983 ± 0.478	71.91	38.889 ± 0.439	76.12
7	0 (55)	-1.41 (8.08)	32.936 ± 0.081	62.02	32.303 ± 0.108	60.52
8	0 (55)	1.41 (8.92)	34.965 ± 0.398	66.83	38.193 ± 0.267	74.47
9	0 (55)	0 (8.5)	37.526 ± 0.101	72.89	33.075 ± 0.057	62.35
10	0 (55)	0 (8.5)	36.402 ± 0.606	70.23	33.065 ± 0.009	62.32
11	0 (55)	0 (8.5)	36.549 ± 0.789	70.58	32.949 ± 0.121	62.05

*Fixed independent variables: enzyme concentration (2.5%) and reaction time (3 h).

The Figure 2 shows the response surface of the degree of hydrolysis by function of pH and temperature from MSCM heat-treated (a) and not heat-treated (b).

Figure 2. Surface response of degree of hydrolysis by function of pH and temperature from MSCM heat-treated (a) and not heat-treated (b).



In Figure 2a, a convergence at a center point can be noted clearly. The low curvature still indicates that there is not much variation in the degree of hydrolysis for small changes in temperature and pH. This would be a good indicator for large scale processes where precise values of pH and temperature need to be kept constant during hydrolysis, becoming operations less complex.

However, the result of the tests with the MSCM without thermal treatment showed a response surface (Figure 2b) with indications that the degree of hydrolysis process can be improved, since the protein content and the degree of hydrolysis increase with the increase of temperature and pH, enabling a more efficient process. The use of MSCM without thermal treatment can also represent one step less in the process of obtaining protein hydrolysate, as well as reducing energy costs.

Considering that the best results for hydrolysis were obtained with MSCM not heat-treated using the highest values of temperature and pH, it was decided to evaluate the influence of the enzyme concentration and reaction time in relation to protein content and degree of hydrolysis, and the results are shown in Table 2.

Table 2. Protein content and degree of hydrolysis of chicken MSM not thermally treated in relation to enzyme concentration and reaction time.

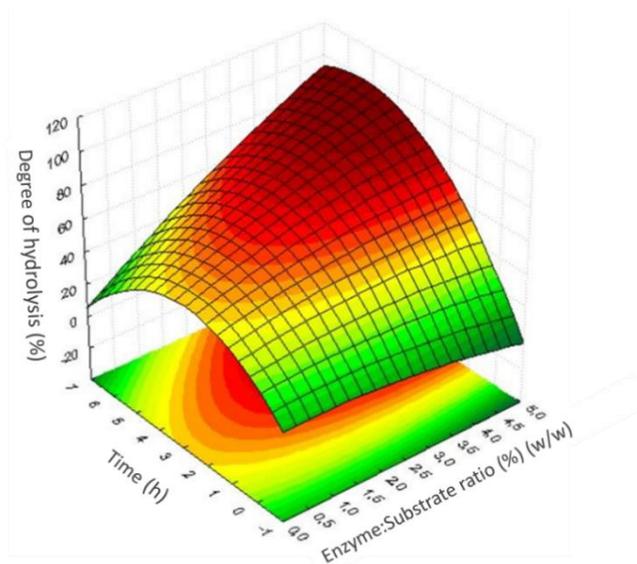
Runs	Enzyme % (w/w)	Time (h)	Protein (mg/mL)	Degree of hydrolysis (%)
1	-1 (1)	-1 (1)	21.922 ± 2.490 ^a	35.93
2	-1 (1)	1 (5)	24.936 ± 0.013 ^a	43.07
3	1 (4)	-1 (1)	26.351 ± 2.824 ^{ab}	46.42
4	1 (4)	1 (5)	43.078 ± 0.497 ^d	86.04
5	-1.41 (0.38)	0 (3)	33.840 ± 0.347 ^{bc}	64.16
6	1.41 (4.62)	0 (3)	43.487 ± 3.184 ^d	87.01
7	0 (2.5)	-1.41 (0.17)	25.835 ± 3.123 ^{ab}	45.20
8	0 (2.5)	1.41 (5.83)	38.486 ± 2.760 ^{cd}	75.16
9	0 (2.5)	0 (3)	36.605 ± 1.493 ^{cd}	70.71
10	0 (2.5)	0 (3)	35.966 ± 2.403 ^{cd}	69.20
11	0 (2.5)	0 (3)	38.876 ± 0.516 ^{cd}	76.09

* independent fixed variables: temperature (58 °C) and pH (8.5).

The best degree of hydrolysis achieved under the conditions studied are given in the runs 6, using enzyme concentration of 4.62% (w/w) and 3 h, followed by run 4 using enzyme concentration 4% and 5 h.

The influence of the enzyme concentration and time were positive, the higher, the better the results. In Figure 3 it is possible to see this development, the increase in enzyme concentration (runs 6 and 4) reduces the hydrolysis time.

Figure 3. Response surface of the degree of hydrolysis as a function of the enzyme concentration and hydrolysis time of chicken MSM not heat-treated.

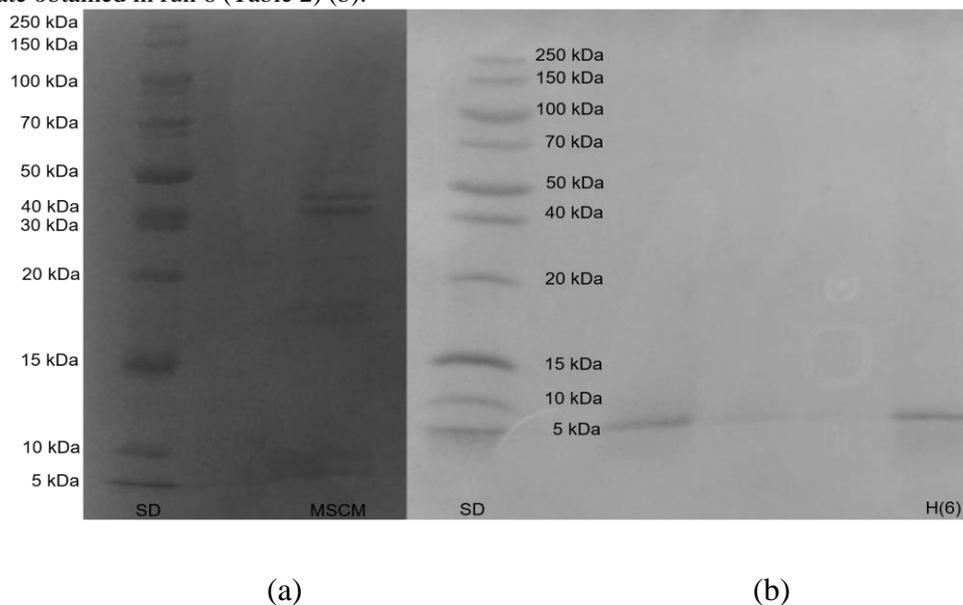


The hydrolysis process using temperature of 58°C, pH of 8.5, enzyme concentration of 4.62 and reaction time of 3 h present the best protein content and degree of hydrolysis.

From the results of run 6 (Table 2) that presented the higher protein content and degree of hydrolysis, where analyzed the protein fractions and antioxidant activity, results shown in Figure 4 and Table 3, respectively. The protein fractions were analyzed also for crude MSCM and the test held at bands between 5 kDa and 250 kDa.

Is observed in the Figure 4 (a) for crude MSCM not heat-treated that are three ranges of bands: 5-10; 17-18; 40-45 kDa. This profile may represent proteins commonly found in bird muscles, possibly represented by actin (46 kDa) and α -tropomyosin (36 kDa) (Porzio & Pearson, 1977).

Figure 4. Image of the SDS-PAGE electrophoresis gels from crude chicken MSM not heat-treated (a) and the hydrolysate obtained in run 6 (Table 2) (b).



The hydrolysate sample showed only bands for 5kDa, this being the limit of detection of the standard used, also demonstrate that hydrolysis process was efficient. It's important to note that hydrolysis for a long time can generate many peptides with molecular weight than those presented.

The results shown that the percentage of antioxidant activity increases proportionally with the concentration of hydrolysate, reaching 89.15% with a concentration of 25% (10.871 mg/mL). To obtain the Equation of the IC₅₀ line, graphs of the antioxidant activity in relation to the hydrolyzate concentration were constructed. The Equation was $Y = 5.5505x + 27.798$, $R^2 =$. An

IC₅₀ of 4 mg/mL was calculated using a correlation between antioxidant activity in % and the protein concentration in the hydrolysate (indicating that this amount is capable of neutralizing 50% of the DPPH radical).

The concentration of IC₅₀ from the present study is below to those found by Sun et al. (2012) that used membrane for purify hydrolyzed of chicken breast (1.28 mg/mL) indicating that a process of purification with membrane can result in a hydrolysate with better antioxidant activity. This is justified since that in proteins of animal origin, fractions with a lower molecular weight <5kDa have the greatest hydroxyl radical elimination activities when compared to larger fractions, object of this study. Fractions <3kDa have the best antioxidant potential for reducing power and the ability to eliminate DPPH, hydroxyl and superoxide. And likewise, peptides between 500Da and 1500Da have better antioxidant activity than > 1500Da (Liu *et al.*, 2016; Xiong *et al.*, 2020).

There are many factors involved in enzymatic hydrolysis process as reactor models, agitation modes, pH, temperature, time, enzyme ratio or types and raw material condition and composition. All these factors add numerous interferences could lead to different behaviors, mainly in antioxidant activity, making comparison with the literature difficult.

4 CONCLUSION

The best results of degree of hydrolysis were obtained without previous heat treatment of mechanically separated chicken meat on the conditions of 58°C of temperature, pH of 8.5, enzyme concentration of 4.62 and reaction time of 3 h. The hydrolysed presented protein fractions of 5kDa and IC₅₀ of 4 mg/mL. The mechanically separated chicken meat hydrolyzed in these conditions is alternative of product with antioxidant properties with potential of application in foods.

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COMPLIANCE WITH ETHICAL STANDARDS

The authors declare no potential conflicts of interest.

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