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Research Article

# DIGESTIBILITY AND ENZYMATIC ACTIVITY *IN VITRO* OF HEN EGG WHITE LYSOZYME

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**ABSTRACT**

**Objective:** The aim of this study was to evaluate the protein digestibility and analyze the residual enzymatic activity of lysozyme.

**Methods:** Protein digestibility was evaluated hydrolyzing the protein with pepsin at pH 1.2, 2.0, and 3.2 during 60, 90, and 120 minutes of incubation. These hydrolysates were analyzed with sodium dodecyl sulfate-polyacrylamide gel electrophoresis and reversed-phase high-performance liquid chromatography. Residual enzymatic activity was evaluated with the spectrophotometric method at 450 nm.

**Results:** Lysozyme was totally hydrolyzed with pepsin at pH 1.2. At pH 2.0, lysozyme was partially hydrolyzed and at pH 3.2 hydrolysis was absent at all times of the assay.

**Conclusions:** Lysozyme was hydrolyzed with pepsin at low pH. Residual enzymatic method can be used to determine the grade of hydrolysis in lysozyme.

**Keywords:** Lysozyme, Enzymatic hydrolysis, Muramidase activity, Antimicrobial activity.

## INTRODUCTION

Lysozyme (E.C.3.2.17, N-acetyl-muramic-hydrolase) is a globular basic protein found in nature and is characterized by its high enzymatic activity. It was first discovered in nasal mucous by Alexander Fleming, who named it "lysozyme" as he observed it is lytic activity toward bacterial cocci [1]. The egg albumen is known to have an exceptionally high amount of lysozyme, normally referred to as hen's egg lysozyme, representing 3.5% of the egg white protein content [2-4]. The lysozyme is a basic protein consisting of 129 amino acids and a molecular weight of 14.3 kDa. This enzyme acts by lysing the cell walls of certain Gram-positive bacteria such as lactic acid bacteria and *Clostridium* sp. by splitting β (1-4) linkages between N-acetylmuramic acid and N-acetylglucosamine of the peptidoglycan of bacterial cell walls. This enzymatic activity is named muramidase activity or lytic activity [5-8]. The eight foods traditionally considered to be responsible for more than 90% of food allergy are milk products, eggs, fish, shellfish, peanuts, tree nuts, wheat, and soy [9]. Among food allergies, allergy to egg is, together with peanut and milk, the most common in children and infants with a prevalence that varies between 7.9% and 10% [10,11]. It is generally believed that protein, which is resistant to proteolytic digestion in the digestive tract, retains sufficient structural integrity to have an increased probability to stimulating immune reactions. Small amounts of intact partially digested proteins are absorbed in the intestine and enter the circulatory system. Simulate gastric fluid (SGF) is used to determine the digestibility and allergenicity of a protein [12]. In this study, the digestibility of hen egg white lysozyme was investigated and characterized using pepsin enzyme. Protein digestion was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) method, and residual enzymatic activity was analyzed with the spectrophotometric method.

## METHODS

### Lysozyme and materials

Lysozyme (L2879, chloride from chicken egg white Grade VI, 40,000 units/mg protein, EC 3.2.1.17) and pepsin crystalline (4500 units/mg obtained from porcine stomach mucus and *Micrococcus lysodeikticus*) were purchased from Sigma Chemical Co. (Saint Louis, MO, USA).

### Enzymatic hydrolysis of lysozyme

Commercial, isolate lysozyme and hydrolysates were initially dissolved at 5 mg/mL in potassium phosphate buffer 10 mM (pH 1.5). 1 ml of this lysozyme solution was mixed with 50 μmL of pepsin solution of 200 U/mg. 5 mg/mL in solution of 0.035 M NaCl SGF at pH 1.2, 2.0, and 3.2 to obtain an enzyme-to-substrate ratio of 1:20 w/w. This mixture was incubated at 37°C during 1 hr. The reaction was stopped by heating at 80°C for 15 minutes, and the pH was adjusted at 7.0 by addition of 1 M NaOH [7].

### SDS-PAGE analysis

Samples were dissolved in 10 mM Tris-HCl buffer, pH 8, 2.5% SDS, and 10 mM ethylenediaminetetraacetic acid (non-reducing conditions) or the same buffer containing 5% b-mercaptoethanol (reducing conditions), and heated at 95°C for 10 minutes [13]. Analysis by SDS-PAGE used Mini-Protean Tetra Cell (Bio-Rad, USA) electrophoresis apparatus at 200 V. The electrophoretic and silver staining conditions were carried out according to the manufacturer instructions.

### Reversed-phase high-performance liquid chromatography (RP-HPLC) analysis

Lysozyme hydrolysates, at a concentration of 2.0 mg/mL, were analyzed using a Hi-Pore® RP-318 (250 × 4.6 mm i.d.) column (Waters, Milford, MA) in a Waters 600 HPLC system. Solvent A was 0.37% (v/v) trifluoroacetic acid (Scharlau Chemie, Barcelona, Spain) in double-distilled water, and solvent B was 0.27% (v/v) trifluoroacetic acid in HPLC-grade acetonitrile (Lab-Scan, Gliwice, Poland). The chromatographic conditions were as in Martos *et al*. [14]. Detection was at 220 nm, and data were processed using Empower 2 Software (Waters).

### Muramidase activity assay

The lytic activity of lysozyme was determined by monitoring the decrease in turbidity of a suspension of *M. lysodeikticus* cell spectrophotometrically at 450 nm at 25°C, according to Shugar's method [15]. One unit of lysozyme was defined as a decrease in the absorbance at 450 nm of 0.001/minutes. The muramidase activity of each sample was assayed by triplicate.

**Statistical analysis**

Results are presented as means±standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance. The *post-hoc* analysis was performed by the Tukey test. All tests were considered significant at $p<0.05$. Statistical analysis was performed using the software package Prism 4 for Windows, version 4.3 (GraphPad Software Inc., www.graphpad.com).

## RESULTS

**Gastric hydrolysis**

Hen egg white lysozyme was hydrolyzed with pepsin at different pHs in SGF (NaCl 0.35 M) with a relation enzyme/substrate of 1/20 during 60, 90, and 120 minutes at 37°C, 400 rpm. SDS-PAGE electrophoresis analysis shows that lysozyme was totally hydrolyzed with pepsin at a low pH 1.2 at all times assays. The 14.400 Da band was not found at pH 1.2, the band with molecular weight of 6.500 Da was found corresponding to peptides produced of hydrolysis with pepsin, at this pH 1.2. (Fig. 1)

At pH 2.0, the lysozyme was partially hydrolyzed. The band with molecular weight of 14.400 Da was found, this band corresponds to lysozyme. At this same pH 2.0, the hydrolysis was more effective at a time of 120 minutes of incubation with pepsin, compared to the bands using the 60 and 90 minutes incubation. (Fig. 1)

At pH 2.0, the molecular weight bands of 6.500 Da were found, corresponding to peptides produced with hydrolysis with pepsin. On the other hand, lysozyme was not hydrolyzed with pepsin at pH 3.2, at all times assay. Lysozyme has resistance at gastric hydrolysis with pepsin at pH 2.0 and pH 3.2 in these conditions of the assay.

**RP-HPLC**

RP-HPLC analysis of hydrolysates of lysozyme during 60 minutes shows that lysozyme was totally hydrolyzed at pH 1.2. At pH 2.0, hydrolysis was partially obtained. Finally, at pH 3.2 hydrolysis was not present (Fig. 2). These results were similar in all times assay. (Fig. 1)

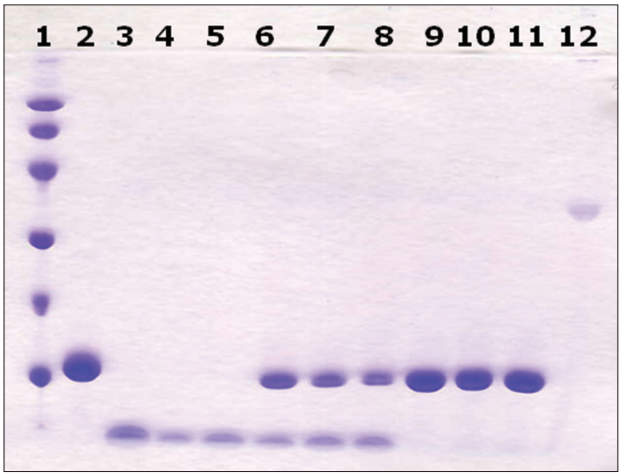


**Fig. 1: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of lysozyme hydrolyzed. Lane 1: Molecular weight, Lane 2: Lysozyme without pepsin, Lane 3: Lysozyme with pepsin at pH 1.2 (60 minutes), Lane 4: Lysozyme with pepsin at pH 1.2 (90 minutes), Lane 5: Lysozyme with pepsin at pH 1.2 (120 minutes), Lane 6: Lysozyme with pepsin at pH 2.0 (60 minutes), Lane 7: Lysozyme with pepsin at pH 2.0 (90 minutes), Lane 8: Lysozyme with pepsin at pH 2.0 (120 minutes), Lane 9: Lysozyme with pepsin at pH 3.2 (60 minutes), Lane 10: Lysozyme with pepsin at pH 3.2 (90 minutes), Lane 11: Lysozyme with pepsin at pH 3.2 (120 minutes) and Lane 12: Pepsin blank**

**Enzymatic activity**

Enzymatic activity was tested to determine the degree of gastric hydrolysis at pH 1.2, 2.0, and 3.2. Lysozyme enzyme needs its active site to bind to the substrate. The site can be modified through hydrolysis with pepsin. The residual activity was evaluated with the spectrophotometric method at 450 nm with a solution of *M. lysodeikticus* ATCC 4698. Fig. 3 shows the enzymatic activity of lysozymes. Native lysozyme was used as positive control. Fig. 3a shows the residual enzymatic activity of hydrolyzed lysozyme during 60 minutes with pepsin at pH 1.2. We can see that hydrolyzed lysozyme has no enzymatic activity, and it only conserves 1.0% of its natural activity. At pH 2.0, during 60 minutes, hydrolyzed lysozyme conserved 45% of its enzymatic activity. At pH 3.2, hydrolyzed lysozyme conserved 98% of its enzymatic activity compared to control lysozyme. The 90 minutes hydrolysates present residual enzymatic activity with values of 0.8%, 38%, and 90 % at pH 1.2, pH 2.0, and pH 3.2, respectively (Fig. 3b).

Hydrolysates lysozyme during 120 minutes present residual enzymatic activity with values of 0.6%, 35%, and 87% at pH 1.2, 2.0, and 3.2, respectively (Fig. 3c). These results show that the enzymatic activity was proportional to the time of hydrolysis with pepsin.

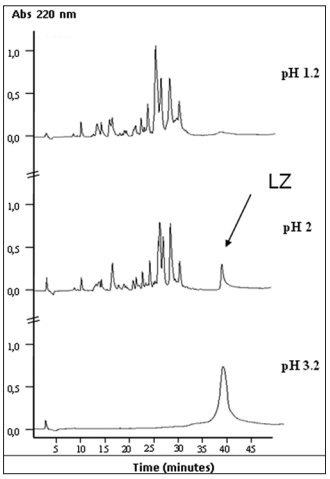


**Fig. 2: Reversed-phase high-performance liquid chromatography analysis of hydrolysates of lysozyme during 60 minutes**

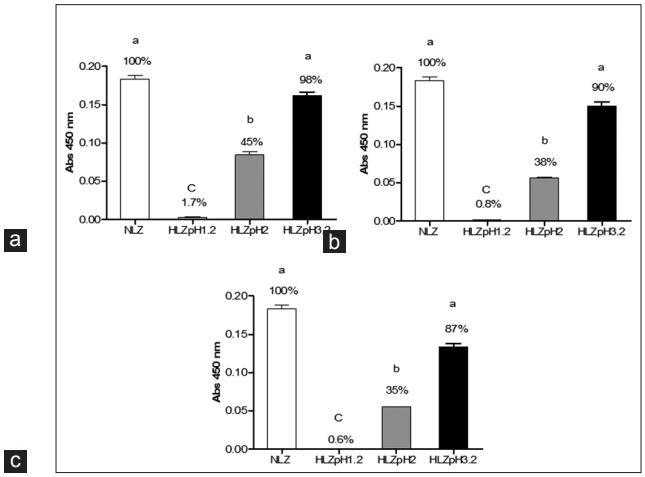


**Fig. 3: Residual enzymatic activity of lysozyme, (a) Hydrolysates of lysozyme with pepsin at pH 1.2, 2.0, and 3.2 during 60 minutes, (b) hydrolysates of lysozyme with pepsin at pH 1.2, 2.0, and 3.2 during 90 minutes, (c) hydrolysates of lysozyme with pepsin at pH 1.2, 2.0, and 3.2 during 120 minutes**

## DISCUSSION

Hen egg white lysozyme is a potent allergen named Gal d4 with resistance to hydrolysis with pepsin. In this study, lysozyme was totally hydrolyzed with pepsin at low pHs. However, it has been recently described that lysozyme at pH 1.2 has a total susceptibility to the hydrolysis with pepsin [16-18]. Fu *et al*. [19] have reported that lysozyme resisted more than 60 minutes at pH 1.2, at an E:S of (13:1) wt:wt. Thomas *et al*. described that hen egg white lysozyme is resistant to hydrolysis with pepsin at pH 2.0. Ibrahim *et al*. [20] found that 40% of the original lysozyme was hydrolyzed after 120 minutes of digestion at an E:S of 1:50 (wt:wt) at pH 4.0. Lysozyme is also a major allergen of egg white. It is generally accepted that resistance to digestion is a common feature to food allergens. There is controversy about the hydrolysis of hen egg white lysozyme, but this might be due to the different methods used.

On the other hand, we have reported hydrolysates of lysozyme without muramidase activity. These results are in agreement with different studies where hydrolyzed lysozyme with no muramidase activity has been described. Mine *et al*., 2004 [4] have described hydrolyzed of lysozyme with antimicrobial activity without muramidase activity. On the other hand, You *et al*., 2010 [2] described hydrolyzed and peptides from lysozyme without muramidase activity. Other authors such as Ibrahim *et al*., 2005 [20] have also described hydrolyzed of lysozyme with antibacterial activity but without muramidase activity.

## CONCLUSIONS

Hen egg white lysozyme was hydrolyzed with pepsin at low pHs. Hydrolysates of lysozyme at pH 1.2 do not present enzymatic activity. Hydrolysates at pH 3.2 conserve their enzymatic activity as in native lysozyme. The residual enzymatic activity method can be used to determine the degree of hydrolysis of the enzyme.

## ACKNOWLEDGMENTS

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