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# APPLICATION OF PLANT PROTEOLYTIC ENZYMES FOR TENDERIZATION OF RABBIT MEAT

**Maria Doneva, Iliana Nacheva, Svetla Dyankova, Petya Metodieva, Daniela Miteva**

Institute of Cryobiology and Food Technology, Cherni Vrah 53, 1407, Sofia, Bulgaria
Corresponding author: Maria Doneva, e-mail: maria.doneva@ikht.bg
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**Abstract:** The purpose of this study is to assess the tenderizing effect of plant proteolytic enzymes upon raw rabbit meat. Tests are performed on rabbit meat samples treated with papain and two vegetal sources of natural proteases (extracts of kiwifruit and ginger root). Two variants of marinade solutions are prepared from each vegetable raw materials– 50% (w/w) and 100 % (w/w), with a duration of processing 2h, 24h, and 48h. Changes in the following physico-chemical characteristics of meat have been observed: pH, water-holding capacity, cooking losses and quantity of free amino acids. Differences in values of these characteristics have been observed, both between control and test samples, as well as depending of treatment duration. For meat samples marinated with papain and ginger extracts, the water-holding capacity reached to 6.74 ± 0.04 % (papain), 5.58 ± 0.09 % (variant 1) and 6.80 ± 0.11 % (variant 2) after 48 hours treatment. In rabbit meat marinated with kiwifruit extracts, a significant increase in WHC was observed at 48 hours, 3.37 ± 0.07 (variant 3) and 6.84 ± 0.11 (variant 4).
The test samples also have reduced cooking losses compared to control samples. In control samples, cooking loss is increased from 13.79% (2 h) to 20.78 % (48 h). SDS-PAGE of meat samples after 48 h of treatment shows a reduction in the intensity of actin and myosin bands in all variants with papain and vegetal extracts. Electrophoretic pattern of test samples depicts proteolysis and degradation of muscle proteins.

**Key words**: tenderization, rabbit meat, papain, kiwifruit, ginger root

## Introduction

Quality of meat is determined as a combination of sensory and technological characteristics like tenderness, color, water-holding capacity and texture (*Istrati et al., 2014).*

Tenderness has been identified as the most important factor affecting consumer satisfaction and perception of taste *(Naveena et al., 2004).*

Toughness of meat depends on the amount of intramuscular connective tissue, the length of sarcomere, and also the activity of endogenous proteolytic enzymes. There are two different components to meat toughness: actomyosin toughness and background toughness. Actomyosin toughness is attributed to myofibrillar proteins, whereas background toughness is due to connective tissue presence *(Chen et al., 2006)*. Reduction of meat toughness during maturing "post mortem" or by additional treatment is a process characterized by changes in the actomyosin complex and connective tissue and is defined as tenderization (*Bekhit et al., 2012; Rawdkuen and Benjakul, 2012; Kemp and Parr, 2012).*

Proteolytic enzymes like papain, bromelain, ficain, zingibain, actinidain, etc., derived from plants are widely used in most parts of the world as tenderizers for meat. The most frequently used vegetal sources of proteolytic enzymes are papaya, ginger, pineapple, kiwi *(Ahlawat et al., 2008; Naveena and Mendiratta, 2001; Liu and Hongjun, 2001).*

These natural proteases have the potential to reduce toughness of meat so it may acquire the desired organoleptic characteristics (*Naveena and Anjaneyulu, 2004).*

Natural products containing proteolytic enzymes are defined as natural tenderizers. It has been proven that such natural products may be used for processing meat.

Juices or extracts of numerous fruits, vegetables or vegetal materials containing proteolytic enzymes may be successfully applied as marinade for various types of meat and meat products. In this case marination is a process of soaking in or injecting in meat a proteases-containing solution for achieving improved quality of the meat *(Maiti et al., 2008).*

The purpose of this study is to investigate, evaluate and compare the tenderizing effect of natural vegetal proteases upon raw rabbit meat.

## Materials and Methods

***Materials***

Meat – rabbit (*Oryctolagus cuniculus*) *biceps femoris* muscle, from the local market.

Enzyme preparations – papain (Merck)

Vegetal material: Kiwifruit (*Actinidia deliciosa)*, ginger root (*Zingiber officinale*) from the local market.

***Methods***

*Enzyme solutions marinade type*

The two vegetal sources of natural proteases (kiwifruit and ginger roots) are pilled, cut and homogenized for 1-2 minutes. This homogenate is filtered through four layers of lint. The following solutions are prepared: *variant 1* (50% w/w) and

*variant 2* (100 %w/w) from ginger and *variant 3* (50%w/w) and *variant 4* (100 % w/w) from kiwifruit. Additionally, a papain solution with 50 U/ml caseinolytic activity is prepared. Distilled water is used for the control.

*Enzyme activity*

Caseinolytic activity of proteolytic enzymes is determined using casein as substrate in 50 mM Tris / HCl buffer at pH 7.0 with 1 mM $CaCl_2$, as per the Chen et al. (2003) method. One unit of enzyme activity is defined as the quantity of enzymes needed to release 1μg tyrosine from casein for 1 min. (*Chen et al., 2003).*

*Marination of rabbit meat samples*

Pieces of rabbit meat, about 3x3x3 cm in dimension, are weighted and then soaked in already prepared marinade solutions with different proteolytic activity. After stirring, the samples are placed in plastic containers and kept refrigerated at 4°C for 2, 24 and 48 hours. Besides the test variants, controls are prepared for the three different durations of processing.

*pH measurement*

10 g meat samples are being weighted from every variant and then homogenized with 50 ml of cold distilled water. pH values are measured using pH – meter (Jenway 3310).

*Determining water-holding capacity (WHC)*

Meat samples with weight of 3-5 g are wiped from surface water using filter paper and weighted. This value is marked initial weight. Samples are treated with the corresponding marinade solutions for 2, 24 and 48 hours and then surface water is removed using filter paper. Besides the test variants, controls are prepared for the three different durations of processing. Processed meat is weighted and this value shows the weight after enzyme treatment (final weight). Percentage of water holding capacity is calculated with the following formula:

WHC% = 100 x (final weight – initial weight) / final weight

*Cooking loss*

Samples weight is measured before and after thermal processing in order to determine cooking losses defined as weight of every sample subtracted from initial weight, then divided by initial weight and multiplied by 100. The parameter "cooking loss" is calculated in percent.

Concentration of free amino acids in soluble fractions post enzyme hydrolysis is determined by ninhydrin test *(Murariu et al., 2003).*

*Polyacrylamide gel electrophoresis (SDS-PAGE)*

SDS-PAGE is performed using the Laemmli method (1970).

Polyacrylamide gel - 6% stacking gel and 10 % separating gel. Electrolyte buffer: Tris – glycine, pH 8.5 with 0.1 % SDS. Electrophoresis is performed at 25 mA current. Gel is colored using 0.1% Coomassie blue (30-40 min), and zones in gel with no protein bands are discolored for 24 h. (*Laemmli, 1970).* Bovine serum albumin (BSA) and LMW protein marker was purchased from SERVA.

*Statistical analysis* - All experiments were conducted in five replications. The data are presented as means ±SD (standard deviation). Statistical analysis was performed using two-sample t-test. The results are considered to be significant when $P<0.05$. All statistical analyses were performed using Microsoft Excel 2013.

## Results and Discussion

Rabbit meat samples are treated with papain and vegetal extracts from ginger and kiwi. A change has been observed in the following physical and chemical characteristics of meat during tenderization: pH, water-holding capacity, loss in cooking and quantity of free amino acids. Tables 1, 2 and 3 list the physical and chemical characteristics of treated rabbit meat.

**Table 1. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 2 hours**

| Properties | Control | Papain | Variant 1 ginger 50% | Variant 2 ginger 100% | Variant 3 kiwi 50% | Variant 4 kiwi 100% |
|---|---|---|---|---|---|---|
| pH | 6.22±0.02 | 6.21±0.02 | 6.23±0.02 | 6.19±0.05 | 5.83±0.01 *** | 5.50±0.01 *** |
| WHC % | 3.11±0.03 | 3.91±0.04 *** | 3.07±0.02 * | 3.42±0.09 *** | 0.99±0.04 *** | 1.45±0.06 *** |
| Cooking loss % | 13.79±0.21 | 35.18±0.56 *** | 25.65±0.65 *** | 27.52±0.88 *** | 29.25±0.37 *** | 28.40±1.29 *** |
| Free amino acids mg/ml | 0.566±0.033 | 0.906±0.01 *** | 0.752±0.053 *** | 0.791±0.005 *** | 0.822±0.018 *** | 0.804±0.015 *** |

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

**Table 2. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 24 hours**

| Properties | Control | Papain | Variant 1 ginger 50% | Variant 2 ginger 100% | Variant 3 kiwi 50% | Variant 4 kiwi 100% |
|---|---|---|---|---|---|---|
| pH | 6.22±0.05 | 6.17±0.01 | 6.18±0.02 | 6.13±0.03 | 5.64±0.03 *** | 5.42±0.02 *** |
| WHC % | 3.30±0.10 | 6.38±0.04 *** | 4.95±0.04 *** | 5.53±0.06 *** | 1.37±0.03 *** | 2.31±0.01 *** |
| Cooking loss % | 14.27±0.25 | 23.89±0.67 *** | 22.28±0.68 *** | 25.25±0.50 *** | 20.68±0.86 *** | 22.30±0.89 *** |
| Free amino acids mg/ml | 0.641±0.003 | 1.315±0.070 *** | 0.988±0.074 ** | 1.119±0.033 *** | 1.043±0.019 ** | 1.070±0.083 *** |

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

**Table 3. Physico-chemical characteristics of rabbit meat samples treated with the corresponding solutions for 48 hours.**

| Properties | Control | Papain | Variant 1 ginger 50% | Variant 2 ginger 100% | Variant 3 kiwi 50% | Variant 4 kiwi 100% |
|---|---|---|---|---|---|---|
| pH | 6.22±0.02 | 6.17±0.01 ** | 6.13±0.07 | 6.11±0.04 *** | 5.63±0.02 *** | 5.37±0.02 *** |
| WHC % | 5.91±0.08 | 6.74±0.04 *** | 5.58±0.09 *** | 6.80±0.11 *** | 3.37±0.07 *** | 6.84±0.11 *** |
| Cooking loss % | 20.78±0.98 | 22.28±0.74 * | 18.70±0.58 *** | 23.76±0.50 * | 20.23±0.30 *** | 20.16±0.52 * |
| Free amino acids mg/ml | 0.631±0.008 | 1.672±0.007 *** | 1.297±0.015 *** | 1.344±0.023 *** | 1.182±0.029 *** | 1.283±0.096 *** |

Note: Values are presented at mean ± standard deviation. Significant differences between test groups and control (*p<0.05; ** p<0.01;*** p<0.001)

One of the main properties determining the quality of meat is water-holding capacity – its ability to retain inherent water. Other factors like juiciness, aroma and color are directly related with water-holding capacity (*Gokuglu et al., 2017*).

The percentage of water-holding capacity increases in rabbit meat samples treated with papain and vegetal extracts, when duration of treatment is increased. In the samples marinated with papain and ginger, this effect was observed at 2 h of treatment and the tendency was maintained within 48 hours reaching to 6.74 ± 0.04 (papain), 5.58 ± 0.09 (variant 1) and 6.80 ± 0.11 (variant 2). In rabbit meat marinated with kiwifruit, a significant increase in WHC % was observed at 48 hours - 3.37 ± 0.07 (variant 3) and 6.84 ± 0.11 (variant 4).

Data about effect of enzyme tenderization upon meat WHC is contradictory. Some authors establish a significant increase of this indicator after 48-hour treatment of buffalo meat using ginger homogenate (*Naveena et al 2004). Gokuglu et al. (2017*) report about insignificant increase in water-holding capacity in tenderizing squid (*Loligo vulgaris*) muscle using bromelain and papain solutions. Other authors report significant reduction of this indicator when tenderizing various types of meat using bromelain extracts (*Ketnawa and Rawdkuen, 2011).*

Higher WHC percentage in test samples treated for 48 hours might be due to increase of protein-reactive groups that are available for bonding with water after partial enzymatic hydrolysis. These samples (after 48 h processing) also have reduced cooking losses compared to control. In control samples cooking loss is increased from 13.79% (2 h) to 20.78 % (48 h). On the other hand, some researchers have found higher cooking loss in meat samples treated with higher protease dosage. *Zhang et al. (2017)* investigates the tendering effects of actinidin and the commercial papain preparation using rabbit muscle. They consider that higher cooking loss is due to more extensive rupture of the muscle tissue at higher dosage.

In variants 3 and 4 a lower WHC percentage is observed compared with control and samples treated with papain and variants 1 and 2. This might be due to lower pH, which results in reduction of protein-reactive groups available for bonding with water.

A test for determining free amino acids in reaction solutions was performed in order to identify whether and to what extent enzyme solutions in selected concentration provoke protein hydrolysis to amino acids. Obtained results are listed in tables 1, 2 and 3.

Statistically significant differences in concentration of free amino acids are established between control and test variants. Similar results were noted in our previous study of tenderization of buffalo meat using papain and bromelain (*Doneva et al., 2016)*.

In test variants similar content of free amino acids in reaction solution after enzyme hydrolysis is observed. Highest content of free amino acids is observed in samples treated with papain. Also, this indicator increases when duration of meat samples treatment is increased. More intense hydrolysis of meat proteins leads to worsening both the appearance and taste qualities of meat.

The color of meat is of great importance as this is the first qualitative freshness indicator for customers. In images presented on figure 1 and 2 it is visible that meat samples treated with ginger extracts preserve their color and fresh appearance, and being thermally processed they have preserved their juiciness compared to control samples. In samples treated with papain there is deformation, meat color has faded and its surface becomes slimy.

The plant proteolytic enzyme papain is probably the most effective tenderizing agent with the highest rate of tenderization. However, papain has a tendency to over-tenderize the meat surface since it has broad specificity and indiscriminately decompose connective tissue and myofibrillar proteins, worsens the quality of the meat (*Ashie et al., 2002)*. The vegetal extracts from ginger and kiwifruit can be used as alternatives to papain for tenderization of rabbit meat.

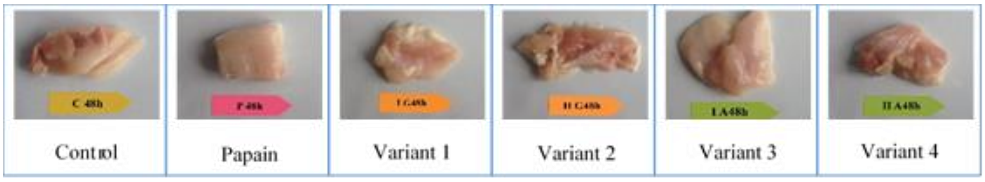


**Figure 1. Control and test variants treated with vegetal juices marinade type**


**Figure 2. Control and test variants treated with vegetal juices marinade type after thermal processing**

Figure 3 shows the result from electrophoresis of meat samples after treatment with solutions of papain and vegetal extracts for 2 and 48 hours. SDS-PAGE analysis is used for assessment of the effect of enzyme treatment upon electrophoretic profile of rabbit meat. SDS-PAGE specifies two main bands with molecular mass corresponding to contractile protein – actin and myosin. Actin and myosin are the two predominant proteins in muscles. Proteolysis of these meat proteins plays a significant part in tenderization of meat (*Kanat, et al., 2015).*

The improved meat tenderization with vegetable cysteine proteases is due to the higher breakdown of myofibril proteins and the disruption of the muscular fibril structure in the experimental samples compared to the control ones *(Jorgova et al.,1989).*

Myosin heavy chain has a molecular weight of about 200 kDa. Actin is a globular protein with molecular weight of about 44 kDa. In all variants of samples treated for 48 hours a reduction in the intensity of actin and myosin bands is observed. Intensity reduction of these two bands is more expressed when using papain solutions and ginger extracts. Also, in these samples there is an increase in meat proteins fragmentation.

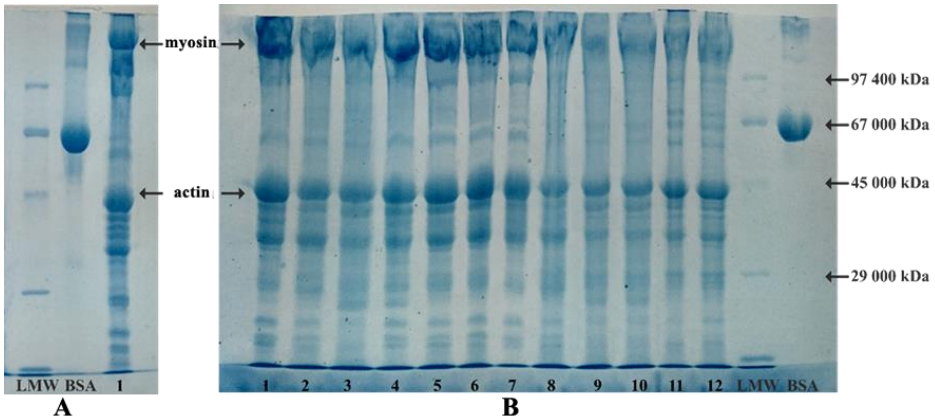


**Figure 3. A - LMW - a protein marker; BSA - standard - bovine serum albumin, 1-control rabbit meat**
**B – (1 to 6 - samples treated for 2 hours): 1-control, 2-papain, 3-variant 1, 4-variant 2, 5-variant 3, 6- variant 4; (7 to 12 - samples treated for 48 hours): 7 - control, 8-papain, 9- variant 1, 10 - variant 2, 11- variant 3, 12- variant 4**

# Conclusion

The effect of tenderizing enzymes upon the appearance of meat is a primary factor for choosing enzymes and methods of treatment. Preservation of juicy and fresh appearance is an important indicator for customers. Treatment of

rabbit meat with papain and kiwi fruit and ginger root extracts has a significant effect upon the physico-chemical characteristics of meat: pH, water-holding capacity, cooking loss and quantity of free amino acids. Differences in values of these characteristics have been established both between control and test variants and between treatment duration. In raw rabbit meat samples treated with papain and ginger and kiwi homogenates, the percentage of water-holding capacity increases when duration of treatment is increased. Statistically significant differences in concentration of free amino acids between control and test samples have been observed, as the highest values are in samples tenderized with papain.

Electrophoretic profile of control and tenderized meat samples shows reduction in intensity of actin and myosin bands in all variants treated with papain and vegetal extracts. Tests results show that ginger and kiwi extracts may be successfully used for enzyme tenderization of rabbit meat.

## Primena biljnih proteolitičkih enzima u tenderizaciji mesa zeca

*Maria Doneva, Iliana Nacheva, Svetla Dyankova, Petya Metodieva, Daniela Miteva*

## Rezime

Cilj ove studije je bio procena efekta biljnih proteolitičkih enzima na sirovo meso zeca. Ispitivanje je urađeno na uzorcima mesa zeca tretiranim papainom i biljnim prirodnim proteazama (ekstrakti kivija i korena đumbira). Dve varijante rastvora za mariniranje su bile pripremljene od svake biljne sirovine - 50% (w/w) i 100% w/w), sa trajanjem tretmana od 2h, 24h i 48h. Uočene su promene sledećih fizičko-hemijskih karakteristika mesa: pH, kapacitet zadržavanja vode, kalo kuvanja i količina slobodnih aminokiselina. Razlike u vrednostima ovih karakteristika su zabeležene, kako između kontrolnih i ispitnih uzoraka, tako i zavisno od trajanja terapije. Uzorci mesa marinirani ekstraktima papaine i đumbira, kapacitet zadržavanja vode dostigao je 6.74 ± 0.04% (papain), 5.58 ± 0.09% (varijanta 1) i 6.80 ± 0.11% (varijanta 2) nakon 48 sati tretmana. Kod mesa zeca mariniranog ekstraktima kivija, značajno povećanje kapaciteta zadržavanje vode je primećeno nakon 48 sati, 3.37 ± 0.07 (varijanta 3) i 6.84 ± 0,11 (varijanta 4).
Uzorci korišćeni za testiranje su takođe pokazali manji kalo kuvanja u poređenju sa kontrolnim uzorcima. U kontrolnim uzorcima, kalo kuvanja se povećao sa 13.79% (2 sata) na 20.78% (48 sati). SDS-PAGE uzoraka mesa posle 48 h tretmana pokazuje smanjenje intenziteta aktina i miozinskih opsega u svim varijantama sa

papain i biljnim ekstraktima. Elektroforetski obrazac uzoraka prikazuje proteolizu i degradaciju mišićnih proteina.

**Ključne reči:** tenderizacija, meso zeca, papain, kivi, koren đumbira

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