The effect of freezing rate, frozen storage time and thawing methods on the concentration of thymosin proteins in pork meat

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Proteins are the most important ingredients in meat. They are found in meat in different forms and with different functions. Thymosins are small proteins present in many animal tissues, with molecular weights of 1000-15000 Da. The technological processing of meat causes changes in the amount and shape of the basic ingredients. During the freezing of meat and its storage in the frozen state, various chemical reactions take place causing changes in the proteins. The aim of this paper was to examine the influence of the freezing rate and thawing methods on the changes and behaviour of thymosin proteins in pork meat (M. longissimus thoracis et lumborum) during storage. Protein analyses were performed using capillary gel electrophoresis (CGE) and the SDS-MW Analysis Kit (Beckman Coulter). The meat samples were frozen at 10 different rates (from 0.23 cm/h to 1.43 cm/h). The samples were tested at different times during 60 days of storage at -20°C (after 1, 15, 30, 45, 60 days). Before the analysis, the samples were thawed in the refrigerator, at room temperature and in the microwave. After one day of storing frozen meat samples, the relative concentration of thymosin was less than 1% in all tested samples. During 60 days of storage, most samples had a slight increase in the relative concentration of thymosin. The highest relative concentration of thymosin after 60 days of storage (1.67%) was recorded in a meat sample that was frozen at a rate of 1.10 cm/h and thawed in a microwave oven.

INTRODUCTION

Proteins are the most important building blocks of muscle tissue. Proteins in muscles (flesh) account for about 18.5%. Meat proteins are divided into three basic groups: myofibrillar, sarcoplasmic and connective tissue proteins. The first group consists of myofibrillar proteins, which include proteins soluble in lowconcentrated salt solutions, then proteins soluble in concentrated salt solutions, and insoluble myofibrillar and cytoskeletal or myofibrillarcytoskeletal proteins. Myofibrillar proteins are organized in myofibrils and their substructures, (thick and thin) myofilaments. Myofibrillar proteins constitute about 50-60% of total muscle proteins (Huff - Lonergan, 2010). The second group of meat proteins are sarcoplasmic proteins, which are soluble in water (albumins) or highly dilute solutions of salts (globulins). These proteins are characterized by crystallization and are very

sensitive to denaturation. These include myoglobin and enzymes of energy metabolism, which make up about a third of total muscle proteins (~ 30%) (Lawrie & Ledward, 2006). About 10% of muscle proteins are stromal proteins, which are predominantly found in structural connective tissue proteins – collagen and elastin (Huff – Lonergan, 2010; Rede & Petrović, 1997; Xiong, 2004).

Thymosins are small proteins present in many animal tissues. They constitute three families: the prothymosin, parathymosin, and β -thymosin families (Hannappel & Huff, 2003). The thymosin proteins are all short, highly charged, intrinsically unstructured proteins under natural conditions with molecular weights of 1000–15000 Da. The thymosin beta proteins are major intracellular G-actin-sequestering peptide, thus allowing cells to have a high concentration of G-actin, ready for quick use (Hoch &Volk, 2016; Litwack, 2018; Tokura et al., 2011). Since thymosins modulate cell migration, angiogenesis and immune responses, they have been categorized as biological response modifiers, and β -thymosin, in particular, is a key regulator of tissue regeneration (Hara, 2011).

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During the freezing and storage of frozen products, various chemical changes of ingredients take place, which leads to destabilization of protein gels and emulsions, and increased release of water during thawing (Savanović & Grujić, 2017). The factors influencing the change of meat and meat products during freezing and storage are ice formation rate and recrystallization, dehydration, salt concentration, ionic strength, oxidation processes, lipid changes, and release of certain cellular metabolites (Zaritzky, 2008). Studying the changes in food products during processing and storage, and especially in the processing of raw materials at low temperatures, showed that the quality and physicochemical properties of food products significantly depended on changes taking place in proteins (Delgado & Sun, 2010). The aim of this paper was to examine the influence of the freezing rate and thawing methods on the changes and behaviour of thymosin in pork meat during storage.

MATERIAL AND METHODS

The freezing of meat samples

The test was performed on pig meat - samples of the back muscles of M. longissimus thoracis et lumborum pigs below one year of age, and the average gross weight of about 130-140 kg, after cooling for 24 hours. After the separation of the muscles from the trunk, the pieces were cut into 2.0 cm thick slices, packed in polythene bags, and, after marking, frozen at different rates (Table 1). The freezing of fresh meat samples was performed at different temperatures, ranging from -20°C to -80°C. Arctiko UPUL 580 Ultra Low Temperature Freezer was used for the freezing. The temperature at the centre of the product was monitored throughout the freezing process, using Testo AG 922 digital thermocouple thermometer. The freezing was performed until the temperature of -20°C was reached in the centre of the product, after which the samples were kept at -20°C until the analysis. The samples were tested at different times during storage (1, 15, 30, 45 and 60 days after freezing). Before analysis, the samples were thawed in one of the following ways: 10 hours at 4°C (in the refrigerator), 2 hours at 20°C (at room temperature), or in the microwave oven (2450 MHz, 700 W).

The extraction of protein from meat

Protein extracts were obtained according to the method of Toldra et al. (1993). Two grams of pork meat was homogenized with 20 ml of 0.03 M sodium phosphate buffer (pH=7.40), at 4°C for 2 minutes, using a homogenizer (Ultraturax, IKA). The homogenate was centrifuged for fifteen minutes, 5000 rpm, at 4°C. The supernatant containing sarcoplasmic proteins was drained, and the myofibrillar proteins were extracted from the residue by homogenization with a solution containing 8 M urea and 1% β -mercaptoethanol, at 4°C for 2 minutes, using a homogenizer (Ultraturax, IKA). The homogenate was centrifuged again, under the same conditions, to produce a supernatant containing myofibrillar proteins.

Protein separation

Protein separation was performed by capillary electrophoresis (Agilent, CE 7100) using the SDS-MW Analysis Kit (Beckman Coulter). The preparation of the apparatus and separation of proteins is described in detail by Grujić & Savanović (2018).

The separation of known molecular weight proteins (Mw standards) containing seven known molecular weight proteins (10 kDa – 225 kDa) was performed for 30 minutes. Based on the migration times and known molecular weights, a calibration curve was obtained to be used to estimate the molecular weights of the extracted myofibrillar proteins. A regression line was obtained:

 $y (log M_{w}) = 0.088X - 0.0286, R^{2} = 0.9874$ (1)

The separation of proteins was performed in a capillary with an inner diameter of 50 μ m, a total length of 33 cm, and an effective length of 23.50 cm. SDS gel buffer of appropriate composition was used to fill the capillary. Injecting was performed electrokinetically using -5 kV voltage for 20 seconds. Separation is performed for 30 minutes, using a voltage of -16.5 kV. The detection wavelength was 220 nm with 20 nm permeability (without reference wavelength), and the response time was 1 second. Protein identification was performed by comparing the obtained molecular weights with the relevant data published in the literature (Gallego et al., 2015; Ngapo & Alexander, 1999; Porzio & Pearson, 1977; Sotelo et al., 2000), and the procedure is explained in detail in the work of Grujić & Savanović (2018). The identified proteins are shown in Figure 1.

Table 1. Conditions for freezing meat

Freezing rate (cm/h)	0.23	0.30	0.40	0.50	0.60	0.90	1.00	1.10	1.25	1.43
Freezing time (min)	257	200	150	120	104	65	60	54	48	42

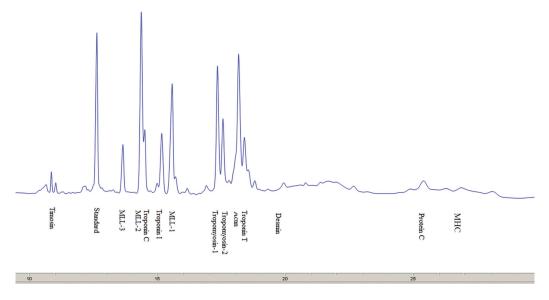


Figure 1. The electropherogram obtained after separating myofibrillar proteins from pork meat (M. longissimus thoracis et lumborum)

The changes and behaviour of the thymosin protein were monitored during the research for this work. The peak areas and relative protein concentrations were determined by ChemStation Software (Agilent). The areas of peaks in the electropherograms, determined by indirect detection, allowed us to automatically quantify the relative concentration of protein thymosin in the mixture, using the capillary instrument software.

RESULTS AND DISCUSSION

Table 2. shows the relative concentration of thymosin protein in pork meat after one day, and after 60 days of storage. After one day of storing frozen meat samples

at -20°C, the relative concentration of thymosin was less than 1% in all tested samples. During 60 days of storage, the relative concentration of thymosin slightly increased in most samples. The analysis of the obtained electropherograms of samples after one day of storage and thawed at room temperature showed the lowest relative concentration of thymosin in samples frozen at 1.25 cm/h (0.68%), while the highest concentration of thymosin was observed in samples frozen at the maximum rate 1.43 cm/h (0.95%) and a minimum rate of 0.23 cm/h (0.94%). For samples thawed in the refrigerator, the relative concentrations of thymosin protein ranged from 0.77% (rate 0.23 cm/h) to 0.96% (rates 0.60 and 1.00 cm/h). The samples of pork meat

Table 2. The influence of freezing rate and thawing method on the relative concentration of thymosin protein in pork meat after 1 and 60 days of storage

Freezing rate (cm/h) —	Relative co	oncentration of thy after 1 day	vmosin (%),	Relative concentration of thymosin (%), after 60 days			
	А	В	С	А	В	С	
0.23	0.94	0.77	0.75	1.02	1.30	1.02	
0.30	0.83	0.88	0.96	0.91	1.01	1.00	
0.40	0.85	0.84	0.91	0.92	0.98	0.75	
0.50	0.87	0.84	0.93	1.22	0.99	0.92	
0.60	0.90	0.96	0.72	0.94	0.98	0.94	
0.90	0.91	0.82	0.76	0.89	1.18	0.81	
1.00	0.89	0.96	0.97	1.06	1.65	0.89	
1.10	0.81	0.92	0.96	0.89	1.09	1.67	
1.25	0.68	0.78	0.81	1.09	1.16	1.05	
1.43	0.95	0.88	0.76	0.99	1.11	0.87	

A- thawing at room temperature; B- thawing in the refrigerator; C- thawing in the microwave oven

thawed in a microwave had a relative concentration of thymosin from 0.72% (0.60 cm/h) to 0.97% (rate 1.00 cm/h).

After 60 days of storage, the analysis of the obtained electropherograms of samples thawed at room temperature showed the lowest relative concentration in samples frozen at 1.10 cm/h and 0.90 cm/h (0.89%), while the highest concentration was observed in samples frozen at a rate of 0.50 cm/h (1.22%). For samples thawed in the refrigerator, the relative concentrations of thymosin protein ranged from 0.98% (rates 0.40 and 0.60 cm/h) to 1.65% (rate 1.00 cm/h). The samples of pork meat thawed in a microwave had a relative concentration of thymosin from 0.75% (0.40 cm/h) to 1.67% (rate 1.10 cm/h).

Figure 2. shows the influence of freezing rate and storage time on the trend of change in the relative concentration of thymosin protein in pork meat, thawed at room temperature. Pork meat samples frozen at 0.23 cm/h and the samples frozen at 1.43 cm/h showed an increase in thymosin concentration during the first 30 days of storage (from 0.94% and 0.95% to 1.24% and 1.25%). After 60 days of storage at -20°C, the relative concentration of thymosin was 1.02% in pork meat samples frozen at 0.23 cm/h and 0.99% in samples frozen at 1.43 cm/h. In the samples frozen at 0.60 cm/h and thawed at room temperature, the lowest thymosin concentration was recorded after 45 days of storage (0.85%), and the highest after 15 days of storage (1.05%).

Figure 3. shows the influence of freezing rate and storage time on the relative concentration of thymosin protein in pork meat thawed in refrigerator. In the samples frozen at 0.23 cm/h, thymosin concentration of 0.77% after one day of storage rose to 1.30% after 60 days of storage. Pork meat samples frozen at 0.60 cm/h had the lowest concentration of thymosin after 45 days of storage (0.82%), while the highest concentration was recorded after 60 days of storage (0.98%). During the storage of samples frozen at a maximum rate of 1.43 cm/h, the lowest concentrations of thymosin were observed after 45 days (0.77%), and the highest concentrations of thymosin were observed after 30 days (1.38%).

Figure 4. shows the influence of freezing rate and storage time on the relative concentration of thymosin protein in pork meat thawed in a microwave. In samples frozen at 0.23 cm/h, the lowest concentration of thymosin was recorded after 45 days of storage (0.69%), while the highest relative concentration of thymosin (1.07%) was recorded after 15 days of storage. Samples frozen at 0.60 cm/h had the lowest thymosin concentration after one day of storage (0.72%), and after 60 days of storage, the thymosin concentration increased to 0.94%. Samples frozen at

the maximum rate (1.43 cm/h) during the first 45 days of storage demonstrated an increase in the relative concentration of thymosin from 0.76% (after 1 day) to 1.77% (after 45 days), and after 60 days of storage, the relative concentration of thymosin was 0.87%.

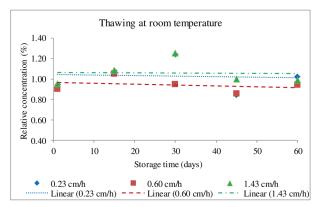


Figure 2. The influence of freezing rate and storage time on the relative concentration of thymosin protein in pork meat, thawed at room temperature

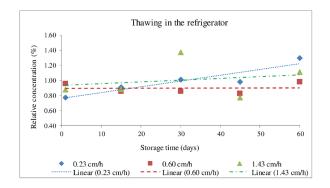


Figure 3. The influence of freezing rate and storage time on the relative concentration of thymosin protein in pork meat, thawed in the refrigerator

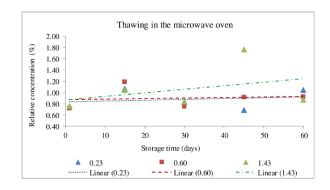


Figure 4. The influence of freezing rate and storage time on the relative concentration of thymosin protein in pork meat, thawed in a microwave oven

Based on the obtained results, an increase in thymosin concentration after 60 days of storage was noticeable in most samples. Since thymosin is a low molecular weight protein, it is assumed that, during storage, higher molecular weight proteins split into many lower molecular weight proteins.

During the freezing and storage in the frozen state, various chemical reactions take place in meat causing changes in its proteins. Decreasing the amount of water in the liquid state and increasing the concentration of electrolytes during freezing can lead to a change in the structure of the protein (Savanović & Grujić, 2017). It is known that the ability of plant and animal tissues to bind water as well as the ability of water to resorb during thawing depends on the state of the protein (Chan et al., 2011; Wang et al., 2013). If during the freezing and storage of the product in the frozen state the state of the protein changes, the thawing tissue cannot absorb all the water formed during the thawing of ice crystals. This leads to an unwanted release of water from the product and a change in appearance and texture (Nesvadba, 2008). In frozen products, the interactions between protein and water are reduced, while those between proteins are intensified, which causes a change in the state of the protein in food products (Blond & Le Meste, 2004). The most significant changes in protein during the freezing and storage of frozen products are denaturation, oxidation, and modification of the amino acid chain, the formation of protein polymers, a decrease in protein solubility, an increase in proteolytic activity, the aggregation or fragmentation of proteins, and an increase in carbonyl concentration. These changes are associated with a decrease in the functional properties of proteins and a decrease in the quality of meat and meat products after thawing (Savanović & Grujić, 2017). Coggins & Chamul (2004) reported that the most significant physicochemical changes occurring during the storage of frozen food were lipid oxidation, protein denaturation, product discolouration, ice sublimation, and the recrystallization of ice crystals.

CONCLUSION

During the freezing of meat and storage in the frozen state, a change in the structure of proteins takes place. Based on the results obtained by examining the influence of the freezing rate and thawing methods on the relative concentration of thymosin, it can be concluded that freezing, thawing and storage time affect the concentration of thymosin in pork meat. After one day of storing frozen meat samples at -20°C, the relative concentration of thymosin was less than 1% in all tested samples. During 60 days of storage, the relative concentration of thymosin slightly increased in most samples. The highest relative concentration of thymosin after 60 days of storage (1.67%) was recorded in a meat sample frozen at a rate of 1.10 cm/h and thawed in a microwave oven.

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Uticaj brzine smrzavanja, dužine skladištenja i načina odmrzavanja na koncentraciju proteina timozina u svinjskom mesu

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Ključne riječi: timozin, smrzavanje, svinjsko meso.

Proteini su najznačajniji sastojci mesa i oni se u mesu nalaze u različitim oblicima i imaju različite funkcije. Timozini su mali proteini prisutni u mnogim životinjskim tkivima sa molekulskom masom od 1000–15000 Da. Tokom različitih tehnoloških postupaka u mesu dolazi do promjena količine i stanja osnovnih sastojaka. U toku smrzavanja mesa i skladištenja u smrznutom stanju, u mesu se odvijaju različite hemijske reakcije, koje uzrokuju promjene proteina. Cilj ovog rada je bio da se ispita uticaj brzine smrzavanja i načina odmrzavanja na promjene i ponašanje proteina timozina u svinjskom mesu (M. longissimus thoracis et lumborum), u toku skladištenja. Analize proteina su vršene pomoću kapilarne gel elektroforeze, uz korištenje SDS-MW Analysis Kit-a (Beckman Coulter). Uzorci mesa su smrznuti na 10 različitih brzina (od 0.23 cm/h do 1.43 cm/h) i ispitivani u različito vrijeme tokom 60 dana skladištenja na -20°C (nakon 1, 15, 30, 45, 60 dana). Prije analize uzorci su odmrznuti u frižideru, na sobnoj temperaturi i u mikrotalasnoj pećnici. Na osnovu dobijenih rezultata može se zaključiti da brzina smrzavanja, način odmrzavanja i dužina skladištenja utiču na koncentraciju timozina u svinjskom mesu. Nakon 1 dan skladištenja smrznutih uzoraka mesa relativna koncentracija timozina je iznosila manje od 1% u svim ispitivanim uzorcima. Tokom 60 dana skladištenja kod većine uzoraka došlo je do blagog povećanja relativne koncentracije timozina. Budući da su timozini proteini male molekulske mase, pretpostavlja se da se tokom skladištenja proteini veće molekulske mase cijepaju u veći broj proteina manje molekulske mase. Najveća relativna koncentracija timozina nakon 60 dana skladištenja, u iznosu od 1,67% zabilježena je kod uzorka mesa koji je smrznut brzinom 1,10 cm/h i odmrznut u mikrotalasnoj pećnici.