INVESTIGATION INTO THE INFLUENCE OF EXOGENIC CALCIUM ON DEGRADATION PROCESSES OF CONNECTIN OF MUSCULAR TISSUE

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Introduction

In the early 80s first experimental data showing possibility of non-enzymatic destruction of myofibrillar structures under the influence of calcium ions were published (Hattori A., Takahashi K., 1982). This allowed to Japanese scientists to formulate a calcium theory of the mechanism of meat tenderization during postslaughter aging (Takahashi K., 1999). However, it should be noted that this theory is based mainly on the experimental data obtained during investigations of model objects - isolated microfilaments or other myofibrillar structures.

This work is one of the first attempts of using the commercial raw materials (hot beef) for studying the influence of exogenic calcium on processes of degradation of one of the main structure-forming proteins of muscular tissue – connectin and thus on meat tenderization.

The objective of the present work is to study the influence of calcium ions on connectin of muscular tissue.

Materials and methods

The object of the investigation was the L. dorsi of beef. Hot meat within 1 –1.5 hours post-mortem was trimmed with separation of visible fat and connective tissue. Then the muscular tissue was chopped in a chopper in cold. 3 specimens of the obtained ground meat were used for the experiments. Each specimen was homogenized in the homogenizer in cold in the appropriate buffer during 2 – 3 minutes.

The following buffer solutions with pH 5.8 were used :

1 – phosphate buffer (containing 0.01 KH_2PO_4, 0.75M KCl, 0.02% NaN_3) + 1mM EDTA;

2 – phosphate buffer + 0.1mM $CaCl_2$;

3 – phosphate buffer + 0.1mM $CaCl_2$ + 10mM iodoacetamide (proteases inhibitor).

Then from each homogenate the aliquots of the suspension were selected and centrifuged at 3000 rev./min during 5 minutes.

The remaining part of the homogenate was placed into refrigerator and kept at 4±2°C.

For further experiments, the aliquots were selected after 5, 20, 40 and 60 hours and were centrifuged under the same conditions.

Immediately after centrifuging the sediment was separated and further experiments were carried out in supernatants.

Total protein in the specimens was determined according to M.M. Bradford (1976).

For electrophoretic investigations from supernatants, immediately after centrifugation aliquots were selected which were mixed in equal volumes with the solubilizing buffer, containing 8M of urea, 2M of thiourea, 3%SDS, 75mM dithiotreitole, 25mM Tris-HCl, pH = 6.8 (Fritz J.D. et al., 1993) and 20 μl 0.1 of bromphenol blue.

Then the prepared specimens were heated during 3 minutes on a boiling water bath and after cooling to room temperature they were frozen and stored in frozen state until used for electrophoresis.

4 μl of the specimen was introduced into gel for electrophoresis. Electrophoresis was carried out in polyacrylamide gel with density gradient from 4 to 7.5%. The strength of the gel for concentration was 2.56%. Electrophoresis was carried out in a buffer solution of the composition as follows: 0.05M Tris, 0.384 M of glycine, 0.1% SDS, pH=8.8 (the upper electrode tank contained 10mM of β- mercaptoethanol) during 1.5 hour at a room temperature and amperage from 10mA (at the beginning of the process) to 40 mA (at the end).

Treatment, dyeing and washing of gels was carried out according to Fritz J.D. et al (1989, 1993).

Results and discussion

At the present time it is known that during post slaughter aging of muscle tissue the molecule of connectin (molecular mass – 3000 kDa) falls into β-connectin (2800 kDa) and S-fragment with molecular mass 1200 kDA (Takahashi K. et al., 1992).

Using the polyacrylamide gel electrophoresis with SDS we tried to follow the quantitative changes of connectin and its degradation products during incubation under different conditions:

1. Calcium ions fully absent.
2. In the presence of 0.1mM $CaCl_2$
3. In the presence of the same $CaCl_2$ concentration and iodoacetamide (inhibitor of proteases)

First of all it should be noted that the initial homogenates of muscular tissue (zero samples) had some amounts (from 1% to 3% from total protein) of β-connectin and S-fragment. This could be explained both by the age of the animal and its physiological state at the time of slaughter. For a more clear presentation of the results obtained and their interpretation all the experimental data on the change in the amount of the studied protein and products of its degradation were recalculated in relation to their concentrations in the initial samples (zero points). These concentrations were assumed as 100%.

First let's consider the behavior of the studied protein at full absence of calcium in the incubation medium. These conditions were created by adding a complexon EDTA in the tissue homogenate. In this case there were practically no changes in the amount of connectin and its derivatives – β-connectin and S-fragment (Figs A, B, C, curves 1) during 60 hours of incubation. The obtained results allow to make a conclusion that the complete removal of calcium ions stops possible processes of connectin degradation associated with its proteolysis by the tissue calcium-activated proteases and calcium-induced splitting of peptide bonds. Different picture can be observed when introducing 0.1 mM $CaCl_2$ into the incubation medium. It should be mentioned that such concentration of calcium ions is by factor of one higher as compared to that which is present in hot muscular tissue (Takahashi K., 1999). Under these conditions a noticeable reduction in the amount of connectin takes place (Fig.A; curve 2) and a significant increase in the amount of S-fragment (Fig. C; curve 2). These data allow to conclude that destruction of connectin is directly induced by introduction of calcium ions into the meat system. While interacting with calcium-binding centres in connectin molecules the calcium ions ensure their splitting into- β-connectin and S-fragment (Takahashi K., 1999). β-connectin that also possesses calcium-binding centers, can further split into S-fragment and polypeptide with molecular mass 400 kDa (Takahashi K., 1999). This can explain quite a substantial increase in the concentration of S-fragment during incubation (Fig. C, curve 2).

Analysis of results on degradation of connectin in the presence of calcium ions and the inhibitor of calcium- activated and SH proteases of iodoacetamide has revealed a number of peculiarities of these processes. From the data presented it is seen that the amount of connectin decreases under these conditions somewhat faster than in the previous case (Fig.A, curve 3). The increase in the concentration of S-fragment is comparable with the decrease in connectin content but considerably less pronounced than in the absence of iodoacetamide (Fig. C, curve 3). And the amount of β-connectin in this case doesn't increase, but on the contrary, falls sharply (Fig. B, curve 3). The presented results don't allow to give a valid explanation to this fact. One can only suppose that iodoacetamide is in some way initiates the acceleration of degradation of β-connectin to less fragments – polypeptides with molecular weights 400 and 1000 kDa (Takahashi K., 1999), which in this work have not been identified. One can't also exclude the possibility that this phenomenon was the result of removal of β-connectin from the reaction zone due to aggregation and non-specific bonding with other myofibrillar proteins under the action of iodacetamide. A similar phenomenon was discovered recently when investigating the effect of proteases inhibitors - calpastatin and leupeptin on connectin and nebulin filaments (Tatsumi R. et al., 1998).

Conclusions.

1. It was found that introduction into meat system of calcium ions in the amounts higher than physiological concentration, accelerates calcium-induced splitting of the main structure-forming proteins of muscular tissue - connectin and nebulin, that can accelerate the process of meat tenderization.
2. It was revealed that proteases inhibitor – iodoacetamide significantly decreases degradation of nebulin filaments and changes the mechanism of calcium-induced splitting of connectin.
3. It is shown that removal of calcium ions from tissue homogenates leads to practically full inhibition of destruction of structure-forming proteins.

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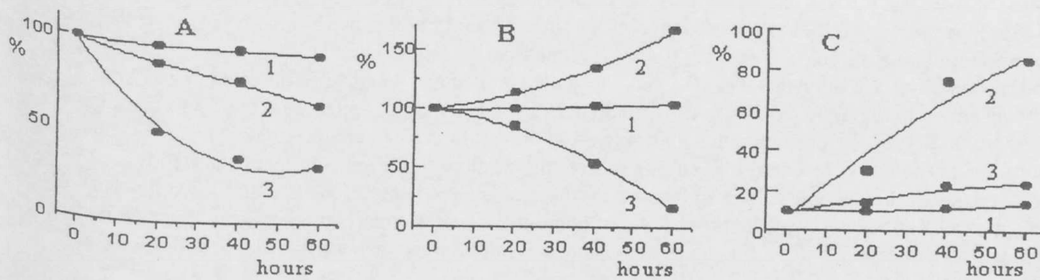

Dynamics of quantitative change of connecin (A) and products of its degradation, β-connectin (B) and S-fragment (C) during incubation of muscular tissue (beef) in the absence of calcium ions (1), in the presence of calcium ions (2) and in the presence of calcium ions and iodoacetamide (3).