DETECTION OF MEAT FREEZING USING MITOCHONDRIAL ENZYMES AND CHANGES DURING STORAGE

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Abstract – Measurement of enzyme activity in exudate from meat was studied for the detection of freezing of pork meat. Citrate synthase and aconitase are released from mitochondria damaged by ice crystals. Whereas citrate synthase activity offers no reliable and significant differences between frozen and refrigerated pork, the aconitase activity proved to be a reliable marker of previous freezing of pork meat.

I. INTRODUCTION

The freezing of meat causes the loss of its fresh appearance, drip loss, and other changes that have negative effects on the sensory characteristics of the meat; such changes are greater than in meat stored at refrigeration temperatures [1][2]. Many methods were suggested for the detection of previously frozen meat [3][4][5], several of them focused on the damage of intracellular organelles by ice crystals that are formed during the freezing process. Some specific enzymes that are released from mitochondria can be detected and used as markers of freezing [6]. Such enzymes are *β*-hydroxyacyl CoA-dehydrogenase (HADH), N-acetyl-β-glucosaminidase [7], citrate synthase, aconitase [8] and other.

In our earlier study on chicken meat [9][10], citrate synthase was not proved to be quite a reliable marker for the detection of freezing, whereas aconitase showed to be more suitable. Oxidation of unsaturated lipids leads to the formation of reactive malondialdehyde that could lower the activity of some enzymes [11]. In opposite to chicken meat pork and beef have lower content of polyunsaturated fatty acids that undergo oxidative changes, especially linoleic and arachidonic acid [12]. Thus it can be supposed that the enzyme activity will be less influenced by the oxidation products. Due to this reason we tested the use of aconitase and citrate synthase activity on pork to compare these two enzymatic methods and assess which one is more suitable for the distinguishing fresh and frozen pork.

II. MATERIALS AND METHODS

Material

Pork *m. longissimus lumborum et thoracis* was cut into slices of approximate weight 150 g. Each slice was packed under vacuum in polyethylene foil. The samples were divided in two halves; one half was stored at 4 °C and the other at -22 °C. All refrigerated samples were stored until analysis; frozen samples were thawed in a refrigerator approximately 24 hours before measurement.

For the repeatability of done experiments, a reproduced experiment was carried out.

Methods

The activity of enzymes was determined in the exudate of meat from both chilled and frozen/thawed meat. According to previous findings [9], the released exudate was filtered by syringe filters (CHS FilterPure Nylon Syringe Filter, 0.45 µm, 25 mm) and the total exudate amount was taken in account.

Aconitase activity

Aconitase activity was measured by the Spekol® 1300 spectrophotometer (Analytik Jena AG, Germany) on the basis of absorbance changes induced by the production of NADPH, which is detected at the wavelength of 340 nm. NADPH is produced during a reaction in which the isocitric acid is oxidized by decarboxylation and α-ketoglutaric acid is formed. This reaction is preceded by the reversible isomerisation of citric acid via cis-aconitic acid as an intermediate. The activity of aconitase is directly proportional to the variance of absorbance value per minute.

The released exudate from meat was diluted in ratio 1:9 and filtered. 200 µl of this prepared solution was pipetted to a cuvette and 200 µl of NADP, 200 µl of substrate and 200 µl of isocitrate dehydrogenase were added. The mixture was shook carefully to avoid bubbles

that might interfere during measuring and the reaction mixture was incubated in spectrophotometer at 37 °C for 15 minutes. After that, the measurement itself lasted 5 minutes. The interval of recording the absorbance was 20 seconds. For each sample, three parallel measurements were done.

Citrate synthase activity

Citrate synthase catalyses the reaction of acetyl-CoA and oxaloacetate to citrate and CoA-SH. This thiol reacts with added DTNB (5,5´-dithio-bis(2-nitrobenzoic)) and forms CoA-S-S-TNB. The activity of citrate synthase is based on the absorbance of yellow product TNB (5-thio-2-nitrobenzoic acid) at 412 nm.

Samples were prepared as follow: 0.5 ml of exudate and 10 µl of bicine buffer (N,N-bis-(2-hydroxyethyl)-glycine) was mixed and diluted with demineralised water (1:9). Further the sample was tempered to laboratory temperature (25 °C) and 10 µl of DTNB, 10 µl of acetyl-CoA and 920 µl of a test solution for citrate synthase were added.

First the endogenic activity was determined, when the sample was incubated for 20 seconds. After that the measurement started and the absorbance was recorded every 10 seconds for the total time of 90 seconds. Followed by the addition of 50 µl of oxaloacetic acid, once again the solution was incubated for 20 seconds and the absorbance was measured for another 90 seconds - the overall activity was estimated.

Statistical analysis

Measurements were performed three times for each sample. The data from the frozen and refrigerated meat were compared by the Student test (StatSoft CR, Prague, Czech Republic). The experiment was twice repeated.

III. RESULTS AND DISCUSSION

The goal of the study was to establish if specific enzymes of Krebs cycle that should only be present in exudates of thawed meat, and should therefore not be present in fresh meat can be used as a marker of freezing of meat.

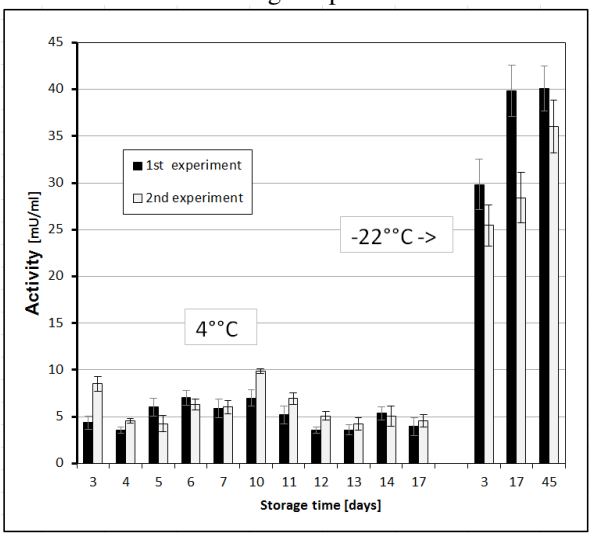
The frozen samples were measured to the day 45, whereas chilled samples were measured only to the day 17; after this time pork meat started to decay. The activity of citrate synthase and aconitase should be zero for samples stored in a refrigerator; since they are intracellular enzymes they should be released only when the membrane of cells is disrupted [13]. However, non-zero activity for intracellular enzymes was recorded in other papers [1][8][9][14][15].

Aconitase

The measurement was done separately on two sets of samples (See Fig. 1). The values from first and second series closely correlated ($r = 0.98$). During refrigerated storage the activity values ranged between 3.5 and 8.0 $mU.ml^{-1}$. The duration of frozen storage showed an increasing trend of measured values from 25 to 40 $mU.ml^{-1}$.

The average error of measuring was 12 % for refrigerated samples and higher for samples stored under freezing conditions. Cut-off limit was set to the twice the amount of average

Fig.1. Aconitase activity during refrigeration and frozen storage of pork meat



value and as it could be seen in Fig. 1, none of the recorded values extended this limit.

The statistical evaluation of the divergence between the two ways of storage was performed by the assessment of the normal value distribution of each type of storage. It showed that both sets had normal distribution ($p < 0.003$) and could therefore be used as one. Data were then subjected to Student t-test and according to the results it could be stated that the values of the aconitase activity between

refrigerated and frozen samples were statistically highly significantly different (p <<< 0.001).

Citrate synthase

The experiment with citrate synthase was carried out on the same samples as were for the aconitase. However first and second series did not demonstrated same results (r = 0.20) as for the aconitase (r = 0.98) (see above). The cut-off limit was similarly set to

Table I: Activity of citrate synthase during refrigerated and frozen storage of pork meat

Time of storage [days]	Citrate synthase activity [µmol·min^{-1}·ml^{-1}]	
	1. experiment	2. experiment
Cold storage		
3	0,196 ± 0,125	0,441 ± 0,427
4	0,295 ± 0,074	0,662 ± 0,147
5	0,931 ± 0,211	0,460 ± 0,217
6	0,147 ± 0,060	0,221 ± 0,187
7	-	0,147 ± 0,168
10	0,257 ± 0,160	0,294 ± 0,347
11	0,386 ± 0,240	0,514 ± 0,472
12	0,637 ± 0,173	0,529 ± 0,358
13	0,417 ± 0,489	0,386 ± 0,342
14	0,429 ± 0,307	0,613 ± 0,451
17	0,319 ± 0,069	1,074 ± 0,635
Frozen storage		
3	0,882 ± 0,147	0,809 ± 0,309
17	1,667 ± 0,665	0,515 ± 0,309
45	0,926 ± 0,489	0,897 ± 0,327

the twice the amount of mean citrate synthase value for unfrozen samples. As can be seen in tab. I, some of the samples might be falsely classified. The activity of citrate synthase for fresh samples ranged between 0.00 and 1.35 U.ml^{-1}, while for the frozen samples the values ranged from 0.2 to 2.4 U.ml^{-1}.

Results from first try-out were feasible to be distinguished with significance level $p < 0.001$, but in the second series the significance level decreased to p = 0.05. The biggest problem in classifying recorded values was the high measurement error that in some cases exceeded 100%; the mean measuring error for citrate synthase was 60.9%.

Comparison of the methods

Comparing both methods showed obvious higher measuring error for citrate synthase (60.9%) than for aconitase (12.1%). Activity values of citrate synthase for refrigerated samples exceeded the given cut-off limit and at the same time frozen samples did not come up to this limit. This fluctuation of values was not present for aconitase and the refrigerated and frozen samples could therefore be distinguished with higher significance level (p <<< 0.001).

Compared to frequently published method, where the reference enzyme is β-hydroxyacyl-CoA-dehydrogenase, the refrigerated and frozen samples can be distinguished by aconitase activity with higher significance level; the significance level for the use of HADH is lower than 0.001 [1] [16].

IV. CONCLUSION

The measurement of the aconitase activity in exudate of meat proved to be a suitable method for distinguishing fresh and frozen/thawed pork meat. The difference between both storage modes was statistically highly significant (p <<< 0.001). The citrate synthase seems to be less suitable because of low significance level and high measuring error.

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