Differentiation between Fresh and Thawed Chicken Meat by the Measurement of Aconitase Activity

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Abstract

Škorpilová T., Šimoniová A., Rohlík B.-A., Pipek P. (2014): **Differentiation between fresh and thawed chicken meat by the measurement of aconitase activity**. Czech J. Food Sci., **32**: 509–513.

The presence of mitochondrial enzymes in the exudate of meat might indicate former freezing procedures. Based on this hypothesis, a method was developed to distinguish fresh meat from frozen/thawed meat. Another goal of this study was to compare the activity of mitochondrial enzyme aconitase in meat under different storage conditions. Despite the theory, the detected enzyme was despite the theory also found in the exudate of fresh meat. However, comparing the enzyme activity measured in fresh meat towith that measured in thawed meat, it is obvious that the enzyme activity in frozen/thawed meat is significantly higher. It was further found that the values of aconitase activity also vary in different anatomical parts of chicken meat. This might be caused by slightly different chemical composition of breasts and thighs and by the presence of bone and skin in the samples of chicken thighs

Keywords: mitochondrial enzymes; adulteration; detection; freezing, meat

Although people try to keep raw meat in its best quality for as long as possible, it is not possible to apply any known preserving method to ensure its freshness for an arbitrarily long period of time. However, one of the commonly used methods to preserve food is freezing and it allows storing meat for several months at least. Nonetheless, meat unfortunately not only loses its freshness during the frozen storage, but also many other changes (chemical, physical or microbial) might occur, which negatively affect the meat quality. For example, freezing and subsequent thawing changes the overall water content in meat. The drip loss after thawing is higher than in unfrozen meat, so this meat is drier and less tasty after cooking and loses nutritive compounds with its exudate (Leygonie *et al*. 2012).

This may be abused by retailers who may take an advantage of the fact that it is nearly impossible to visually distinguish fresh meat from thawed meat, and therefore they might sell thawed meat labelled as fresh, which is more expensive. The possible deception of consumers has led to an attempt of finding fast, non-destructive and reliable techniques to detect if meat was previously frozen.

Ice crystals, formed during the freezing process, cause cell damage, especially the rupture of cell membranes and organelles. Therefore, indigenous enzymes are released from the cells. Based on this release, enzyme activity detecting methods have been suggested. However, not all enzymes are suitable for the detection of previous freezing procedures, since they are not specific (intracellular), e.g. succinyl dehydrogenase and fumarase (Hamm 1979). On the other hand, enzymes such as citrate synthase and aconitase can be considered as specific intracellular enzymes and are present in thawed meat exudate, which therefore predetermines these enzymes as suitable markers for detecting frozen/thawed meat tissue (Hamm & Gottesmann 1984). Although a number of publications present results concerning this problem (e.g. Fernandez *et al*. 1999; Diaz *et al*. 2002; Ballin & Lametsch 2008), only a very few of them were aimed on poultry.

Supported by the Ministry of Agriculture of the Czech Republic, Project No. QI111B053.

In a previous paper (Šimoniová *et al*. 2013) citrate synthase was used in order to find out if this enzyme is suitable for detection of meat freezing. Parallelly, the same experiments were carried out with aconitase.

Thus this study served as a follow-up to the research field of using mitochondrial enzymes to differentiate between fresh and thawed chicken meat.

MATERIAL AND METHODS

Damage to the meat tissue, caused by freezing process and storage period, was determined as the catalytic activity of aconitase, which was evaluated from the absorbance measured in the meat exudate after adding specific reagents from an OxisResearch® enzyme kit. The detection method was applied to beef, pork, and fish meat; this particular experiment focused on using the enzyme aconitase for chicken meat.

Material. Four specific parts from each of 22 chicken carcasses were considered as samples for the measurement. The activity of aconitase was determined in the exudate of the meat.

To determine the effect of refrigerated and frozen storage of chicken meat the following samples were prepared. Intact chicken carcasses, with defined origin, were divided into two halves (left and right side), of which the breasts and thighs were separated from the carcass. Apart from the breasts, which were skinned, the chicken thighs were separated from the carcass with bones (femur, fibula, and tibia) and skin. All samples were vacuum packed (96%) into low-density polyethylene (LDPE) plastic foils. One half of the samples (½ thighs, ½ breasts – left and right) was stored at 4°C and the other half was stored at –22°C. Thighs and breasts (from the right and left part), which were stored at –22°C, were divided into two groups, of which one represented a long-term storage period (45 days at –22°C) and the other served to demonstrate the effect of repeated freezing/thawing cycles on the activity of aconitase.

Each measurement sample set consisted of four different parts of chicken (left breast and thigh and right breast and thigh). The activity of aconitase was measured in the meat exudates of these samples in defined intervals. The thawing process took place in the refrigerator at 4°C for approximately 24 hours. The enzyme activity in frozen/thawed meat exudate was determined in defined intervals (day 3, 17, and 45) to the 45th day *post mortem*; samples stored at 4°C were analysed on day 2, 3, 5, 6, 7, 8, 9, to the 10th day *post mortem*, since further analysis was not possible due to significant microbial growth and spoilage of meat.

Methods. The activity of aconitase was determined in the exudate of meat from both refrigerator stored meat and from frozen/thawed meat. It was further necessary to determine microbial activity and its contribution to the aconitase activity; therefore, the total counts of aerobic bacteria were measured.

Aconitase activity. Aconitase catalyses the reversible isomerisation of citric acid to isocitric through the *cis*-aconitic acid in the Krebs cycle.

citrate → *cis*-aconitate → isocitrate

The next reaction is the oxidative decarboxylation of isocitric acid to α-ketoglutaric acid. During this oxidation process, NADP is changed to NADPH.

isocitrate → α-ketoglutarate

Since the reaction takes place, the concentration of NADPH is increasing and can be detected with a spectrophotometer at 340 nm (Spekol® 1300; Analytik, Jena AG, Germany). The activity of aconitase is directly proportional to the variance of absorbance value per minute. The advantage of this method is that aconitase is specific only to mitochondria, so the overvaluation of measurement output is absent (OxisResearch™ 2012).

Samples. Samples were prepared as follows: the exudate released from meat was diluted with demineralised water at a ratio of 1 : 9 and filtered with syringe filter (CHS FilterPure Nylon Syringe Filter, 0.45 µm, 25 mm). 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 shaken carefully to avoid bubbles that might interfere during measuring and the reaction mixture was incubated in a 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.

The activity of aconitase was calculated by the following formula:

$$mU = ((A_{340}/min)/(\varepsilon \times c)) \times d \text{ (nmol/ml/min)}$$

where: ε – molar absorption coefficient (6220 M/cm); *c* – temperature corrective coefficient of absorption (2.4435), d – dilution factor (1 : 9)

Microbial counts. Approximately 10 g of the peripheral part of the sample was taken in a standard way and put into 100 ml of saline with Tween and was then homogenised in a Stomacher. The solution was then diluted into defined fractions, which were inoculated onto the below-mentioned agar.

The total count of mesophilic microorganisms (CFU) was determined according to ISO 2293:1996 on plate count agar cultivated at 30°C for 2 days.

Statistical analysis. Tests were performed three times for each sample. The frozen storage data were compared with the refrigerated storage data using Student's test with a significance level of 5%. Statistical analysis was performed by the STATISTICA 10.0 software (StatSoft CR, Prague, Czech Republic).

RESULTS AND DISCUSSIONS

The experiment was divided into three steps, similar to the previous study (Šimoniová *et al*. 2013). The first experiment served to verify the method, if it is even possible to determine the enzyme activity of aconitase in the exudate of meat. Therefore the amount of released exudate was not taken in account. Based on the results of the first experiment, the second measurement considered the amount of released exudate and whether the exudate was filtrated or not, since the purity of the exudate also played a significant role. The third experiment was based on the data and knowledge gained during the second experiment and so all interfering factors were considered.

First verifying experiment. The first experiment served above all to find out if the enzyme is detectable in meat exudate and how its activity reflects the use of different ways of storage. Activity of aconitase was measured in exudate from both thawed and refrigerated meat. Alizadeh *et al*. (2007) indicated that specific enzymes are present only in exudate from thawed meat and so they are not to be found in fresh meat exudate. Many studies showed that the values of enzyme activity detected in the exudate from fresh meat are not always zero, but there was some increase in the enzyme activity in a drip from previously frozen meat. According to these findings, we tried to establish the differences between fresh and frozen/thawed meat by a change in the absorbance value of measured aconitase activity. As the time of storage prolonged, the measured values of activity increased. It is not clear why the activity of enzyme was found even in refrigerated meat, but it is in agreement with previous measurements (Šimoniová *et al*. 2013).

During storage of both refrigerated and frozen meat the activity of aconitase in exudate gradually increased (Figure 1). In the case of frozen meat it might be due to the formation of new ice crystals which damaged cellular and intracellular membranes and so the enzyme was released into the exudate. Moreover, the cell damage might be extended during frozen storage, since ice crystals might increase in size during the storage period, due to temperature fluctuation in the freezer, which also leads to repeated partial thawing and recrystallisation of the ice. On the other hand, the increase of enzyme activity in refrigerated meat could be explained by the ongoing postmortal changes and higher microbial count, since microorganisms have their own aconitase and may therefore influence the final values. The total microbial count analysis was therefore included in subsequent experiments.

Poultry breasts released more exudate than thighs during both types of storage. Unlike the breasts the thighs were stored with bones that decreased the area of measuring, and skin that could absorb a part of released exudate. This fact was therefore considered in subsequent experiments and the activity of aconitase was related to the given volume of released exudate.

Second experiment. According to various amounts of exudate released from different anatomical parts of the chicken and considering the different way of storage, this volume of exudate was measured so the activity of aconitase could be recalculated. As the samples were packed in an LDPE foil from which the exudate was

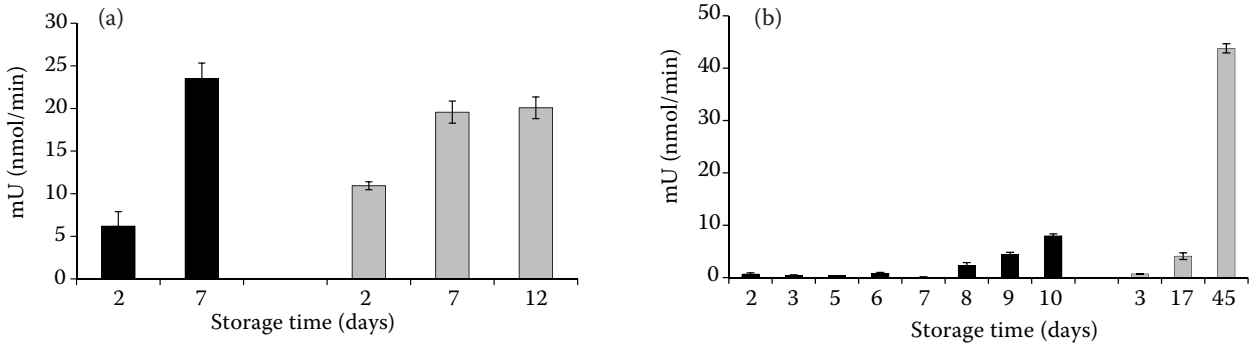

Figure 1. Effect of (a) refrigerated and thawing storage and (b) refrigerated and frozen storage on the activity of aconitase in chicken meat (mean value of breasts and thighs)

directly taken, microparticles of tissue might have contaminated the exudate. Therefore the next step before measurement itself was the filtration of the exudate.

Although the measured values varied, the trend of an increase was similar to the previously performed experiment (Figure 2). The activity of aconitase was higher in exudate from thawed samples and increased with storage time. The aconitase activity also increased under refrigeration conditions and was higher at the end of storage. Chicken breasts lost more exudate than thighs both during refrigeration and after thawing. Thighs were stored with skin, which might absorb a certain amount of exudate.

Third repeated experiment. Due to the possible overvaluation of results caused by bacterial aconitase, we conducted an analysis of total microbial count. Like in prior experiments, the activity of aconitase increased during both ways of storage, being clearer after the recalculation related to the released amount of exudate.

There was no significant difference between breasts and thighs at the beginning of refrigeration storage, the breaking point occurred after 10th day of storage, when the activity of aconitase in breasts was almost three times higher than in thighs (Table 1). After this measurement, the refrigeration storage was stopped due to the obvious microbial contamination, so it was not necessary to determine the freshness of the meat any more. This particular increase after a long time of chilling might be explained exactly like in First verifying experiment, by the release of mitochondrial enzymes as a consequence of significant postmortal changes.

As mentioned in Šimoniová *et al*. (2013), the microbial counts are more likely related to the surface, as there was a relatively small proportion of the sample (Figure 3). The extent of the influence of microbial aconitase is not therefore clear.

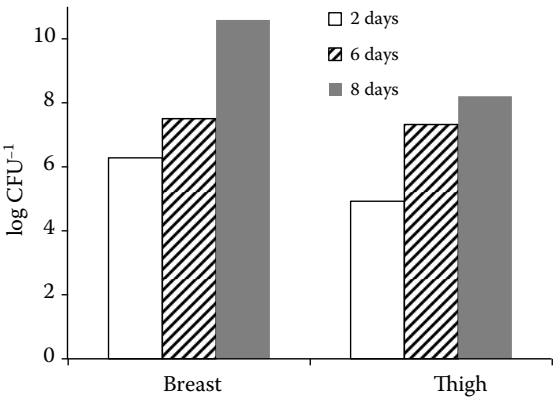

Figure 3. Total microbial counts during refrigeration storage of chicken meat

On the contrary, noticeable differences were recorded between anatomical parts already at the beginning of the frozen storage, being again higher in breasts. However, the measured values of the activity from frozen samples were not distinguishable from the values recorded on the same day of measuring in refrigerated meat. The obvious difference was measured after 17th day of frozen storage and the sharp increase continued.

Comparison of aconitase and citrate synthase. Similar results were obtained after parallel measuring with citrate synthase (Šimoniová *et al*. 2013), where the enzyme activity also increased during storage under both refrigeration and frozen storage, but being significantly higher in the latter case.

Compared to citrate synthase, aconitase activity was higher in breasts than in thighs, and, further-

Table 1. Effect of the refrigerated and frozen storage on the activity U (mU/ml) of aconitase in chicken breast and thihgs

Storage time (days)	Refrigerated				Frozen			
	breast		thighs		breast		thighs	
	left	right	left	right	left	right	left	right
2	1.916 ± 0.430	0.000 ± 0.000	0.439 ± 0.000	0.445 ± 0.430	–	–	–	–
3	0.000 ± 0.000	0.000 ± 0.000	0.903 ± 0.248	0.681 ± 0.248	0.987 ± 0.000	1.173 ± 0.000	0.000 ± 0.000	0.660 ± 0.000
5	0.000 ± 0.000	0.000 ± 0.000	0.645 ± 0.000	0.877 ± 0.000	–	–	–	–
6	0.000 ± 0.000	3.443 ± 0.496	0.000 ± 0.000	0.000 ± 0.000	–	–	–	–
7	0.378 ± 0.248	0.000 ± 0.000	0.000 ± 0.000	0.000 ± 0.000	–	–	–	–
8	1.355 ± 0.656	2.171 ± 0.744	4.452 ± 0.656	1.446 ± 0.000	–	–	–	–
9	3.376 ± 0.248	5.373 ± 0.656	2.459 ± 0.263	6.690 ± 0.263	–	–	–	–
10	17.047 ± 0.248	6.242 ± 0.263	1.855 ± 0.248	6.966 ± 0.496	–	–	–	–
17	–	–	–	–	4.613 ± 0.744	9.719 ± 1.382	0.362 ± 0.186	1.705 ± 0.215
45	–	–	–	–	79.148 ± 0.430	78.123 ± 2.274	9.957 ± 0.569	7.940 ± 0.224

more, the activity of aconitase did not decrease at the end of the frozen storage. This fact makes the use of aconitase more suitable than the use of citrate synthase, because there are no techniques to find out how long the meat has been frozen yet, and the decrease in the citrate synthase activity may cause a falsely negative outcome.

CONCLUSION

The activity of aconitase increased during both ways of storage, with significantly higher measured values for thawed meat after a few days of storage. The chicken breasts showed higher values of the enzyme activity and the increase was more precipitous than in the thighs. After longer frozen storage the activity of aconitase continued to increase.

Aconitase proved to be a suitable enzyme for the detection of meat freezing, which was evident also in comparison with the experiment with citrate synthase.

The experiments were carried out on the samples of whole parts of meat, where the activity was measured in exudate spontaneously released from the sample. The question is whether it is possible to obtain released mitochondrial enzymes directly from the muscle tissue. The answer to this question is the subject of further research.

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Received for publication July 29, 2013
Accepted after corrections January 27, 2014

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