Differentiation Between Fresh and Thawed Chicken Meats

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ABSTRACT

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Adulteration of fresh meat and its possible substitution with frozen and thawed meat represents a problem, since thawed meat has not only lower sensory qualities than fresh meat but also a lower price. Commercial enzymatic kits seem to be a solution to reveal such unfair practises and were applied to detect the activity of specific enzymes, citrate synthase, mitochondrial enzymes that are released from the organelles destroyed by frost. We determined, whether the meat of slaughtered chicken was fresh or frozen/thawed, and to provide convincing results. The absolute results vary with the type of meat and depend on the enzyme used. However, the enzyme activity in the exudates of frozen/thawed meat is always higher than in fresh meat. This value further increases with each subsequent freezing cycle. The determination of citrate synthase activity was done only in the exudate released from the examined meat samples. However, to determine the enzymes activity directly in unpacked meat, which have not released any exudate, is the subject of further research.

Keywords: citrate synthase; meat; freezing; detection

Fresh meat is a sensitive material, which is not able to maintain its desired sensory qualities and food microbial safety for a longer time. Long-term storage and transport between slaughterhouses, meat processors and consumers may take days or even several months (overseas import). To keep meat in fresh state requires not only excellent hygiene conditions but also high financial costs, which some producers try to reduce by transporting the meat in frozen state and thaw it at the final destination. However, frozen/thawed meat does not meet the standard quality parameters of fresh meat, for which it is bought by many consumers.

Moreover, meat on thawing loses exudate thus the substances, which add the characteristic flavour and nutritional value (vitamins, minerals) to meat, are partially lost, and the meat becomes less tasty. The texture of meat is also affected by the formation of ice crystals, which damages the muscle structure and so increases the water activity on the meat surface and subsequently supports the growth of microorganisms, which contributes to a shorter shelf life. In addition, adulteration of fresh chicken meat occurs during foreign trade affairs, when the meat is shipped overseas in the frozen state and is subsequently thawed at the final destination.

Due to the above defined reasons, it is necessary to detect whether or not the meat was frozen. Thus different methods for the detection of adulteration with frozen meat have been suggested. Many methods have been developed to examine enzyme activity in the meat exudate, which contains certain enzymes present only after freezing. These specific enzymes are usually found in cellular organelles, and following the damage made by ice

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crystals, which were formed during the freezing process, these enzymes are released from the cellular organelles into the exudate (Hamm 1979). Therefore, it is possible to determine their activity. Yet long time ago the detection of mitochondrial enzymes, especially aconitase, in exudate was suggested (Hamm & Gottesmann 1984). Analytical methods dealing with the detection of frozen meat are based on the determination of mitochondrial enzymes activity, which is usually performed on assessing the freshness of fish (Hoz *et al*. 1993).

During the freezing process, the cell structure of the fish meat tissue is destroyed by ice crystals and specific mitochondrial enzymes are released into the meat exudate on thawing. Enzymes such as citrate synthase and β-hydroxyacyl-CoA dehydrogenase (HADH) are typical enzymes present in the thawed meat exudate and are not present in the exudate of fresh meat, therefore, their detection may indicate whether or not the meat was previously frozen (Alizadeh 2007).

These enzymes come particularly from the Krebs cycle and are situated in the mitochondria. The methods proving whether the meat was frozen are based on the determination of the specific enzyme activity of citrate synthase, aconitase, ATP synthase, fumarase (Gottesmann & Hamm 1984a), lipoamiddehydrogenase, and 3-β-hydroxyacyl-CoA dehydrogenase (HADH) (Gottesmann & Hamm 1984b).

Although a number of publications present results concerning enzyme freezing detection of fish, pork, and beef (e.g. Fernandez *et al*. 1999; Diaz *et al*. 2002; Ballin & Lametsch 2008), only very few of them concern poultry.

One of the mentioned enzymes detected to confirm meat freshness is citrate synthase. The detection of this specific enzyme has got two undoubtable advantages. Since this enzyme is specific to mitochondria, no result overstatements occur and furthermore, it is possible to determine the enzyme activity in a relatively small amount of exudate (5 µl). In previous studies, it was found that it is possible to distinguish frozen/thawed meat from fresh, especially pork, meat by the detection of specific enzymes (Pipek 2010).

However, these enzymatic methods have also some drawbacks, e.g. the HADH method is suitable for detecting frozen/thawed meat, which was frozen at temperatures below –12°C, and is not suitable for detecting thawed minced meat since the grinding process destroys the cell structure and HADH is consequently released into the meat exudate irrespective of previous freezing. It is therefore more suitable to apply a spectrophotometric method in the case of minced meat.

As mentioned before, the HADH method is a reliable method to distinguish fresh meat from frozen/thawed meat only if the meat was frozen at temperatures below –12°C, however, due to a long storage period or higher proteolytic activity in meat, it is possible that HADH undergoes structural changes and its activity will not be detected in the final stage (Ballin 2008).

Similarly it is necessary to reckon with the fact that the activity of citrate synthase in the meat samples, stored for a long period at a low temperature, does increase in time and can reach the same value as in those samples, which had been previously frozen and then thawed. However, the time at which the activity of citrate synthase reaches these values, the fresh meat has already exceeded its shelf life and is spoiled. Therefore, this method can be applicable since the spoiled meat does not need to be distinguished from fresh meat, whether it was frozen or not (Pipek 2010).

Citrate synthase (acetyl-CoA-oxaloacetate-C-acetyltranspherase) catalyses the condensation reaction of the two-carbon acetate residue of acetyl coenzyme A and a molecule of four-carbon oxaloacetate to form the six-carbon citrate (Eq. 1).

$$\text{Acetyl-CoA + oxaloacetate} \rightarrow \text{citrate + CoA-SH + } H^+ + H_2O \quad (1)$$

CoA-SH with a thiol group is formed by hydrolysis of the thioester of acetyl-CoA. This thiol reacts with 5,5'-dithio-bis(2-nitrobenzoic) acid (DTNB) and forms 5-thio-2-nitro benzoic acid (TNB) (Eq. 2). The absorbance of TNB is measured by the spectrophotometer at 412 nm.

$$\text{CoA-SH + DTNB} \rightarrow \text{TNB + CoA-S-S-TNB} \quad (2)$$

The activity of citrate synthase is given by the difference between its endogenic activity and the overall activity.

MATERIAL AND METHODS

The activities of specific mitochondrial enzymes were determined in meat exudate to distinguish fresh meat from the frozen/thawed. The study focused on the impact of attributes such as the

freezing procedures, or a long period of refrigerated/freezing storage, which significantly affect the degree of cell destruction in the meat tissue. The damage to the meat tissue, caused by the freezing process and storage period, was determined in terms of the catalytic activity of citrate synthase evaluated from the absorbance measured in the meat exudate after adding specific reagents from a Sigma Aldrich enzyme set (Sigma-Aldrich, Schenelldorf, Germany).

Samples

The samples used for the measurements comprised four parts from a chicken, and the activity of citrate synthase was determined in the exudate of the meat.

To determine the effects of refrigerated and freeze storage of chicken**,** the following samples were prepared. Intact chicken carcasses, of defined origin, were divided into two halves (left and right side) from which the breasts and thighs were separated. 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 packed into LDPE plastic bags under 96% of vacuum. 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. The thighs and breasts (from the right and left parts), which had been stored at –22°C, were divided into two groups, of which one represented a long-term storage period at –22°C and the second one was used to demonstrate the effecs of repeated freezing/thawing cycles on the activity of citrate synthase.

Each measurement sample set consisted of four different parts (left breast and thigh and right breast and thigh). The activity of citrate synthase 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 samples of the repeated freezing cycle test were repeatedly thawed at 4°C and frozen at –22°C for a defined amount of cycles. The enzyme activity in the frozen/thawed meat exudate was determined in defined intervals to the 45th day *post mortem;* the samples stored at 4°C were also analysed in defined intervals to the 10th day *post mortem*, since further analysis was not possible due to a significant microbial growth and meat spoilage.

The experiment was repeated three times. The first experiment served to verify the method, i.e. to show if it is possible at all to determine the enzyme activity of citrate synthase in the exudate of chicken meat. Therefore the amount of the exudate was not taken into account. Based on the results of the first experiment, the second measurement considered the amount of the released exudate and whether or not the exudate had been filtred, since the purity of the exudate also played a significant role. The third experiment was based on the data and knowledge received from the second experiment, and so it considered all interfering factors. It therefore served as a repeated experiment of those previously done.

Methods

To evaluate how the citrate synthase (CS) activity in meat is influenced by freezing and refrigeration storage, meat samples were prepared of muscle tissue from chicken. The activity of citrate synthase was determined in the exudate of meat from both refrigerator-stored meat and frozen/thawed meat. Also, to distinguish the influence of microorganisms on the CS activity, total counts of aerobic bacteria were measured.

Citrate synthase activity. The samples were packed in LDPE plastic bags and stored in a refrigerator at 4°C until the required amount of exudate was released. This explains why the chilled samples were not measured directly from day 1, but after several days of storage.

The activity of citrate synthase was measured using a spectrophotometric method, that is based on the absorbance of a yellow product formed during the enzymatic reactions of 5-thio-2-nitrobenzoic acid (TNB) and CoA-S-S-TNB, when coenzyme A is released from acetyl-CoA and 5,5'-dithio-bis (2-nitrobenzoic) acid (DTNB) is added. The absorbance of TNB is measured at 412 nm in a glass cell (optical path length 10 mm).

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

First, the endogenic activity was determined after the sample was incubated for 20 seconds. After that, the measurement started and the absorbance was recorded every 10 s for the total time

of 90 seconds. This was followed by the addition of 50 µl of oxaloacetate acid; the solution was incubated again for 20 s and the absorbance was measured again for 90 s, and the overall activity was estimated. The final activity of citrate synthase was calculated according to the following formula (Eq. 3).

$$U = \frac{\Delta A_{412}/\text{min} \times V \times \text{dil}}{\varepsilon^{mM} \times L \times V_{enz}} \quad (\mu\text{mol/ml/min}) \quad (3)$$

where:

ΔA_{412}/min – difference between the endogenic and overall activity of citrate synthase at 0 and 60 s

V – total volume = 1 ml (ml)

ε^{mM} – (mM^{-1}/cm)

dil – sample dilution, molar absorption coefficient TNB at 412 nm = 13.6mM^{-1}/cm

L – cell length = 1 cm (cm)

V_{enz} – sample volume = 10 µl (ml)

Microbial counts. Approximately 10 g of the final part of the sample was taken in a standard way and put into 100 ml of saline with Tween and the mixture was then homogenised in a Stomacher. Followingly, the solution was diluted into defined fractions, which were then inoculated onto the above mentioned agars.

The total count of mesophilic microorganisms (CFU) was determined according to ISO 2293:2009 on plate-count-agar cultivated for 2 days at 30°C.

RESULTS AND DISCUSSION

The goal of this study was divided into three stages. First, it was necessary to determine whether it is possible, at all to detect specific mitochondrial enzymes in chicken meat exudate. Freezing of the meat samples induced, as expected, an increase of the citrate synthase activity; this activity increased during freeze storage. However a certain increase of enzyme activity was observed also during the cooling storage and it even increased during the storage period.

Based on the data obtained from the first experiment, the second stage was to define the precise amount of the released exudate and to focus on the impacts of attributes such as freezing procedures, long period refrigerated/freezing storage, and exudate purity. The damage to the meat tissue, caused by the freezing process and storage period, was determined as the catalytic activity of citrate synthase, which was evaluated from the absorbance measured after adding specific reagents to the meat exudate. The third stage served as a verification measurement of the previously done experiments.

First verification experiment

The goal of the first verification experiment was to determine whether it is possible to detect the specific mitochondrial enzyme in the chicken meat exudate. The first measurements were focused on the detection of citrate synthase in the meat exudate and the results showed that it is possible to detect this specific enzyme in such a medium as chicken meat exudate. The results also showed that it is possible to detect this specific mitochondrial enzyme not only in the exudate of frozen/thawed meat but also in the exudate of only refrigerated meat, with which it had been assumed that these enzymes are not present. Moreover, the activity of citrate synthase even increased during the storage period and these results set the base for further examination.

The activity of citrate synthase was studied in the defined chicken muscles (breasts and thighs).

When fresh chicken meat was stored in the refrigerator (7 days), the activity of citrate synthase in the exudate increased (Figure 1). The results showed that these enzymatic changes develop diversely in different anatomical parts of the animal (chicken), but do have a similar increasing trend in all muscle parts. The activity of citrate synthase increased during the refrigerator storage most probably due to the disintegration of the cellular and subcellular structures of the meat during the aging processes in the meat itself. This leads to an

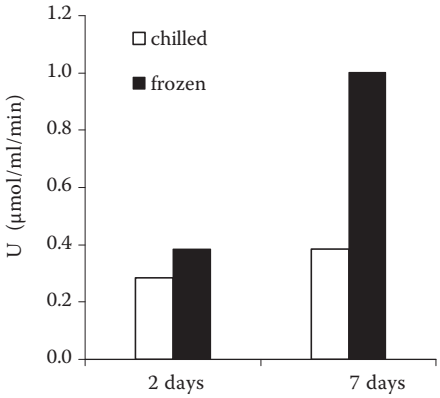

Figure 1. The effect of freezing and storage on the activity of citrate synthase at chicken meat

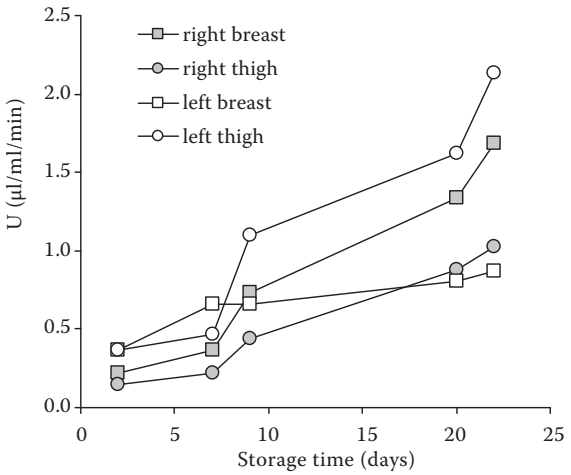

Figure 2. Activity of citrate synthase in chicken muscles as influenced by freezing and freeze storage

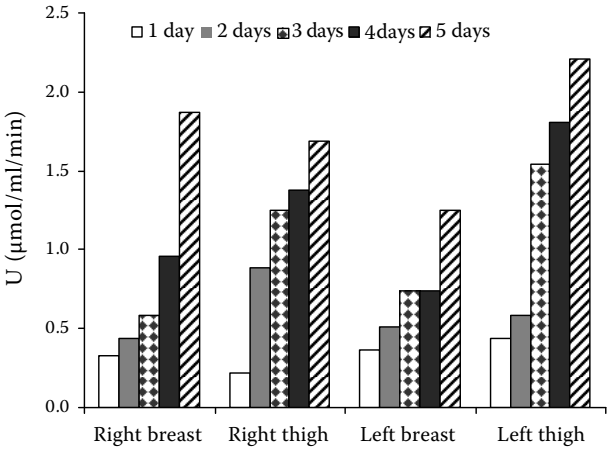

Figure 3. Affect of repeated freezing/thawing on the activity of citrate synthase in chicken muscles

extended amount of the enzyme in sarcoplasm and thus a higher recorded activity of citrate synthase during the refrigeration storage. Since the chicken had been slaughtered in a standard way, without any special antimicrobial arrangement, the presence and growth of microflora could also have an influence on the meat structure during the storage process.

However, at such point of the storage time, when the microorganisms are in high counts, the meat already begins to spoil and it is not necessary to prove its freshness. On the other hand, citrate synthase activity in freshly frozen meat samples is similar to its activity in long-term stored refrigerated meat; but it is also irrelevant to detect whether it was frozen/thawed.

When the meat is frozen, the enzyme activity rapidly increases in the exudates of thawed meat in all muscle parts (Figure 2). Moreover, the citrate synthase activity even increases during the storage time, since the chemical and enzyme reactions in the meat, which has been long-term stored at temperatures below the freezing point, can change the inside of the cells, which, in consequence, can burst and the specific enzymes are released into the exudate. Therefore, a higher citrate synthase or other enzyme activity can be measured.

Another reason for the increasing activity of citrate synthase during the storage period at temperatures far below the freezing point is most probably the damage to the cell organelles caused by the growing of ice crystals during the freezing process. Moreover, even during the storage period the temperatures need not be constant and, due to their fluctuations, the ice crystals may grow or shrink, which damages the cell structure even more. Thus, the larger the damage to mitochondria, the higher the activity of citrate synthase.

Repetitive freezing/thawing cycles also had a significant influence on the activity of citrate synthase (Figure 3). It was obvious that with each freezing/thawing cycle the activity of citrate synthase gradually increased. However, the variability of the estimated values between the measured samples was probably affected by two different factors. First, there could have been a slight difference in the properties of each sample, due to the fact that each sample originated from a different anatomical part or a different animal. Second, each part of the muscle also released a quite different amount of exudate, which was at first not taken into account, since the purpose of this experiment was to determine the possibility to detect the specific mitochondrial enzyme, citrate synthase.

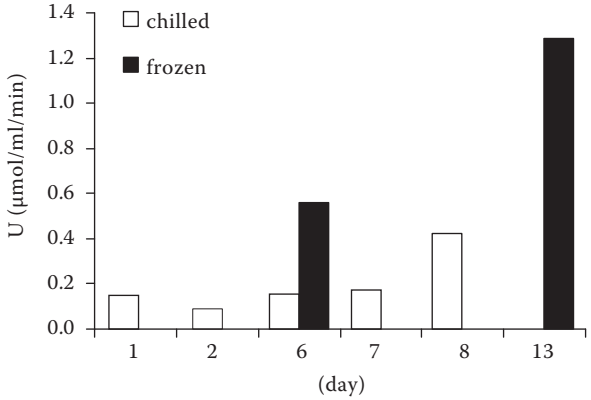

Figure 4. Affect of storage on the activity of citrate synthase – comparison of frozen and refrigerated chicken meat (repeated experiment)

Table 1. The effect of freezing and storage on the activity of citrate synthase U (μmol/ml/min) at chicken meat

Storage time (days)	Refrigerated				Frozen			
	breast		thighs		breast		thighs	
	left	right	left	right	left	right	left	right
2	0.478 ± 0.001	0.221 ± 0.001	0.294 ± 0.001	0.147 ± 0.104	0.441 ± 0.074	0.294 ± 0.074	0.441 ± 0.074	0.368 ± 0.074
7	0.441 ± 0.180	0.478 ± 0.037	0.221 ± 0.104	0.404 ± 0.037	0.809 ± 0.074	0.919 ± 0.037	1.838 ± 0.104	0.441 ± 0.074

It was further found that the breast muscles released more exudate than the thighs, and that with each freezing cycle the volume of the released exudate from the breast muscles even increased. This difference evidently relates to the fact that the thighs were covered with the skin, which absorbed a certain part of the exudate.

Based on these results the following experiments took into account the fact that the volume of the released exudate differed in each sample, so the enzyme activity was put into relation with the amount of the exudates released.

Second (follow-up) experiment

Based on the data obtained from the first experiment, the second experiment focused on the goal to define the precise amount of the released exudate and to put the activity of citrate synthase into relation with the amount of the released exudate. It was also necessary to determine the impacts of attributes such as the freezing procedures, long period refrigerated/freezing storage, and exudate purity. The damage to the meat tissue, caused by the freezing process and storage period, was determined as the catalytic activity of citrate synthase, which was evaluated from the absorbance measured in the meat exudate after adding specific reagents. The measurements were done in shorter intervals to achieve more precise results, which showed that during the refrigerated storage the activity of citrate synthase increased (Figure 4 and Table 1).

The increase of enzyme activity of citrate synthase could be probably explained by the activity of microorganisms present in the muscle tissue, which might damage the muscle cell structure, and thus the endogenous enzymes could be released into the meat exudate during the storage period.

Third repeated experiment

The third experiment served as a verification measurement of the previously done experiments, and the data obtained confirmed the results from the second experiment. Again it was found, that there are differences in the citrate synthase activity depending on the specific anatomical part of the

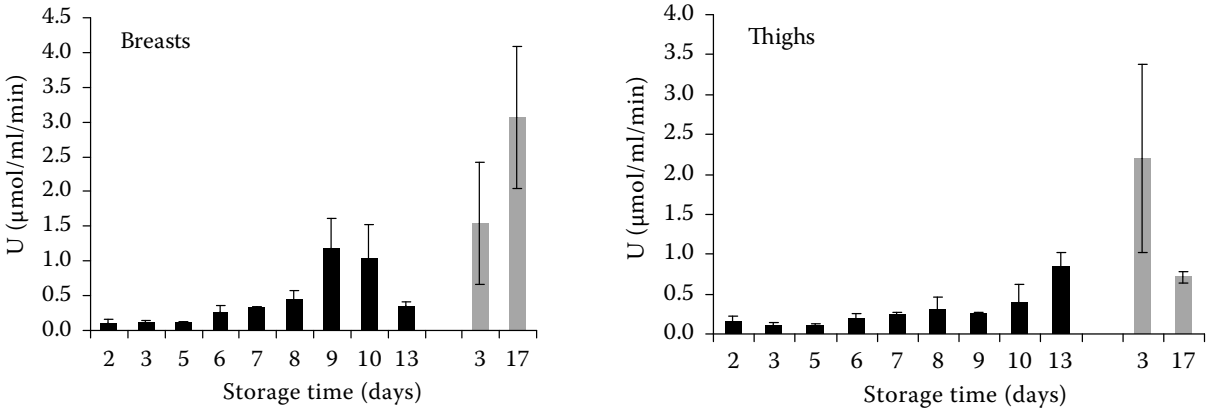

Figure 5. Affect of freezing/thawing on the activity of citrate synthase – comparison of frozen and refrigerated parts of chicken breast and chicken thighs (2nd repeated experiment)

Table 2. The effect of freezing and storage on the activity of citrate synthase U (μmol/ml/min) at chicken meat (repeated experiment)

Storage time (days)	Refrigerated				Frozen			
	breast		thighs		breast		thighs	
	left	right	left	right	left	right	left	right
1	0.147 ± 0.001	0.147 ± 0.001	–	–	–	–	–	–
2	0.110 ± 0.037	0.074 ± 0.001	–	–	–	–	–	–
6	0.0184 ± 0.110	0.147 ± 0.001	0.147 ± 0.001	0.147 ± 0.001	0.588 ± 0.074	0.625 ± 0.037	0.809 ± 0.074	0.221 ± 0.001
7	0.294 ± 0.001	0.074 ± 0.001	0.184 ± 0.110	0.147 ± 0.074	–	–	–	–
8	0.294 ± 0.074	0.147 ± 0.001	0.772 ± 0.037	0.478 ± 0.037	–	–	–	–
13	–	–	–	–	0.515 ± 0.221	1.507 ± 0.110	1.728 ± 0.331	1.397 ± 0.294

animal (breast or thigh) during both refrigerated and freezing storage. However, the differences observed were not so significant, due to the large variance of the measured values (Figure 5).

It was further found that at the end of the freezing storage period the enzyme activity decreased (Figure 5), which corresponds to the results of Fernandez (1999) and Ballin and Lametsch (2008).

The third experiment also tried to relate the enzyme activity in fresh meat with the total count of microorganisms during the storage period. The measured data showed that, to a large extent, the growth of microorganisms correlates with the increasing activity of citrate synthase in both breast and thigh muscle tissues. Figure 6 demonstrates the microbial growth throughout the storage period and it can be seen that the total counts rapidly increase after 6 days, which represents the common

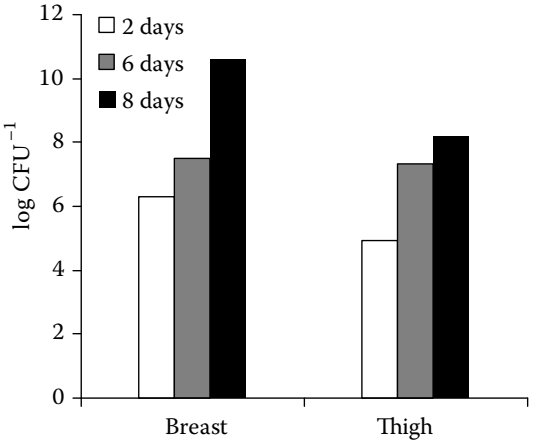

Figure 6. Total counts during cool storage of chicken meat

shelf-life period. This growth dynamics closely correlates with the increase of citrate synthase at the same time. Therefore, it can be suggested that the microbial activity has an influence on the amount of the released mitochondrial enzymes, which could be detected in the exudate of fresh meat stored at 4°C, as mentioned above

Considering all the obtained data and consequential results, it is possible to state that there are certain differences in the activity of citrate synthase between fresh and frozen/thawed meat. However, these differences are not absolutely valid. The problem is, of course, that the analysis can only be done in the exudate, respectively in the exudate of packed meat. Therefore, the question stands whether it is possible to obtain the released enzymes directly from the muscle tissue. On the other hand, though, it offers the possibility to apply other analytical methods.

CONCLUSIONS

The activity of citrate synthase in the frozen chicken samples was significantly higher than in the samples that had been stored under refrigeration. The activity increased during both refrigeration and freezing storage. Subsequent freezing/thawing cycles caused a rapid increase of the citrate synthase activity, due to a larger extent of the tissue damage. Citrate synthase activity in chicken breasts was significantly lower than in chicken thighs.

The evaluation of citrate synthase activity seems to be a relatively appropriate method to prove if the meat was frozen or not, although this is reliable only for a certain storage time of fresh meat. The first testing of this method showed the possibility to distinguish refrigerated chicken meat from frozen meat using the enzyme methods.

The problem is, of course, that the analysis can be only done in the exudate, respectively in the exudate of packed meat. Therefore the question stands, whether it is possible to obtain the released mitochondrial enzymes directly from the muscle tissue.

The answer to this question is the subject of further research.

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