Release of Mitochondrial Enzymes by Freezing and Thawing of Meat: Structural and Analytical Aspects

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INTRODUCTION

Enzyme proteins of the muscle cell are associated with membranes of cell organelles. Damage of membranes or organelles by freezing and thawing of tissue can be recognized by a partial or complete release of enzymes into the sarcoplasmic and extracellular fluid. If the enzyme released is specific for the subcellular organelle in question, the release provides information on the type and extent of damage of the particular organelle. Such biochemical methods sometimes indicate subtile changes which are not visible in electrone micrographs. Investigations of this type are of importance to medical researchers and physiologists, as well as to food scientists. For example, enzymes released from cell organelles by freezing and thawing, might be more active than in the bound state. Knowledge about disintegration of cell membranes is important if deterioration of meat quality by freezing and freeze-storage is to be more fully understood. The presence of membrane-bound enzymes in the muscle press juice after freezing and thawing could lead to methods for differentiation between nonfrozen meat and frozen-thawed meat (F-T test) (1).

In earlier work, we had studied the influence of freezing and thawing of muscle on mitochondria by means of the release of aconitase, fumarase, glutamic dehydrogenase, malate dehydrogenase, glutamic pyruvate transaminase and the mitochondrial isozyme of glutamic oxaloacetic transaminase (GOT_M) (1). On the base of the release of GOT_M a reliable F-T test was developed (2-4). In this test, GOT_M has to be separated from the sarcoplasmic GOT isozyme by membrane electrophoresis. The use of the release of another suitable mitochondrial enzyme, which has not to be separated from other isozymes, would make the test much easier and faster. This was the principal reason of the present work. For these studies the three mitochondrial enzymes lipoamide dehydrogenase (LIPDH; EC 1.6.4.3), citrate synthase (CS; EC 4.1.3.7) and ß-hydroxyacyl-CoA dehydrogenase (HADH; EC 1.1.1.35) were chosen. In a compartimentation study, which is not to be discussed here, we found that these three enzymes are associated with the inner membrane of the mitochondrion. The strength of binding to the membrane increases according to the sequence CS < HADH < LIPDH. The total activities of these enzymes in different muscle types of cattle and pigs is positively correlated with the myoglobin content. At least 90 % of these enzyme activities is membrane-bound i.e. located in the mitochondria.

MATERIAL AND METHODS

Material

Bovine and porcine semimembranaceus muscle was excised from the carcass as soon after slaughter as possible and trimmed of fat and connective tissue.

Freezing at -20°C and thawing at room temperature: 6 treatments were used (100 g samples, at least 3 replications): (1) immediately analyzed ("prerigor, before freezing"); (2) immediately frozen, stored at -20°C for 48 h, thawed ("prerigor, after freezing"; in these samples thaw contracture took place); (3) storage at +2°C for 3 days ("postrigor, before freezing"); (4) storage at +2°C for 3 days, freezing, storage at -20°C for 48 h, thawing ("postrigor, after freezing"); (5) like treatment (3) but storage at +2°C for 14 days ("aged, before freezing"); (6) like treatment (4) but storage at +2°C for 14 days ("aged, after freezing").

Different temperatures and rates of freezing. For freezing, 10.0 x 5.0 x 2.5 cm samples of postrigor bovine muscle were sealed in polyehylene bags and immersed in the methanol bath of a cryostate. In the "fast" freezing experiments, the bath was maintained at the desired temperature (-5°, -10°, -20°, -40°, -60° and -80°C) and the samples were kept in the bath until their center reached the bath temperature. In the "slow" freezing experiments, the samples were immersed in the bath at a bath temperature of +2°C, then the bath temperature was lowered at a rate of 0.05°C/min (to -5°, -10°, -20°C) and 0.2°C/min (to -40°, -60°, -80°C) until the desired final temperature was reached. The change of temperature in the samples was measured by thermocouples and recorded. The rates of cooling applied are indicated in table 1. All samples were thawed at room temperatures.

Table 1: Rate of freezing in the temperature range between 0° and -5°C, measured in the center of the samples

Final temperature in the center of the sample, °C	"fast" freezing min/°C	"slow" freezing min/°C
- 5	18.52	25.54
-10	5.50	15.14
-20	1.65	16.66
-40	0.61	7.94
-60	0.34	7.50
-80	0.13	7.93

Preparation of extract and press juice. 10 g of muscle tissue (unfrozen or frozen-thawed) was homogenized with 40 ml 0.05 M phosphate buffer (pH 7.6) containing 0.1 % Triton X-100. The homogenate was centrifuged at 1000 x g and 4°C for 20 min and filtrated. The enzyme activity

extracted by this way is called "total extractable activity". For the preparation of muscle press juice about 100 g of the tissue (unfrozen or frozen-thawed) was pressed between two plexiglass plates with a maximum pressure of about 10 kg/cm^2. The press juice was filtrated.

Determination of enzyme activities. In the extracts as well as in the muscle press juices the enzyme activities were determined. In the freeze-thaw experiments, aliquotes of the thaw drip collected in the bag were added to the homogenate and press juice. LIPDH: 2.45 ml 0.1 M phosphate buffer (pH 6.5), 0.2 ml 34.4 mM EDTA, 0.05 ml 15 mM NADH, 0.1 ml of the diluted muscle extract or press juice (diluted with the phosphate buffer). The reaction was started by addition of 0.2 ml 50 mM DL lipoamide. Time of reaction: 5 min. The decrease in absorption at 340 nm was measured. The activities of CS and HADH were measured by the methods (5) and (6) respectively. The absolute activities were expressed as international units (micromoles of the metabolized substrate per min) per g wet tissue (U/g) or per ml press juice (U/ml).
Extent of enzyme release: The activity of a given enzyme in the muscle press juice was expressed as a percent of the total extractable activity of that enzyme (percent enzyme released).

Differentiation between unfrozen and frozen thawed meat (F-T tests). The tests are basing on the release of HADH. Spectrophotometric test: 2.6 ml 0.1 M phosphate buffer (pH 6), 0.2 ml 34.4 M EDTA, 0.05 ml 7.5 mM NADH, 0.1 ml diluted muscle press juice (diluted 1:200 with the phosphate buffer). Starting the reaction by addition of 0.05 ml 5.9 mM acetoacetyl-CoA; after 3 min reaction at 25°C absorption measurement at 340 nm. The meat was frozen and thawed if the activity in the press juice exceeds 3.5 U/ml (for beef) or 6.0 U/ml (for pork). - Color test: 2.4 ml 0.1 M phosphate buffer (pH 6.0), 0.2 ml 34.4 M EDTA, 0.2 ml $NADH.Na_2$ (1 mg/ml), 0.12 ml acetoacetyl-CoA, sodium salt (5 mg/ml) and 0.1 ml diluted muscle press juice (1:100 diluted with the phosphate buffer) are mixed in a test tube, shaken and kept in the darkness for 60 min (beef) or 30 min (pork). Then 0.1 ml Meldolablau (Boehringer, Mannheim) solution (28 mg /100 ml) were added. After shaking for 30 sec the solution turns to be blue if the meat was frozen and thawed, and remains colorless if the meat was unfrozen.

RESULTS AND DISCUSSION

1. Influence of freezing and thawing on the release of mitochondrial enzymes.

1. Freezing at -20°C and thawing at room temperature. Freezing of bovine muscle samples before onset of rigor mortis and thawing did not significantly change the total extractable activity of the enzymes investigated (table 2). The same was observed for prerigor frozen pork as well as for postrigor frozen or aged frozen beef and pork.

Freezing and thawing of muscle, however, caused a highly significant release of the three enzymes from the mitochondria into the sarcoplasm (and extracellular fluid). In table 3 the results for postrigor bovine muscle are presented; they were almost the same as the results obtained by freezing prerigor muscle.

Table 2: Enzyme activities (U/g) in the extract of prerigor bovine muscle before and after freezing (-20°C)

Enzyme	Animal No. I		II		III	
	before	after	before	after	before	after
LIPDH	15.9	14.5	13.6	14.3	18.0	19.1
CS	3.1	2.8	1.8	1.3	4.4	3.0
HADH	13.3	14.1	9.7	11.0	15.5	12.3

Table 3: Enzyme activities[a] in the press juice of postrigor bovine muscle before and after freezing (-20°C)

Enzyme	Animal No. I			II			III		
	before	%	after	before	%	after	before	%	after
LIPDH	0		20.5	0		18.5	0		17.7
CS	0		41.1	0		35.0	0		30.8
HADH	7.3		46.3	3.6		37.4	4.2		32.7

[a] Percent of the total extractable activity

From porcine muscle mitochondria more enzyme activity was released by freezing the tissue (Fig. 1) than from bovine muscle mitochondria. After aging of beef or pork the mitochondrial membranes apparently become more labile because more enzyme is released by freezing as it is shown in Fig. 1 for HADH in porcine muscle. It should be mentioned that storage of meat at +2°C up to 14 days did not cause any release of LIPDH, CS and HADH. Repeated freezing and thawing of meat results in an increased release of the mitochondrial enzymes.

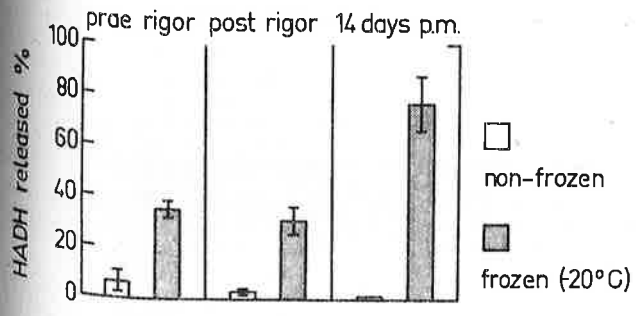

Figure 1:
HADH activity (percent of total extractable activity) in the muscle press juice of porcine muscle before and after freezing (-20°C). The bars indicate the standard deviation.

2. Influence of freezing and thawing conditions. Freezing conditions have a remarkable influence on the release of the mitochondrial enzymes. Samples of postrigor bovine muscle were frozen to -5°, -10°, -20°, -40°, -60° and -80°C at "low" and "fast" cooling rates (table 1) and thawed at room temperature. Figure 2 presents the results for CS and HADH; with LIPDH similar results had been obtained.

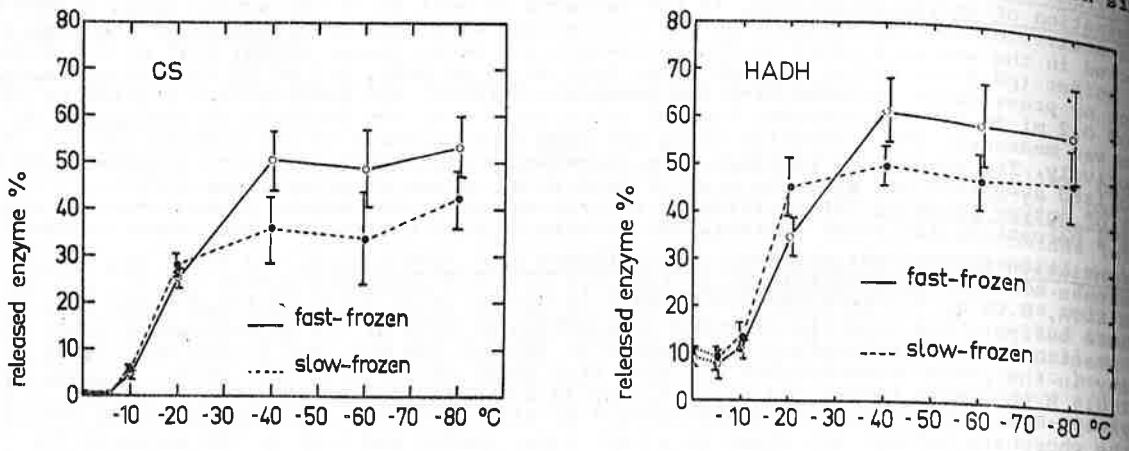

Figure 2: Influence of freezing conditions and thawing on the activities of CS and HADH in the press juice from postrigor bovine muscle. Percent of the total extractable activity. The bars indicate the standard deviation.

At high subfreezing temperatures (-5°, -10°C) only little release of mitochondrial enzymes occured; between -10° and -20°C, however, a considerable release took place. Between -20° and -80°C no significant changes in the enzyme release could be observed in the "slowly" frozen samples. Below -20°C, "fast" freezing caused a stronger release than "slow" freezing. The influence of temperature and rate of freezing on the release of GOT_M and aconitase, studied in our earlier work (1), follows the same pattern. Lowering the temperature from -80° to -196°C in the fast freezing process increased the release of the three enzymes for about 20 percent. The release of the mitochondrial enzymes during "slow" freezing cannot be due to a mechanical damage of the mitochondrial membrane by ice crystals because under these conditions only extracellular ice is formed (7). The most significant increase in the delocalization of the enzymes takes place in the same range of temperature, in which almost all freezable muscle water is transformed into ice (7). This process of ice formation is accompanied by a migration of water from the muscle fibers into the extracellular space, i.e., by a dehydration of the muscle cells. Therefore it is suggested that the enzyme release is due to a damage of the mitochondrial inner membrane by dehydration. Below -20°C only very little additional extracellular ice formation occurs, and that might be the reason that no further injury of the mitochondria takes place (Fig. 2). Under our experimental conditions "fast" freezing at temperatures below -20°C should result in an intracellular ice formation (7). Therefore, the additonal release of enzymes might be due to the formation of small ice crystals in the vicinity of or even inside the mitochondria, the additional membrane damage being due either to mechanical effects or to local dehydration. The phenomena observed cannot be due to changes in the sarcolemma because also in the unfrozen postmortem tissue the cell wall is permeable to enzyme proteins, e.g. to the mitochondrial glutamic dehydrogenase (1). Freeze damage to tissue is often attributed to an increased ion concentration in the unfrozen part of the cell water. However, this factor might not play a dominant role because in this case the injury would be expected to be maximal around -5°C (8) whereas in our experiments it was minimal at this temperature.

Apparently also the rate of defrosting influences mitochondrial membrane damage because slow thawing of "fast" frozen muscle results in a significantly stronger release of the mitochondrial enzymes, particular CS and HADH, than fast thawing (Fig. 3). This could be due to a certain growth of intracellular ice crystals by recrystalization during slow thawing. It should be mentioned, however, that a similar effect of this type - although of minor extent - was observed with "slowly" frozen muscle. It would be also conceivable that the effect of slow thawing is caused by a certain denaturation of the membrane lipoproteins due to a longer exposure to the high ionic strength in the non-frozen cell water within the critical temperature range between -5° and -1°C. This, however, is not probable because storage of slowly frozen (to -20°C) beef at -3°C for 5 days did not result in a significant additional release of LIPDH, CS or HADH.

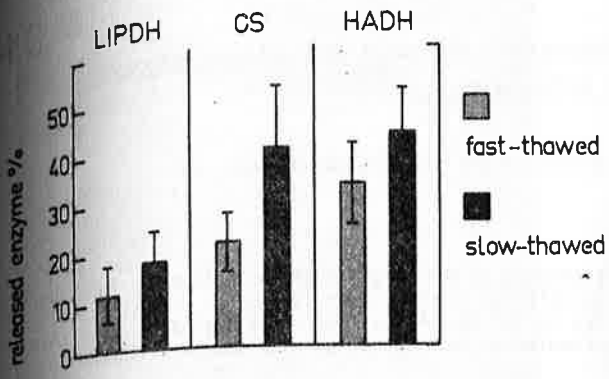

Figure 3:

Influence of thawing conditions on the activity of LIPDH, CS and HADH in the muscle press juice from "fast" frozen (-30°C) postrigor bovine muscle. Percent of total extractable activity. Fast thawing: immersion of the sealed samples in water at +20°C; slow thawing: in air at 0°C. The bars indicate the standard deviation.

II. Methods for differentiation between unfrozen meat and frozen-thawed meat (F-T tests)

The release of mitochondrial enzymes by freezing and thawing can be used to differentiate fresh meat and frozen thawed meat in practical situations. For this purpose, the enzyme chosen should fulfill three requirements: (a) it should be released by freezing and thawing under commercial conditions but not by aging of meat at above-freezing temperatures; (b) the total enzyme activity should not decrease markedly during storage of meat either unfrozen or frozen; (c) the enzyme should be easily detectable in the muscle press juice. Among the three enzymes investigated only HADH meets all these criteria whereas the activity of LIPDH decreases considerably during storage of the unfrozen meat; CS is partially released in PSE pork but would be appropriate for beef (a simple color test could not be developed).

On the basis of the release of HADH a spectrophotometric test as well as a colour test were developed. The procedures are described under "MATERIAL AND METHODS". These tests are applicable to beef, veal and pork and, with minor variations (differences in dilution of press juice, reaction times and/or minimum values of U/ml press juice), also to mutton, poultry (chicken, turkey, geese, ducks) and to liver (beef and pork). Electrical stimulation of beef carcasses and PSE conditions of pork do not interfer. The tests are not applicable to comminuted meat (release of mitochondrial enzymes by mincing) and fish (release of the enzyme during storage at above-freezing temperatures).

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