PHYSICAL AND BIOCHEMICAL METHODS FOR THE DIFFERENTIATION BETWEEN FRESH AND FROZEN-THAWED FISH OR FILLETS

RICONOSCIMENTO TRA PESCE O FILETTI DI PESCE FRESCHI E SCONGELATI MEDIANTE METODI FISICI E BIOCHIMICI

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ABSTRACT

This paper reviews the physical and biochemical methods used for differentiating between unfrozen and frozen-thawed fish or fillets. These techniques include: 1. Measurement of the electric properties of fish tissues. 2. Visual inspection of the eye lens. 3. Judgement of the integrity of red blood cells by microscopy or estimation of the hematocrit value. 4. Determination of the release of enzymes originally bound to mitochondria, lysosomes or red blood cells. The possibilities of applying these techniques either to fish or to fillets are discussed.

RIASSUNTO

Il presente lavoro prende in esame i metodi fisici e biochimici attualmente utilizzati per differenziare i prodotti ittici freschi da quelli scongelati. Tali metodi comprendono: 1. Misurazione delle proprietà elettriche dei tessuti del pesce; 2. Ispezione visiva dell'occhio del pesce; 3. Valutazione dell'integrità dei globuli rossi mediante microscopia o stima del valore ematocrito; 4. Determinazione della liberazione degli enzimi presenti nei mitocondri, nei lisosomi o nei globuli rossi. Vengono discusse le possibilità di applicazione di queste tecniche sia al prodotto intero che ai filetti.

- Key words: Fish Tester, Torrymeter, eye lens, lysosomes, mitochondria, red blood cells. -

INTRODUCTION

It is generally accepted that fresh fish (or fillets) and frozen-thawed fish are types of products which should be differentiated.

The Food and Agriculture Organization (FAO, 1982) Code of Practice defines fresh fish as "freshly caught fish, which have received no preserving treatment or which have been preserved only by chilling" and frozen fish as "fish, which have been subjected to a freezing process sufficient to reduce the temperature of the whole product to a level low enough to preserve the inherent quality of the fish and which have been maintained at this low temperature during transportation, storage and distribution up to and including the time of final sale".

Spoilage of chilled fish and deterioration of frozen stored fish follow different routes. The loss of quality of ice-stored fish is mainly caused by bacteria. It is characterized by the development of ammonia, various amines (di- and trimethylamine, histamine, cadaverine, etc.), ethanol and other low molecular weight compounds producing off-flavours (HUSS, 1988); the texture of the flesh often becomes soft.

On the other hand, the texture of frozen-stored fish may be firm and dry and so-called "cold storage flavour" has been described.

The extent of deterioration depends on the temperature, storage time and fish species as well as on several other parameters (CONNELL, 1990). It cannot be claimed that one of the two types of fish (or fillets), fresh or frozen-thawed, generally has a higher quality than the other.

VYNCKE (1983) compared the shelf life of fresh or thawed cod (*Gadus morhua*) fillets kept in ice for up to 13 days. Sensory tests (raw odour and cooked flavour) showed that the shelf life of thawed fillets was 2-4 days longer than fresh fillets.

The increase in pH, total volatile bases-nitrogen (TVB-N), trimethylamine and volatile acids was significantly delayed in thawed fillets. A similar observation was made for thawed fillets of red fish, where the content of trimethylamine (TMA) was very low (less than 2 mg TMA-N / 100 g wet weight) during ice-storage of the fillets for several days (REHBEIN, 1979a).

In Germany, as well as in other countries, fish or fillets, which have been thawed and put in ice, are sold as fresh goods (HERBORG, 1986).

This practice is prohibited by a legal instruction but not by law.

For the benfit of the consumer and prevention of unfair competition in the trade of fishery products, correct labelling of frozen-thawed fish or fillets is desirable.

Control of labelling is only possible, if rapid and reliable methods exist, which allow food control authorities to distinguish between fresh and frozen-thawed fish or fillets.

In the last years a great number of different methods have been published, which are compiled and evaluated in this review.

Recently, these methods have become interesting from quite another point of view. The nematode larvae, which are frequently found in the flesh of herring, saithe and other fish species are killed by deep-freezing of the fish (KARL and LEINEMANN, 1989).

Some products, e.g. matje in the Netherlands and matje or marinated herring in Germany, have to be produced from frozen-thawed fish unless special conditions of curing are applied (KARL, 1988). The methods developed for differentiating between fresh and thawed fish may therefore be used to guarantee that herring has been frozen (REHBEIN, 1991).

PHYSICAL METHODS

Measurement of the electrical resistance

The measurement of the electrical resistance of fish is widely used to evaluate its freshness (HENNINGS, 1963; GR INTERNATIONAL ELECTRONICS LTD, 1978). During spoilage of fish the electrical resistance of the tissues (skin, muscle) steadily decreases due to the destruction of the membrane system. As membranes are also destroyed by freezing and thawing, the electrical resistance of thawed fish is as low as that of spoiled fish. This can be used to identify thawed fish unless the sample is spoiled. Loss of freshness or degree of spoilage may be estimated by the determination of chemical parameters like the K-value (the proportion of hypoxanthine and inosine in the total amount of ATP and its degradation products) or the TVB-N value (HUSS, 1988).

Two instruments are available on the market: the Torrymeter (GR International Electronics, Camberley) and the Fish-Tester (Intellectron International Electronics, Hamburg). They have different scales (Torrymeter: 0-15; Fish-Tester: 0-100), but for both instruments the readings are positively correlated with freshness, i.e. high values indicate high quality and thawed fish is characterized by values near zero.

Differentiation between fresh and thawed trout was achieved with the Fish-Tester (FRITTOLI and RUGGERI, 1968) and the Torrymeter (REHBEIN and AUST, 1980). Torrymeter readings for fresh rainbow trout were from 8-15, whereas frozen and thawed trout gave values from 1-4.5.

The Torrymeter was also successfully used for differentiating frozen-thawed from unfrozen unskinned fillets of yellowtail (*Seriola quinqueradiata*) (KIM et al., 1987). The Torrymeter readings of frozen-thawed fillets measured on the bone side as well as on the skin side were much lower than those of fresh fish fillets (0-3 versus 11-15). During ice storage of the unfrozen fillets the Torrymeter readings decreased to zero within a period of 18 days. Those ice stored fillets were differentiated from frozen-thawed ones by the additional determination of the K-value: the frozen-thawed fillets had low K-values, while the unfrozen fillets had high K-values after storage in ice for 18 days.

In contrast to deep freezing (-20°C or less) partial freezing (-3°C) resulted in only moderate reduction in the Torrymeter readings. Differentiation between unfrozen or partially frozen yellowtail fillets was not possible (RAZAVI-SHIRAZI et al., 1990). Recently it was shown by SAKAGUCHI et al. (1989) that the results obtained for yellowtail fillets cannot be generalized.

When fillets from carp (*Cyprinus carpio*) were frozen at -20°C for 18 h and thawed, the Torrymeter readings decreased only by 2-3 units. On the other hand, the values for unfrozen, ice stored fillets decreased very slowly; the decrement was as low as approximately 1.0 after 15 days. The difference between yellowtail and carp might be attributed to the difference in the fat content, influencing the dielectric property of the surface layers of the fish bodies (GR INTERNATIONAL ELECTRONICS LTD, 1978). Carp fillets which had been subjected to repeated freeze-thaw cycles exhibited a decline in the Torrymeter readings by ca. 3 units for the first cycle, followed by ca. 2 units each for the second and the third cycles. The effect of prolonged frozen storage has not been studied.

In comparison to carp fillets, fillets from cod, a lean marine fish species, showed quite the opposite behaviour. Torrymeter readings, taken on the bone side of the fillets, fell rapidly during ice storage of fresh fillets, from 15 to 7 units within 50 h (REHBEIN and AUST, 1980). Recently a similar ice storage trial was

performed using the Fish-Tester. Table 1 shows that the values for skinned fillets dropped rapidly, whereas the readings for unskinned fillets decreased only slowly (Table 2) (REHBEIN, 1990; unpublished data).

The following conclusions can be drawn from the results described in this section:

1. Reliable differentiation between fresh and frozen fish is possible by using either the Torrymeter or the Fish-Tester.
2. Both instruments may also be applied to distinguish between unfrozen and frozen-thawed fillets, especially if the fillets have not been skinned.
3. In the case of skinned fillets the results depend on the fish species as well as on the ice storage period. Even after a few days of ice storage, the fillets can give readings which fall in the range of values for frozen-thawed fish.

Examination of the opacity of the eye lens

The lens of the fish eye consists of the central part (medulla) and an outer layer, the cortex. The medulla of fresh fish is transparent, but freezing of the fish leads to turbidity; this was first used for differentiation of frozen-thawed fish from fresh fish by LOVE (1956) and then by several other research groups (CARACCIOLO and PETRIS, 1962; CIANI and SALERNI, 1965). Later on the technique of examination was improved by YOSHIOKA and KITAMIKADO (1983); they also found that the small medullae of eel and flatfish did not become opaque as a result of freezing and thawing.

Determination of the hematocrit value; examination of erythrocytes

Both methods rely on the destruction of the red blood cells due to freezing and thawing. Obviously they cannot be applied to those fish fillets possessing only minmal amounts of blood or red muscle, e.g. fillets from cod, redfish (*Sebastes marinus*) and many other white-fleshed species.

The hematocrit value is defined as the proportion of the erythrocytes on blood volume. It is expressed as percent of volume and determined by centrifugation of blood. The hematocrit value of

Table 1 - Fish-Tester values during ice storage of skinned cod fillets. On board a trawler, 5 specimens of cod were filleted directly after the catch; the fillets were skinned and stored in melting ice for the time indicated. Each fillet was measured daily at 3 positions (front, middle, and end) with the Fish-Tester. The mean value and standard deviation are reported.

Ice storage time (days)	Fish-Tester value
0	100
1	77 ± 15
2	34 ± 9
3	20 ± 6
4	13 ± 5
5	11 ± 4

Table 2 - Fish-Tester values during ice storage of unskinned cod fillets. On board a trawler, cod was filleted directly after the catch. The fillets were stored in melting ice for the time indicated; each day 3 fillets were measured with the Fish-Tester (the electrodes were placed in the middle of the fillet) and removed from the trial; the mean value and standard deviation are reported.

Ice storage time (days)	Fish-Tester value
0	100 ± 0
1	78 ± 7
2	68 ± 8
3	63 ± 14
4	70 ± 23
5	58 ± 5
6	56 ± 8
7	63 ± 15 (5 ± 0.6) *
8	48 ± 12 (10 ± 0.6)
9	52 ± 5 (8 ± 1.7)
10	36 ± 4 (15 ± 0.6)

* Values measured after skinning the fillets.

frozen-thawed fish (carp, sea bream and Pacific mackerel) was zero, whereas fresh fish gave values between 21 and 43%, depending on the freshness and the species of fish (YOSHIOKA, 1983).

The same author reported about the differentiation of frozen-thawed fillets from fresh fillets by microscopic examination of stained erythrocytes (YOSHIOKA and KITAMIKADO, 1988). A small volume of blood (20 μL or less) was withdrawn with a capillary pipette from the central or dorsal aorta of fillets from red sea bream (*Chrysophrys major*) or Pacific mackerel (*Pneumatophorus j. japonicus*), smeared on a slide glass, dried, stained with Giemsa solution and inspected microscopically. Besides, a small amount of dark surface muscle was taken from the samples and processed as described above.

All erythrocytes were destroyed in frozen-thawed samples, but intact erythrocytes were found in the blood and dark muscle of fresh and refrigerated (for 1 day at + 5°C) samples. The method was also successfully applied to commercial fillets of several other fish species (e.g., sardine (*Sardinops melanosticta*) and tuna (*Thunnus thynnus orientalis*)).

Enzymatic methods

The cells of fish muscle and their organelles are destroyed by freezing and thawing of muscle (KARVINEN et al., 1982). Enzymes located inside the particles or bound to the membranes are released into thaw drip and press juice (WARRIER et al., 1985; REHBEIN, 1979b).

When muscle of fresh fish was carefully extracted with isotonic solution (0.25- 0.30 M sucrose), only low activities of lysosomal and mitochondrial enzymes were detectable in the soluble fraction (supernatant after high-speed centrifugation); the same treatment applied to frozen-thawed fish muscle gave considerably enhanced activities of these enzymes in this fraction (KARVINEN et al., 1982; WERKMEISTER and DEMMER, 1986).

The appearance of originally particle bound enzymes in drip, press juice or isotonic extract has been used by a number of research groups to differentiate between fresh and frozen-thawed fish fillets. However, not all of the methods reported in this section fulfill the following requirements (GOTTESMANN and HAMM, 1987):

1. The enzyme should be released by freezing and thawing, but not during storage of fish or fillets under refrigeration (in most cases, storage in melting ice).
2. The total activity should not decrease too much during storage of fillets either fresh or frozen. For this reason sarcoplasmic reticulum adenosinetriphosphatase can not be used. The activity was lost when cod was stored in ice over 2 weeks or for only 1 week at -15°C (YAMANAKA and MACKIE, 1971).
3. The enzyme should be easily detectable in muscle press juice or isotonic extract.

Considering these postulates the literature of the last 15 years is reviewed and divided into 3 parts. First mitochondrial enzymes are treated, then lysosomal ones and finally those enzymes which are released from the red blood cells of fish.

MITOCHONDRIAL ENZYMES

Cytochrome c oxidase (EC 1.9.3.1)

Cytochrome c oxidase is a component of the inner mitochondrial membrane (VOET and VOET, 1990). The enzyme catalyses the oxidation of reduced cytochrome c with molecular oxygen. The assay system which was used to distinguish between unfrozen and frozen-thawed trout muscle is relatively laborious (BARBAGLI and CRESCENZI, 1981). Muscle of trout

(*Salmo trutta gairdneri*) was homogenized with 0.25 M sucrose by means of a Potter homogenizer. The suspension was centrifuged at 10,000 rpm and cytochrome c oxidase activity was measured in both supernatant and sediment suspension. For unfrozen trout muscle the percentage of enzyme activity of supernatant with respect to total (supernatant plus sediment suspension) was about 30%, whereas this ratio was 58-82% for frozen-thawed trout muscle. In unfrozen trout the activities of supernatant and sediment suspension did not change after 4 days of storage at +4°C, but the influence of longer periods on refrigerated or iced trout has not been investigated.

Cytochrome c oxidase was also used as a mitochondrial marker in a study of the changes in muscle subcellular fractions of Baltic herring (*Clupea harengus membras*) during cold and frozen storage (KARVINEN et al., 1982). The nuclear (N), mitochondrial (M), lysosomal (L), microsomal (P) as well as the soluble (S) fraction were prepared by differential centrifugation of extract from muscle of fresh and frozen herring.

Cytochrome c oxidase activity in the fresh fish was mainly found in the M and L fractions, whereas the frozen fish contained low activity in the M fraction, but high activity in the P fraction.

Thus the shift of cytochrome c oxidase activity from the M and L fractions to the lighter P and S fractions may be used to detect frozen-thawed fish (or fillets), but the technique is not applicable to routine analysis.

Glutamate aspartate aminotransferase (EC 2.6.1.1)

Isoenzymes of glutamate aspartate aminotransferase (also named glutamate oxaloacetate transaminase, GOT) exist in mitochondria and cytosol (GOT_M and GOT_S).

The GOT_M is located in the mitochondrial matrix (VOET and VOET, 1990). The enzyme is set free by destruction of mitochondria as a consequence of freezing and thawing. This has been used to develop a method for the differentiation of fresh and frozen fish muscle consisting in mild extraction with isotonic medium, separation of GOT_M and GOT_S by cellulose acetate electrophoresis and specific staining for GOT with 2-ketoglutarate and aspartate as substrates (SALFI et al., 1985).

The method was applied to various freshwater and marine species. In each case fresh fish muscle gave only one GOT_S-band, whereas frozen-thawed muscle showed one or more additional GOT_M-bands. This positive result has the limitation that only very fresh fish were tested.

A former study (HAMM and MASIC, 1971) performed with carp demonstrated that the mitochondria were already destroyed to a high degree with concomitant release of GOT_M during storage of carp fillets in ice. SALFI et al. (1986) also reported that malate dehydrogenase (EC 1.1.1.37) could not be used to establish a method for the detection of frozen-thawed fish.

Compared to enzyme assays the electrophoretic separation of isoenzymes has two disadvantages: (i) electrophoresis, especially when coupled with an enzyme specific staining procedure, is more laborious and (ii) the result is qualitative; only the absence of the GOT_M-band guarantees that the tested fish were fresh.

Succinate dehydrogenase (EC 1.3.99.1)

This enzyme, which catalyses the dehydrogenation of succinate to fumarate, is embedded in the inner mitochondrial membrane (VOET and VOET, 1990). If mitochondria are destroyed by freezing and thawing, succinate dehydrogenase may

not be released but, like cytochrome c oxidase, it remains bound to membrane debris. The enzyme was utilized without success to differentiate between fresh and frozen squid (*Octopus vulgaris*) (FRIGERIO et al., 1980). Both, cooling as well as freezing, reduced the enzyme activity to a similar degree.

Beta-hydroxyacyl-CoA dehydrogenase, HADH (EC 1.1.1.35)

HADH is located on the matrix side of the inner mitochondrial membrane (GOTTESMANN and HAMM, 1984). The enzyme catalyses the following reaction:

Acetoacetyl-CoA + NADH + H^+ —>
—> Beta-hydroxyacyl-CoA + NAD^+

Acetoacetyl-CoA can be replaced by the artificial substrate N-acetyl-acetoacetyl- cysteamine (DEMMER and WERKMEISTER, 1985).

The applicability of HADH as an indicator enzyme for the differentiation between fresh and frozen-thawed fish was tested by WERKMEISTER and DEMMER (1986) with trout.

Muscle was gently homogenized in isotonic buffered sucrose with a household mixer. Mitochondria were precipitated by centrifugation and the HADH activity of the supernatant was measured. The increase in activity due to freezing (-25°C) and thawing of fresh trout was 7 to 11 fold. Further experiments are needed to demonstrate that HADH is not released during ice-storage of fish or fillets.

LYSOSOMAL ENZYMES

Compared to mitochondria, lysosomes seem to be less structured. Lysosomes are "single membrane-bounded organelles containing numerous hydrolytic enzymes to digest materials ingested by endocytosis and to recycle cellular components" (VOET and VOET, 1990).

However, this view has to be differentiated. At least three types of lysosomes can be distinguished: primary and secondary lysosomes as well as residual bodies (HOLTZMAN, 1989). A great number of lysosomal enzymes have been detected in extracts of fish muscle. Examples from recently published studies are:

Aryl sulfatase (EC 3.1.6.1), beta-glucuronidase (EC 3.2.1.31), RNAse (EC 2.7.7.16), acid phosphatase (EC 3.1.3.2) and acid proteinase (EC 3.4.23) in saithe (*Pollaius virens*) (BEARDALL and JOHNSTON, 1985); acid phosphatase (EC 3.1.3.2) in hake (*Merluccius hubbsi*) (GOLDEMBERG et al., 1987); cathepsin D (EC 3.4.4.23) in herring (KARVINEN et al., 1982); beta-N-acetylglucosaminidase (EC 3.2.1.30) in eight species of fish (UENO and HORIGUCHI, 1984); beta-glucuronidase, beta- N-acetylglucosaminidase, acid phosphatase and cathepsin D in Pacific mackerel (*Scomber japonicus*) (UENO et al., 1986); beta-glucuronidase in Bombay duck (*Harpodon nehereus*) (WARRIER et al., 1985).

These enzymes expressed maximal activity in the acidic pH-range (3-5). This characteristic property can be used to distinguish lysosomal enzymes from their neutral counterpart (CASTILLA et al., 1980).

Three distinct populations of lysosomes were identified in muscle tissue of salmon (*Oncorhynchus kisutch*) and steelhead trout (*Salmo gairdneri*) by electron microscopy: one population was observed in connective tissue cells tentatively identified as macrophages; a second population was detected within but at the periphery of muscle cells and a third population of lysosomes was localized among myofibrils (STEINER et al., 1984).

Some of the above mentioned lysosomal hydrolases have been utilized as a tool to differentiate unfrozen from frozen-thawed fish fillet.

Alpha-glucosidase (EC 3.2.1.20)

Alpha-glucosidase activity was measured in press juice from fresh and frozen- thawed fish of cod (*Gadus morhua*), saithe (*Pollachius virens*), redfish (*Sebastes marinus*) and haddock (*Melanogrammus aeglefinus*) (REHBEIN et al., 1978). The press juice was obtained by high-speed centrifugation of muscle tissue; the enzyme activity was determined using the chromogenic subtrate, p-nitrophenyl- alpha-D-glucopyranoside, at pH 4.5.

The specific activity of alpha-glucosidase was considerably enhanced in press juice from frozen-thawed fillets compared to press juice from fresh ones. This allowed the differentiation of the two categories of fillets. It was difficult, however, to get standard values of the specific alpha-glucosidase activity for both types of press-juice, because the activity in fillets of the same fish species varied to some extent; this might be due to biological factors or to enzyme denaturation during processing and storage.

Therefore the ratio of the specific activities in press juice and sediment extract was determined to reduce the variation. The activity ratios were 0.05 - 0.20 for non-frozen fillets of cod and saithe and 0.50 - 1.20 for frozen-thawed fillets of these species (REHBEIN, 1979c).

During storage of wet fish (cod) in ice the activity ratio for the alpha-glucosidase increased gradually indicating the destruction of lysosomes by fish muscle and bacterial proteases. Spoiled fish had an activity ratio nearly as high as found for unspoiled frozen-thawed fish (REHBEIN et al., 1978), but both products could be differentiated without difficulties by the estimation of parameters of spoilage like total volatile bases-nitrogen or trimethylamine (VYNCKE, 1983).

By means of the estimation of alpha-glucosidase activity in press juice from trout (CATTANEO et al., 1982) or in press juice and sediment extract from rainbow trout (REHBEIN and AUST, 1980), differentiation between unfrozen and frozen-thawed fish was achieved.

A comprehensive study was performed by SHIMOMURA et al. (1987) about the applicability of alpha-glucosidase and other lysosomal enzymes (beta-N-acetylglucosaminidase and cathepsin D) to detect thawed fish.

Press juice and extract were prepared in a similar way as described above by REHBEIN et al. (1978). Fish from seven species was analyzed. The activity ratio increased due to freezing and thawing of fish muscle by 2 to 3 fold for 5 species and by more than 10 fold for 2 species. Thus, differentiation between fresh and thawed fish was possible, but the authors stated that the activity ratio varied considerably from species to species. The absence of generally available standard levels for unfrozen and frozen-thawed fish was considered to be a shortcome of the method.

Beta-N-acetylglucosaminidase (EC 3.2.1.30)

The activity of this enzyme was measured by the release of p-nitrophenol from p-nitrophenyl-N-acetyl-beta-D-glucosaminide at pH 4.5 in press juice and sediment extract as described above for alpha-glucosidase (REHBEIN et al., 1978).

The enzyme was suitable for distinguishing frozen-thawed fillets of haddock and redfish from fresh ones (REHBEIN, 1979a). In the case of trout, however, it could not be used, because the enzyme activities in press juice from fresh and thawed fish were in the same range (CATTANEO et al., 1982).

Comparison of mitochondrial and lysosomal enzymes

Cod fillets were used to compare the release of lysosomal and mitochondrial

enzymes as a consequence of freezing and thawing by determination of enzyme activities in press juice (REHBEIN, 1979b).

Increase in activity was observed for alpha-glucosidase, beta-glucuronidase, beta-galactosidase and beta-N-acetyl-glucosaminidase, but not for acid phosphatase.

The activity of fumarase also increased, but not that of glutamate dehydrogenase (EC 1.4.1.2). As described for porcine skeletal muscle (HAMM et al., 1972), fumarase of cod white muscle may be loosely associated with the inner mitochondrial membrane, whereas glutamate dehydrogenase may be dissolved in the mitochondrial matrix.

Recently several enzymatic and physical methods were tested to detect deep freezing of herring (REHBEIN, 1991). Table 3 shows that alpha-glucosidase, beta-N-acetylglucosaminidase as well as fumarase were useful for the differentiation between unfrozen and frozen-thawed herring. Beta-hydroxyacyl-CoA-dehydrogenase gave inconsistent results.

These two studies demonstrate that enzymes from both cell organelles, mitochondria as well as lysosomes, can be used to differentiate between fresh and thawed fish (or fillets).

Which enzyme gives the best result may depend on the fish species, the product (round fish, fillets with or without skin) and the storage conditions (cooling without ice, storage in refrigerated sea water or in ice).

Occasionally the disorganization of lysosomes (REHBEIN et al., 1982) and mitochondria (HAMM and MASIC, 1971) in muscle of unfrozen, ice-stored fish occurs very rapidly.

In this context it should be mentioned that the latency of fish muscle lysosomal enzymes was lost also due to irradiation of fish (GORE et al., 1982). It was proposed that the increased release of lysosomal enzymes into press juice could serve as an indicator to differentiate irradiated fish from fresh fish.

Table 3 - Differentiation between unfrozen and frozen/thawed herring by measurement of lysosomal and mitochondrial enzyme activities. White muscle was carefully homogenized with 0.3 M saccharose/1 mM K-phosphate pH 7.4 and centrifuged; the sediment was extracted with 0.2% (w/v) Triton X-100. The specific enzyme activities in muscle extract and sediment extract were determined and their ratios were calculated according to the equation: Activity ratio = Specific enzyme activity in muscle extract / Specific enzyme activity in sediment extract. Values are reported as mean ± standard deviation and range in parentheses.
Specific enzyme activity = E * time^{-1} * protein concentration^{-1}

Enzyme	Activity ratio	
	unfrozen, ice stored herring (n=14)	frozen/thawed herring (n=8)
Lysosomal enzymes		
alpha-glucosidase	0.07 ± 0.03 (0.03 - 0.15)	0.36 ± 0.13 (0.18 - 0.52)
beta-N-acetylglucosaminidase	0.16 ± 0.07 (0.05 - 0.28)	0.77 ± 0.28 (0.43 - 1.25)
Mitochondrial enzymes		
fumarase	0.16 ± 0.04 (0.10 - 0.22)	0.57 ± 0.22 (0.32 - 0.98)
beta-hydroxyacyl-CoA-dehydrogenase	0.08 ± 0.07 (0.02 - 0.30)	0.35 ± 0.26 (0.08 - 0.74)

ENZYMES OF RED BLOOD CELLS

The destruction of red blood cells during freezing and thawing of fish has already been mentioned in the section on determination of the hematocrit value. Enzymes located in the cytosol of red blood cells are expected to be released as a result of these processes.

The cytosol of red blood cells of carp contains beta-N-acetylglucosaminidase active at neutral pH (UENO et al., 1987). If unfrozen blood was mixed with an isotonic assay solution (subtrate: 4-methylumbelliferyl-2-acetamido-2-deoxy-beta-D-glucopyranoside), the enzyme activity was practically zero, but it increased more than 100 fold, when frozen-thawed blood was tested (YUAN et al., 1988).

On the basis of this result a fluorimetric method and a rapid paper test were developed to distinguish fresh from frozen-thawed fish.

A small volume of blood was mixed with an isotonic solution containing N-acetyl-D- galactosamine for inhibition of the acidic beta-N-acetylglucosaminidase of the white blood cells.

Then substrate solution was added and the mixture was incubated at 20°C for 10 min. The reaction was stopped and the formed 4-methylumbelliferone was measured by means of a spectrofluorimeter.

More than 30 species of common edible fish were tested and for every species clear-cut differences between the activities of fresh and frozen-thawed fish were found (KITAMIKADO et al., 1990).

For routine analysis a test paper was prepared by soaking filter paper with isotonic solution containing the fluorogenic substrate. A drop of blood was smeared on the test paper and after 10 min the reaction was terminated by addition of alkaline glycine buffer. The colour change was immediately observed under the UV-lamp (360 nm). Blood from fresh fish gave a red colour, while that from frozen-thawed fish changed the colour from red to blue and fluoresced under the UV-lamp.

Two disadvantages of these methods have been reported (KITAMIKADO et al., 1990): first these methods cannot be applied to fish fillets because of the absence of blood; secondly, the results were not reliable for iced fish beginning to spoil, since a considerable proportion of red blood cells in these fish were already hemolyzed.

CONCLUSIONS

On account of the critical evaluation of the numerous methods for differentiating between fresh and frozen-thawed fish or fillets the following recommendations are given:

(i) In the case of whole fish, measurement of the electrical resistance using the Fish-Tester or Torrymeter is superior to other methods, because unambiguous results are obtained in a few minutes.

However, if fish are stored in refrigerated seawater or treated with salt, these instruments cannot be applied for all fish species (e.g., not for herring).

(ii) For differentiation of fillets, one of the above-mentioned enzymatic methods may be used. Which type of enzyme (located in the mitochondria, lysosomes, or red blood cells) gives the best results depends to some extent on the fish species.

It is not sufficient to compare enzyme activities in press juice or extract from thawed and very fresh fillets. The release of enzymes during ice-storage of "fresh" (unfrozen) fillets must also be determined, because during spoilage of fillets particle bound enzymes are gradually set free.

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