

β -hydroxyacyl-CoA-dehydrogenase activity differentiates unfrozen from frozen-thawed Yellowfin tuna (*Thunnus albacares*)

Cristian Bernardi,¹ Erica Tirloni,¹ Simone Stella,¹ Aniello Anastasio,² Patrizia Cattaneo,¹ Fabio Colombo¹

¹Department of Health, Animal Science and Food Safety, University of Milan;

²Department of Veterinary Medicine and Animal Production, University of Naples "Federico II", Naples, Italy

Abstract

Changes in the β -hydroxyacyl-CoA-dehydrogenase (HADH) activity of fresh and frozen-thawed Yellowfin tuna were examined. A statistical approach to HADH activities determined in press juice allowed to set a critical value to differentiate fresh from frozen-thawed Yellowfin tuna: the threshold value was 3.7 U mL⁻¹ at the probability level of 1%. The analysis of 37 tuna (not ready to eat) sampled on retail revealed the unconformity to labelling of 4 samples. A simple statistical algorithm was built to get probabilities from observed values on tuna of being or not frozen/thawed.

Introduction

Frozen fish and thawed-frozen fish have different prices. For this reason and due to a possible lesser quality of the frozen-thawed in comparison to fresh-chilled fish it is important to have a simple and reliable method to differentiate the two types of products. Since Japanese cuisine is fashionable in Italy, fresh tuna entered in food habits of Italians who appreciate tuna either in raw preparations, such as sushi, sushimi and carpaccio, or as tuna steak, while in the past, fresh tuna was eaten only in the coastal regions, and only when available. Atlantic bluefin tuna (*Thunnus thynnus*) is the most appreciated tuna species but, due to the decrease of natural stocks, both in numbers and individual size (Bearzi et al., 2006), the catch limitations, the recovery plans to rebuild the stocks, and consequently its expensive cost, Yellowfin tuna (*Thunnus albacares*) substitutes it very well. Moreover, its typically bright red colour is very attractive for many consumers. Another species more appreciated in Italy is

swordfish, as for Red tuna the captures decrease and the demand increase rise the price.

Fresh Yellowfin tuna (from now on only "tuna") is imported iced in Italy by plane, directly from the place of capture, for example from Maldives, in two-three days, assuring the best freshness. Sometimes it can be vacuum packed as loins, prolonging the shelf-life. There is, however, the possibility that unscrupulous suppliers sell thawed-frozen tuna instead of fresh tuna. According to EC regulations, previously frozen fish, thawed before sale, must be labelled "thawed" and differentiated from the fresh chilled one (Directive 2000/13/EC).

Freezing and thawing and freezer storage may cause a decrease in water holding capacity (WHC) of meat with an increase of drip loss during thawing. Moreover, during prolonged freezer storage, formaldehyde is produced along with dimethylamine (DMA) by enzymatic breakdown of trimethylamine oxide (TMAO), due to TMAO-demethylase (Sothelo & Rehbein, 2000), and this is believed to contribute to protein cross-linking of muscle proteins causing an increase in toughness.

Many physical and enzymatic methods have been studied to distinguish fresh from thawed meat. All enzymatic methods are based on the principle that freezing and thawing damage the cells and their organelles, such as mitochondria and lysosomes, so that enzymes located inside the particles or bound to the membranes are released into the press juice (Rehbein, 1979). So, in the press juice obtained from frozen muscle there is a greater number of enzymes (enzymatic activity) than in the press juice of fresh muscle.

The mitochondrial enzyme β -hydroxyacyl-CoA-dehydrogenase (HADH), located on the matrix side of the inner mitochondrial membrane (Gottesmann and Hamm, 1984), was extensively studied by Gottesmann and Hamm and proved to be optimum for terrestrial animal meat; from their numerous works on this topic we should mention for instance two basic works: Gottesmann & Hamm (1983) and Gottesmann & Hamm (1987).

HADH method was evaluated for several meat species, included game animals and birds, and reported in the manual of manufacturing meat quality (Church & Wood, 1992). HADH activity was studied in a lesser extent in fish and other seafood: in trout (Werkmeister & Demmer, 1986), in crawfish (*Procambarus clarkii*) and trout (*Salmo gairdneri*) (Hoz et al., 1992), in kuruma prawn (*Penaeus japonicus*) (Hoz et al., 1993), in various fish and shellfish (Fernandez et al., 1999) and in albacore

Correspondence: Cristian Bernardi, VESPA, Università degli Studi di Milano, via G. Celoria, 10, 20133 Milano.
Tel.: +39.0250317855 - Fax: +39.0250317870
E-mail: cristian.bernardi@unimi.it

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(*Thunnus alalunga*) (Pavlov et al., 1994), in swordfish (Civera et al., 1996) and seemed to be useful in most cases.

The last proposed method was the near infrared spectroscopy (NIRS) technique, which seemed to have a good potential (Uddin et al., 2005), but, in our opinion, the use of an enzymatic method is still valid, since, mentioning Rehbein (1992), "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".

For this reason fresh and frozen – thawed tuna for HADH were analysed in order to: 1) verify if measuring HADH activity is suitable to differentiate fresh from frozen-thawed tuna and to determine the critical value of HADH activity to state the frozen-thawed condition; 2) verify the frozen-thawed condition and the conformity to labelling in retail tuna samples; 3) state if a very short freezing period could influence the release of the HADH enzyme in the press juice and at which extent. In fact, a second different aspect, not less important, is that deep freezing (at least 24 hours at -20°C) is compulsory (Regulation (EC) N. 853/2004) in fish destined to be consumed raw or undercooked, in some fish species to be cold smoked and in mild marinated or salted fish, to kill live nematode larvae (such as *Anisakis* spp. responsible of human zoonosis), which could be present in fish muscle.

Experimental design

Trial 1: on the sampling day, 16 tuna of about 40 kg, shipped by plane from Maldives, fished in F.A.O were analyzed for HADH activity. Zone n. 51, gutted and iced and in very good condition of freshness. Aliquots of about 100 g were obtained cutting the ventral muscle. After trimming of fat and connective tissue excesses, muscle slices were pressed in a hand press to obtain press juice. HADH activity was executed on 1:100 diluted fish juice. The remaining muscular part was frozen and stored at -24°C for 20-25 days and again analysed after thawing overnight at 2°C.

Trial 2: 5 samples, 200 g each, were obtained as described above. Each sample was divided in 6 parts, which were labelled in order to trace each portion to the initial sample/subject. The first one was pressed fresh and HADH method was performed. The other portions were individually frozen in plastic bags, then thawed after 24, 48 hours, 7, 12 and 28 days freezing storage and HADH was determined.

Trial 3: 37 Yellofin tuna steaks purchased from retails in Milan area were analyzed. Except 3 samples, labelled thawed, the other ones were not labelled and intended as fresh. When possible, TVB-N was determined to assess the sample freshness in order to exclude a possible influence of spoilage on the enzyme release.

Materials and Methods

Chemicals

Acetoacetyl-CoA and NADH were purchased from Sigma-Aldrich (St. Louis, Mo., USA). Monosodium phosphate, disodium phosphate, ethylene-diaminetetraacetic acid, perchloric acid, boric acid, sodium hydroxide, hydrochloric acid were purchased from Scharlab Italia (Riozzo di Cerro al Lambro, Italy).

The HADH activity was measured according to the method reported by Church & Wood (1992): pressed juices were obtained from fresh and thawed samples using a press machine; the meat juice collected was then diluted (1:100) with phosphate buffer (0.1 M, pH 6.0). Pipetted the following solution and diluted meat juice into a methacrylate disposable cuvettes (10 mm light path length, nominal working volume 2.5 mL): 100 µL diluted extract, 200 µL ethylene-diaminetetraacetic acid (EDTA; 34.4 mM) and 2.6 mL phosphate buffer (0.1 M, pH 6.0), 50 µL NADH (7.5 mM), after the mixture was mixed. Finally, 50 µL acetoacetyl-CoA (5.9 mM) were added and the reaction started. The HADH

activity was determined by measuring immediately the absorbance of the mixtures at 340 nm. After 3 minutes the absorbance was recorded and the decrease in extinction per minute ($\Delta E \text{ min}^{-1}$) was calculated from these measurements. HADH activity was expressed in International Units per mL press juice (U mL^{-1}) and was calculated by means of the following formula:

$$\text{U mL}^{-1} = (\text{V E} - \text{d} - \text{v} - 1) \Delta E \text{ min}^{-1} \text{ dilution factor}$$

V = volume cuvette; E = extinction coefficient for β -NADH at 340 nm; d = light path cuvette; v = volume meat juice.

The data of HADH activity were submitted to statistical analysis as described and discussed below.

Total volatile basic nitrogen (TVBN) was determined by direct distillation according to Reg. CE 2074/2005. In brief, the volatile nitrogenous bases were extracted from a sample using a solution of 0.6 M perchloric acid. After alkalisation the extract underwent steam distillation and the volatile base components were absorbed by an acid receiver. The TVB-N concentration was determined by titration of the absorbed bases.

Results and Discussion

The trial 1 was considered a pilot study as it was conducted to get early evidence of difference of HADH activity measures on

tuna between fresh and frozen/thawed and to get estimates of variability, as this kind of information about tuna was lacking in literature. To minimize random variability, the samples (n=16) were analyzed with paired-data approach, *i.e.* every specimen was analyzed in both fresh and thawed condition.

The results of the first trial are reported in Table 1. Results showed statistical significant (critical P-value=0,05) differences between means by paired t-test analysis and yielded a variance estimate.

Trial 2: keeping in account information coming from the pilot study, the second trial was planned implementing a statistical model evaluating effect on HADH values of freezing duration (referring in particular to earlier times, *i.e.* <30 days - see experimental design); moreover, the repeated measures scenario of the pilot study had to be maintained, so a statistical procedure able to appropriately meet the above requirements was searched. Several statistical models could be useful to analyze data, ranging from fairly simple (linear regression) to more complex ones (logistic regression and so on). Final choice was based on minimalist principle ("the simpler the better"); also the need to keep somewhat easy the model-based calculations to judge specimen state was considered. A linear regression model was selected, in the form known as "study of the growth function", applied as a "repeated measures analysis of variance with polynomial contrasts" (Möller, 1995). This procedure is able to test the existence of a trend of HADH activity (in statistical terms to test the null hypothesis of no trend of a single continuous variable) repeatedly

Table 1. First trial results; HADH activities, expressed in U mL^{-1} , of Yellowfin tunas before and after freezing/thawing are reported.

Sample	Fresh	Frozen-Thawed
1	1.428	4.285
2	0.952	4.285
3	0.793	5.079
4	1.111	5.555
5	1.111	3.650
6	2.222	4.285
7	1.269	5.079
8	1.111	3.650
9	0.952	5.238
10	0.952	3.492
11	0.793	3.333
12	1.269	3.650
13	1.111	3.492
14	0.793	3.809
15	0.793	4.761
16	1.269	3.968

Statistical significant (critical P value = 0.05) difference between means by paired t-test analysis and yielded a variance estimate was found.

measured along a (not equally spaced) time-series. Estimate of sample size needed to a trial to be analysed with the chosen model was performed with "G*Power 3", a public-domain software (Faul, Erdfelder, Lang, Buchner, 2007), protocol "Anova: repeated measures, within factors". Two point five (2.5) units difference (99% lower confidence limit for the difference detected in pilot study) was assumed as the minimal effect to be detected between fresh and frozen/thawed. A much lower effect (order of magnitude: 0.2 units) was assumed between freezing times. The variance was cautiously set to nearly twice the variance estimated by pilot study. These settings brought to a suitable total sample size of 30 (five subjects for 6 times) analyses. HADH activities obtained in the second trial are reported in Table 2.

Data shown in Table 2 were analyzed with the previously described statistical procedure and were significant for linear and not linear trends. To enhance the linear component, both time and HADH values were converted to their natural logarithms: the so-transformed data re-analyzed were significant only for linear trend. This linear function was used to predict times, moreover a simple statistical algorithm was built to get probabilities from observed values to be or not frozen/thawed (Table 3).

Model validation: the above linear function was applied to the same data to which it was fitted (internal validation) and to pilot

study data (external validation) to discriminate between specimens; if rounded-to-integer predicted values were 1 or more than 1 they were assigned to the latter category, else if their values were less than 1 they were assigned to the former. It has to be noted that the low inferior limit of the estimated confidence interval of specificity/sensitivity in internal validation was due to small number of subjects, nevertheless the more important external validation gave much higher estimates. According estimated parameters of the adopted linear function, threshold values for an individual sample are 3.7 U mL⁻¹ and 1.8 U mL⁻¹. These values correspond respectively to 1% and 5% probability of being not-frozen/thawed. As threshold (limit value) to identify a freezing-thawing process in market samples the limit was set at 3.7 U mL⁻¹: HADH activities in the press juice up to 3.6 U mL⁻¹ were considered belonging to not frozen-thawed samples; values ≥ 3.7 U mL⁻¹ were considered thawed. Threshold values were rounded to one decimal digit as indicated in the method adopted for the analytical determination.

Our results confirmed HADH as a valid method able to differentiate fresh from thawed tuna, confirming what found for albacore (*Thunnus alalunga*) by Pavlov et al. (1994); due to the different method for the mitochondrial enzyme extraction and the different result calculation, it was not possible to compare the data. According with

Pavlov, it was not possible to establish a unique limit for all species to determine the fresh or thawed state, as there are marked variations in HADH values among different species. In this work for the first time, a useful HADH limit was established; this limit will be useful to distinguish unfrozen from thawed Yellowfin tuna.

Trial 3: the HADH values were evaluated considering the threshold 3.7 U mL⁻¹. Among the tunas sampled on retail, 33 were identified as fresh or thawed confirming the labelling, while 4 (sample 12,18, 23 and 26) had HADH activity levels higher than the critical value (3.7 U mL⁻¹) and did not match with the labelling.

Regarding the TVBN values, this biochemical index was determined on 13 tuna from retail, sampled at random among those of trial 3. The mean value was 31.5 mg N/100g, SD 4.134 range 25.2-39.1 mg N/100g. Legal limits of TVBN are set for selected species of commercial interest (Commission Regulation (EC) No 2074/2005) and there is no legal limit for tuna species. Generally, the recognized maximum limit of acceptability of 30 mgN/100g for fresh fish (Connell, 1995) was exceeded by most samples. Moreover, Oehlenschläger (1992) affirmed that when the concentration of TVBN exceeds 30 mg N/100g flesh, the fish should be considered unfit for consumption. Shakila et al. (2005) reported values of 8 mg N/100g in *Katsuwonus pelamis* freshly procured for the experimental trial, while Ruiz-Capillas and Moral (2005) found in Bigeye tuna (*Thunnus obesus*) initial TVBN values of about 27 mg N/100 g, judged very high. Finally, there was no correlation between TVBN values and the corresponding HADH activity in the press juice, on the basis of the analyzed samples, confirming what pointed out by Gottesmann & Hamm (1985).

Conclusions

The two populations (fresh and frozen-thawed) were well discriminated by HADH.

Table 2. Second trial results; HADH activities, expressed in U mL⁻¹ in Yellowfin tuna during frozen storage are reported. Data shown were significant for linear and not linear trends.

HADH sample	Days					
	0	1	2	7	12	28
1	0.634	2.380	1.746	3.333	3.174	3.492
2	0.317	1.746	3.650	3.412	3.174	3.809
3	0.952	2.539	3.650	3.650	4.603	4.126
4	0.634	1.746	3.174	2.857	3.650	3.015
5	0.793	1.904	2.539	2.619	2.222	3.333
mean	0.666	2.063	2.952	3.174	3.365	3.555
S.D.	0.235	0.372	0.814	0.423	0.865	0.428

Table 3. Predicted freezing times and observed values probabilities in Yellowfin tuna.

Freezing (days)	Calculation to predict time	T value	T value Probability
0	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) + 0.483)/0.389)	DISTRIB.T(T_value;4;2)
1	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) - 0.875)/0.389)	DISTRIB.T(T_value;4;2)
2	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) - 0.956)/0.389)	DISTRIB.T(T_value;4;2)
7	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) - 1.104)/0.389)	DISTRIB.T(T_value;4;2)
12	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) - 1.168)/0.389)	DISTRIB.T(T_value;4;2)
28	EXP (HADH -0,8747)/0,1179	ASS ((ln(HADH) - 1.268)/0.389)	DISTRIB.T(T_value;4;2)

Calculations to predict freezing times and related probabilities - calculations are expressed in Microsoft Excel™ format ; HADH is the observed value. T value probability is calculated by a two-tailed t-test with 4 (= 5-1) degrees of freedom as the predicted values were estimated on 5 observations (by each time point).

The critical level of HADH for differentiating thawed tuna was set at ≥ 3.7 U mL⁻¹. A mathematical model useful to assess the freezing time of tuna was set. This tool may be particularly useful to determine the freezing time; in fact, fishery products to be consumed raw or almost raw must be frozen at a temperature of not more than -20°C in all parts of the product for not less than 24 hours, according to the requirements concerning parasites of the Regulation EC n.853/2004 (Annex II, section VIII, chapter III, point D).

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