EDTA Inhibition of Inosine Monophosphate Dephosphorylation in Refrigerated Fishery Products

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A wide range of dephosphorylation rates of IMP (inosine monophosphate) were measured in the muscle obtained from different species of fish. Treatment of muscle with as little as 0.8 μmole of EDTA [(ethylenedinitrilo)tetraacetic acid] per gram inhibited the dephosphorylation of IMP in muscle from species of fish that had medium or slow rates of dephosphorylation. The apparent reason for the ineffectiveness of EDTA treatment on the muscle from species with a rapid dephosphorylation rate is that there is a lag period between the application of EDTA and inhibition of dephosphorylation. During this lag period, all the IMP in the muscle is dephosphorylated. Sensory evaluation of EDTA-treated muscle showed that the treated muscle had a significantly more acceptable flavor compared with the untreated muscle.

Certain nucleotides have been shown to be flavor potentiators (Hashimoto, 1965; Kuninaka, 1966; Kuninaka *et al.*, 1964; Spinelli and Miyauchi, 1968). The major naturally occurring nucleotide in fish muscle after a few hours post-mortem refrigerated storage is inosine monophosphate (IMP) (Jones, 1963; Kobayashi, 1966). During continued storage of unfrozen fish muscle, the endogenous IMP is dephosphorylated by a phosphomonoesterase.

The rate of dephosphorylation of muscle IMP has been determined for plaice, Atlantic cod, haddock, lemon sole, and coal-fish (Jones, 1963), for plaice and skipjack (Ehira and Masahiko, 1966), for haddock, lemon sole, and plaice (Kassemsarn *et al.*, 1963), for English sole (Guardia and Dollar, 1965), and for halibut (Spinelli, 1967). Only a few West Coast species, however, have been investigated thus far.

The inhibition of IMP dephosphorylation has been studied in carp muscle extracts by the use of (ethylenedinitrilo)tetraacetic acid (EDTA) (Endo *et al.*, 1966). EDTA treatment probably inhibited the dephosphorylation reaction by EDTA chelation of the metal ion that is required for enzymic activity. Also, tests have been reported on fish muscle in which polymetaphosphate and fluoride ions were used as IMP dephosphorylation inhibitors (Kobayashi, 1966).

Apparently, IMP plays an important role in enhancing the flavor of fish (Spinelli and Miyauchi, 1968). The stabilization of endogenous IMP would appear to favor the maintenance of high quality in refrigerated fishery products. The present study was designed to determine the rate of IMP dephosphorylation in the muscle of a number of fish species and to evaluate the effectiveness of EDTA as an inhibitor of this dephosphorylation reaction in several fishery products.

EXPERIMENTAL

Fish. The fish muscle used in this study was obtained from in-rigor fish within about 24 hours after capture. Since the fish of most of the species used were relatively small, dephosphorylation rates and inhibition studies were carried out on the whole muscle (fillet) or on aliquots of the whole ground muscle.

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Storage. All samples were stored at 0° C. in glass containers.

Nucleotide Analysis. Nucleotides were determined by the method of Spinelli and Kemp (1966).

EDTA Treatment. The calcium chelate of EDTA (Geigy Chemical) or the disodium salt of EDTA (J. T. Baker Chemical) were dissolved in water and mixed into ground muscle at a level of 0.8 μmole per gram (300 p.p.m.). Fillets or portion-sized pieces of fillet were treated by shaking them in a plastic bag with a solution of EDTA. The amount of EDTA added, calculated on the basis of the total weight of the fillets or pieces, was 0.8 μmole per gram.

Irradiation. The muscle was cut into 50- to 100-gram pieces, treated with EDTA solution or in the case of the control samples with water, and vacuum-packed in C-enameled No. 2 cans. These samples were irradiated in the Brookhaven National Laboratories Mark II irradiator located at the University of Washington.

Phosphomonoesterase Activity Measurement. The incubation mixture contained 0.5 ml. of 10,000 × G supernatant, 0.4 ml. of 0.1*M* IMP, 2.0 ml. of 0.2*M* Tris buffer, pH 8.1 or 8.5, and water or other additives to a volume of 3.0 ml. At the beginning and at the end of the incubation at 30° or 37° C. an aliquot was removed from the mixture and mixed with 5% TCA (trichloroacetic acid). The method of Fiske and SubbaRow was used to determine inorganic phosphate (Hawk *et al.*, 1954).

Sensory Evaluation. Portions of EDTA-treated and untreated muscle samples were placed in 4.25 × 3.25 × 1.25 inch covered aluminum containers and steamed for 12 to 14 minutes. Triangle tests were made on the steamed samples to determine whether a difference could be detected between treated and untreated muscle. Also, each panel member was asked to give his preference for the odd or paired samples. Eight to 10 persons participated in each test, and the tests were replicated (two times on ground sand sole and once each on fillets of sand sole and lingcod).

Cooking Tests. Weighed amounts of muscle were wrapped in aluminum foil and cooked in an oven at 149° C. and in deep fat at 191° C.

RESULTS AND DISCUSSION

Dephosphorylation Rates. The rates of IMP dephosphorylation varied greatly among species (Figure 1). At melting ice temperatures, the IMP of English sole (*Parophrys vetulus*) and true cod (*Gadus macrocephalus*) was hydrolyzed rapidly; in Dover sole (*Microstomus*

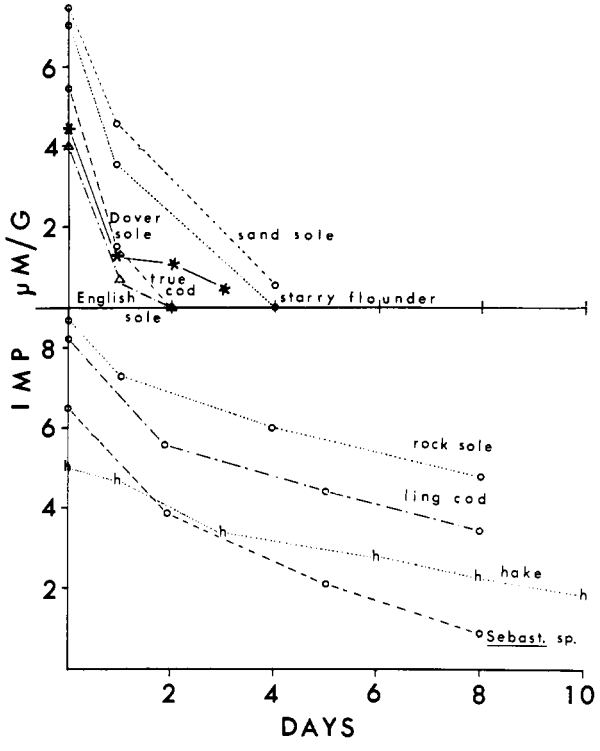

Figure 1. Rates of IMP dephosphorylation in various species

pacificus), sand sole (*Psettichthys melanostictus*), starry flounder (*Platichthys stellatus*), and *Sebastodes* sp., IMP was hydrolyzed more slowly; in rock sole (*Lepidopsetta bilineata*), lingcod (*Ophiodon elongatus*), and hake (*Merluccius productus*), IMP was hydrolyzed very slowly.

When IMP dephosphorylation rates were measured at 37° C. using muscle extracts, the species that exhibited rapid rates and slow rates were, in general, similar to those species that exhibited rapid and slow rates of IMP dephosphorylation in the fillet at melting ice temperatures (Table I*A*). The incubation of the muscle extract of a slow hydrolyzer (halibut) with the extract of a fast hydrolyzer (starry flounder) gave a dephosphorylation rate of only slightly less than that of the fast hydrolyzer alone (Table I*B*). A boiled muscle extract of a fast hydrolyzer (starry flounder) incubated with the muscle extract of a slow hydrolyzer (lingcod) gave a higher dephosphorylation rate than the slow hydrolyzer alone, but the rate was only about 20% that of the rate observed for the unheated starry flounder extract (Table I*B*). These results showed that some activation and inhibition can be demonstrated by incubation of the mixed muscle extracts of slow and fast hydrolyzers. Since the amount of change of the rate of IMP dephosphorylation was small compared with the differences between the slow and fast hydrolyzers, other factors are apparently more important than the presence of enzyme activators in the fast hydrolyzers and inhibitors in the slow hydrolyzers.

Effectiveness of EDTA Treatment. When EDTA was added to muscle extracts at the level of 2.5 mmoles, nearly complete inhibition of IMP dephosphorylation resulted. This inhibition was prevented when an excess of Mg^{2+} was added to the muscle extract at the same time as the EDTA or soon (up to 20 minutes at 30° C.) after addition of EDTA. Under these conditions, EDTA inhibition of IMP dephosphorylation in muscle extracts apparently occurred rapidly and was apparently due to the chelation of an activator metal such as Mg^{2+}. This confirmed the work of Endo *et al.* (1966), who found that EDTA inhibition of IMP dephosphorylation in carp muscle extracts could be removed by the addition of an excess of Mg^{2+}.

When EDTA-treated muscle extracts were incubated 12 hours at 2° C. or 3 hours at 30° C. before addition of Mg^{2+}, the inhibition of dephosphorylation was not reversed by the addition of an excess of Mg^{2+}. Apparently, if the muscle extract remained in contact with the EDTA for a sufficiently long period of time, IMP dephosphorylation activity was irreversibly inactivated. Center and Behal (1966) reported that EDTA treatment of nucleotidase from calf intestinal mucosa irreversibly inactivated the dephosphorylation activity.

When EDTA was used to treat ground or whole muscle, the effectiveness of the treatment was dependent on the rate of dephosphorylation of the species used (Table II). EDTA treatment stabilized the IMP in lingcod, *Sebastodes* sp., and sand sole muscle, but did not in English sole and true cod muscle. EDTA was apparently ineffective as an inhibitor in true cod and English sole because the rate of dephosphorylation in these species is rapid, and there is a lag period between the application of EDTA and the inhibition of dephosphorylation. During this lag period, essentially all of the IMP in the muscle was dephosphorylated. The evidence in favor of a lag period is that, in

Table I. IMP Dephosphorylation[a] Rates of Muscle Extracts

A. **Rates of Different Species Incubated at 37° C., pH 8.5**

Species	Dephosphorylation, μmoles per mg. of protein per hr.
Starry flounder	1.33
Dover sole	0.53
English sole	0.4
Hake	0.23
Lingcod	0.1

B. **Rates for Mixtures of Extracts Incubated at 30° C., pH 8.1**

Species	Dephosphorylation, μmoles per mg. of protein per hr.
Starry flounder	1.16
Starry flounder (boiled)	0.0
Halibut	0.9
Starry flounder + halibut (equal volumes of extract) (rate calculated on basis of protein content of starry flounder only)	1.12
Lingcod	0.15
Lingcod + starry flounder (boiled)	0.23

[a] The amount of orthophosphate liberated in experiments without added IMP was insignificant.

Table II. IMP Content of EDTA-Treated Muscle[a]

Storage Time at 0° C., Days	IMP, μmoles per Gram									
	Sand Sole		Lingcod		*Sebastodes* sp.		English Sole		True Cod	
	Control	EDTA	Control	EDTA	Control	EDTA	Control	EDTA	Control	EDTA
0	7.5	7.5	9.3	9.3	8.0	8.0	4.0	4.0	5.5	5.5
1	...	...	...	...	...	...	...	...	1.6	1.9
2	...	...	8.7	9.0	4.9	5.1	0	0	0	0
5	0.8	5.1	5.4	6.8	2.6	3.6	...	...	...	...
7	...	...	3.8	6.8	1.3	3.6	...	...	...	...
10	...	...	1.6	6.0	0.4	3.1	...	...	...	...

[a] 0.8 μmole of EDTA was added per gram of muscle.

Table III. Sensory Tests (Triangle) on Cooked EDTA-Treated Muscle

Species	Type of Sample	No. of Comparisons	No. of Correct Selections	Significance	No. Preferring Treated Sample
Sand sole	Ground	16	14	0.1% level	14
Sand sole	Whole	8	6	5.0% level	5
Lingcod	Whole irradiated	18	15	0.1% level	15

Table IV. Effect of Cooking on IMP Content

Species	IMP Content, μmoles per Gram		
	Initial	191° C., 5 min., deep fat fried	149° C., 25 min., oven baked
Starry flounder	4.6	3.0	0
True cod	6.2	6.0	1.6
Sand sole	7.5	7.2	3.8

EDTA-treated lingcod and *Sebastodes* sp. muscle, the rate of dephosphorylation was not changed significantly during the first several days of storage at 0° C., but after that time, the rate was decreased, and after about 5 days, the rate dropped to almost zero. Also, dephosphorylation was inhibited in English sole and true cod muscle if the muscle was EDTA-treated and stored for a period of 7 days at 0° C. before the addition of 5 to 10 μmoles per gram of IMP. In the untreated, stored muscle, however, IMP dephosphorylation occurred at a rate similar to that of fresh muscle.

When IMP dephosphorylation was inhibited by EDTA in English sole muscle, addition of excess Mg^{2+} did not reverse the inhibition. This nonreversibility of the inhibition in muscle was similar to that observed for muscle extracts.

Flavor Evaluation of EDTA-Treated Muscle. Sand sole muscle, a medium fast hydrolyzer of IMP, and lingcod, a slow hydrolyzer of IMP, were chosen for these tests because they represent the groups of species to which EDTA treatment might be beneficial. The sand sole was treated as ground and whole muscle and evaluated after 5 to 9 days' storage at 0° C. The lingcod was treated with EDTA as whole muscle and irradiated at 0.15 megarad to extend the shelf life and evaluated after 13 and 21 days' storage at 0° C.

Sensory evaluation of EDTA-treated sand sole and lingcod muscle showed that there were highly significant differences between the treated and untreated samples (Table III). Most of the panel members preferred the treated samples, which indicated that EDTA treatment was effective in controlling some of the flavor change that normally occurs in sand sole and lingcod muscle stored at refrigerated temperatures for long periods of time.

Effect of Cooking on Dephosphorylation Activity. The dephosphorylation of IMP in untreated post-mortem muscle proceeds until the IMP is depleted or the phosphomonoesterase is inactivated. In a fresh product, this loss of IMP will continue until the enzyme is inactivated during cooking. Since the rate of an enzyme reaction approximately doubles for each 10° C. rise in temperature, an important factor to consider in preserving IMP is the length of cooking time required to reach the inactivation temperature of phosphomonoesterase. The levels of the dephosphorylation activity in the muscle of three species during two cooking routines are given in Table IV. The large losses of IMP in both starry flounder and true cod during the slow cooking period resulted from the active phosphomonoesterase in these species. The sand sole muscle, which has a less active phosphomonoesterase, had only a moderate loss of IMP during the slow cooking period. EDTA treatment of muscle immediately before cooking did not prevent the dephosphorylation of IMP during cooking. This result indicated that the lag time between EDTA treatment and inhibition was not shortened significantly at elevated temperatures.

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