Collaborative Study of a Test to Determine Whether Shucked Oysters Have Been Frozen and Thawed

EDITH GOULD
National Marine Fisheries Service, U.S. Department of Commerce, Milford, Conn. 06460

Six laboratories have collaboratively tested a method to determine whether shucked oysters have been frozen and thawed at any time. The method utilizes the differing electrophoretic migration rates of various forms of malic enzyme activity, some of which are not present in the tissue fluid until after the tissue has been frozen and thawed. The technique is enzymography, which combines electrophoresis and a specific histochemical medium. Five laboratories tested *Crassostrea virginica*, obtaining markedly different patterns for the unfrozen oysters compared with the frozen and thawed oysters. The sixth laboratory tested *C. gigas*, and was similarly successful in distinguishing between the storage treatments. In shucked oysters stored up to 3 weeks at 4°C, neither autolysis nor bacterial activity altered the enzymographic patterns. The method has been adopted as official first action.

Occasionally, shucked oysters that have been partially frozen during improper storage procedures are marketed as fresh-shucked oysters, although they have a shorter shelf-life and a greater ratio of fluid to solids than the unfrozen animal. At the request of the oyster industry (F. McGinnis, private communication, 1969), an objective test (1) was developed[1] to determine whether shucked oysters, fresh or spoiled, have been frozen and thawed, or superchilled (−2, −5°C) and thawed, at any time. The test has since been subjected to a collaborative study, the results of which are reported here.

Collaborative Study

Differing electrophoretic migration rates of variously soluble forms of malic enzyme (EC 1.1.1.40) are the basis for the test. A normally soluble form of malic enzyme (free ME) activity is present, but not always in detectable amounts, in the tissue fluid of unfrozen oysters. Additional, latent forms are solubilized by freezing and thawing the tissue, as they are by any gross disruption of cellular structure, such as homogenization. The solubilized latent forms are readily visualized by enzymography, the coupling of polyacrylamide gel electrophoresis, and a specific histochemical medium.

Because the method requires that the centrifuged tissue fluid (CTF) be obtained from whole oyster meats, the problem of uniform sampling arose, further compounded by the difficulty of arranging for the collaborating laboratories to conduct their studies simultaneously, on similarly fresh and on similarly stale samples. One laboratory, therefore, tested 36 coded shucked oysters (*Crassostrea virginica*), whose individual storage history was unspecified; 3 other collaborating laboratories were sent 4 lots (9 oysters each, for triplicate multiple samplings) of similarly coded oysters; and 2 more laboratories collected and treated their own test animals, one set of which was *C. gigas*. The 4 treatment variables were fresh-unfrozen, fresh-frozen/thawed, stale-unfrozen, and stale-frozen/thawed. Stale oysters were those that had been frozen and thawed or left unfrozen, and then stored at 4°C 3 weeks.

For detailed instructions see the first paper reporting the test (1). Keep these 3 requirements especially in mind: (*1*) Prepare the polyacrylamide formularies *according to specifications* in the method rather than purchasing them, so that the buffer systems may be made more sharply discontinuous than those in commercially available formularies. (*2*) Keep the gels *cool* during the electrophoresis, either by putting the entire bath assembly in a cold room or a refrigerator, or by circulating iced water through cooling coils. (*3*) Incubate gels in the histochemical medium *no longer than 30 min* in a dark place, and then rinse immediately. (*4*) Centrifuge only *whole* shucked oysters. (Never mince or homogenize the meats. Any mechanical disruption of the tissue will produce results similar to those obtained by freezing and thawing the tissue.)

[1] The test was developed at Atlantic Fishery Products Technology Center, National Marine Fisheries Service, Gloucester, Mass. 01930.

Table 1. Variables in test procedure for malic enzyme enzymography of oysters

Operation	Lab. 1	Lab. 2	Lab. 3	Lab. 4	Lab. 5	Lab. 6
Speed of centrfgn, $\times g$	20,000	2,500	same as 1	same as 1	14,500	same as 1
Electroph. voltage	1 mA/col./30 min +2 mA/col./2 hr	same as 1	same as 1	1 mA/col./30 min, +2 mA/col./15 min, +5 mA/col./75 min	1 mA/col./1 hr, 3 mA/col./2 hr	same as 1
Temp.	4°C	same as 1	room temp.	same as 1	same as 1	same as 1
Histochem. medium	mixed ≤1 hr before use, except *p*-nitro blue tetrazolium added just before use	same as 1	same as 1	mixed 24 hr before use, except *p*-nitro blue tetrazolium and phenazine methosulfate added just before use	same as 1	same as 1

Table 2. Results of collaborative study of malic enzyme enzymography to determine whether shucked oysters have been frozen and thawed

Lab.	Samples	Correct identifications	Per cent
1	12	12	100
2	12	12	100
3	12	0	0[a]
4	16	16	100
5	36	36	100
6	12	12	100

[a] Laboratory 3 performed the electrophoresis at room temperature. All other laboratories cooled the gels as specified in the method.

A few unavoidable minor changes in procedure at some of the laboratories proved the test to be gratifyingly flexible in some areas (Table 1). (Since the method was first published, the Associate Referee has found that the substrate concentration in the histochemical medium can vary substantially, and that 0.1*M* potassium malate, one-fourth the original concentration, produces a creditable pattern of activity on the gels.) One laboratory, however, neglected to cool the gels during electrophoresis (Table 1) and failed to get a pattern of enzyme-active bands on any gel (Table 2). Very little heat can partially inactivate most enzymes, and 3 hr of unrefrigerated electrophoresis will generate no small amount of heat.

Results and Recommendation

With one exception the collaborating laboratories were able clearly to distinguish between unfrozen oyster meats and those that had been frozen and thawed, solely on the basis of the malic enzyme (ME) enzymograms (Table 2). There were no failures other than in the single laboratory that did not cool the gels during electrophoresis, as specified in the method. Because malic enzyme activity is thermolabile, the latent forms more so than the free form (2), the lack of band deposition on the gels in this case was probably due to some heat-inactivation of the enzyme forms during electrophoresis. The unanimous success of the other collaborating laboratories, all of which either refrigerated the bath assemblies or cooled the gels by circulated ice water, supports this inference.

In the 5 successful laboratories the unfrozen oysters, whether fresh or aged (up to 3 weeks at 4°C), never produced more than a single band (free ME), which was often missing or very faint. The frozen and thawed oysters, by contrast, consistently produced a very broad band (at the site of free ME), plus 2 thinner accessory bands and a thin cathodal band. Figure 1 represents the collective results with *C. virginica;* the pattern for oysters superchilled (−2, −5°C) and thawed is identical to the frozen/thawed pattern, as described earlier (1). Work with *C. gigas* produced a single faint band for unfrozen oysters, and 1 broad band plus a thin cathodal band for frozen and thawed oysters.

Because the possibility of technical error should be guarded against, it is recommended that for any questionable lot of shucked oysters a few be frozen and thawed and tested against some that were untreated. If the resulting ME enzymograms are alike, the lot in question has indeed been frozen and thawed at some point, inadvertently or otherwise; if the enzymograms are markedly different, the lot has not been frozen and thawed at any time.

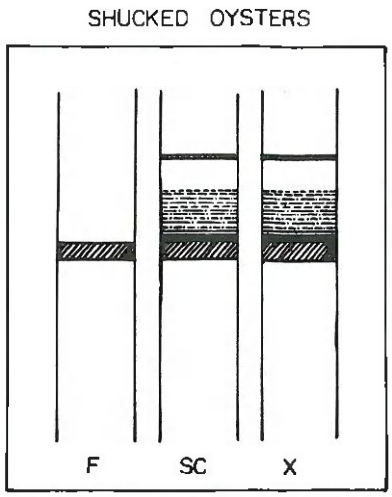

FIG. 1—Representation of malic enzyme enzymograms of centrifuged tissue fluid of oysters, Crassostrea *virginica*, frozen (X) or superchilled (SC) and thawed, and unfrozen (fresh, F).

The method has proved valid with both *C. virginica* and *C. gigas*, the 2 major oysters of American commerce. It can be used successfully either with fresh or with very stale oysters; neither autolysis nor bacterial action significantly alters either the fresh or the frozen pattern. On the basis of the clear-cut results obtained in the collaborative study and presented here, therefore, it is recommended that the method be adopted as official first action.

Acknowledgments

The Associate Referee gratefully acknowledges the time, work, and cooperation of the collaborating laboratory personnel. Those who participated in the study are:

Margaret Dawson, National Marine Fisheries Service, Milford, Conn.

Herman Groninger, National Marine Fisheries Service, Seattle, Wash.

William Hilshey, National Marine Fisheries Service, Gloucester, Mass.

Anthony F. La Terza, Food and Drug Administration, Boston, Mass.

Reba Malman, Maryland Department of Public Health, Baltimore, Md.

Arthur Marin, New York State Food Laboratory, Albany, N.Y.

REFERENCES

(1) Gould, E., & Medler, M. J. (1970) *JAOAC* **53**, 1237–1241

(2) Gould, E. (1968) *J. Fish. Res. Board Can.* **25**, 1581–1589

This report of the Associate Referee was presented at the 86th Annual Meeting of the AOAC, Oct. 9–12, 1972, at Washington, D.C.

The recommendation of the Associate Referee was approved by the General Referee and by Subcommittee C and was adopted by the Association; *see* (1973) *JAOAC* **56**, 398.

Reprinted from the *Journal of the Association of Official Analytical Chemists*, Vol. 56, May 1973.