The Mechanism of the Slime Formation on Sugared Kamaboko. II. Identification of Dextran with Enzyme Preparation from Penicillium funiculosum

Hitoshi UCHIYAMA and Keishi AMANO
(Tokai Regional Fisheries Research Laboratory)

In the first paper of this series (UCHIYAMA and AMANO, 1958) a problem of slime formation, frequently encountered on the surface of sugared Kamaboko after certain period of storage, was described as to the factors being considered to promote this particular kind of spoilage.

The slime, or a substance referred to ‘Neto’ by the people of the industry, can be characterized with its watery transparency and jelly-like viscous property, that is produced only if sucrose or raffinose has been incorporated in Kamaboko products, and is never formed from some other sources of carbohydrate, including glucose, fructose, arabinose, galactose, starch and inulin.

When the spoilage of sugared Kamaboko takes place, a rapid decrease of sucrose is observed. However, after a certain lapse of storage time, the amount of reducing sugar shows a trend of increase, and, eventually the slimy carbohydrate polymer was produced on the surface of this food.

From these biochemical observations as well as chemical and physical properties of the purified product obtained from the slime by repeated precipitation with methanol, the writers presumed that dextran would be the most responsible instigator for the slime formation on a sugared Kamaboko.

In order to make further confirmation for the above conclusion, an identification test specific for dextran, using dextranase prepared from Penicillium funiculosum, was carried out in the present work.

In 1952, JEANES et al already pointed out that Penicillium funiculosum, Penicillium lilacinum, Penicillium verruculosum and other related molds are capable of developing an extracellular enzyme which splits dextran. In submerged culture of these microorganisms, they found free enzyme which brings dextran into cleavage at the site of the α-1, 6 glucosidic linkage of this compound.

With regard to the enzymatic activity of dextranase, KOBAYASHI (1954) reported a case of Penicillium strain which produced an active dextranase when a certain
amount of dextran was added to the culture medium prior to inoculation, meanwhile only a weak activity of the enzyme was noticed when the test strain was grown in dextran-free medium.

So, the microorganism and the cultural medium to be employed was carefully determined in this study from the angle of the above two articles.

Materials and Methods

'Neto' sample

The 'Neto' sample used in this experiment was prepared in a similar manner mentioned in the previous paper. First, 'Neto' was washed off with hot water from spoiled pieces of sugared Kamaboko which were introduced from a commercial plant in Odawara, the place well known for producing sugared Kamaboko. The suspension thus obtained was centrifuged in order to obtain 'Neto' preparations which were free from small fragments of Kamaboko. The supernatant was then added with methanol up to a concentration of 20 per cent to eliminate bacterial cells and minor amount of Kamaboko debris. After centrifugation, methanol was added to the supernatant so as to get final concentration of 50 per cent. The precipitate was then collected and dissolved in small amount of hot water, and further purification with methanol was repeated for three times. Approximately, 3 grams of dried 'Neto' product was obtained from 100 grams of comminuted sample of Kamaboko, which contains about 10 per cent of cane sugar.

Preparation of dextran splitting enzyme.

A 50ml. portion of a modified Czapek's medium, containing 1% of dextran instead of sucrose, was placed in a 100ml. Erlenmeyer flask and culture of Penicillium funiculosum from an agar slant was inoculated in this medium and incubated for 3 or 4 days at 30°C.

With development of the mold on surface of the medium, liquid portion, originally turbid due to the presence of dextran, became clearer which indicates production of dextranase in the medium. And the centrifugate obtained from the liquid portion was used as a crude dextranase solution.

Measurement of the change in specific viscosity of 'Neto' solution by addition of dextranase.

Dried 'Neto' was dissolved in hot water at a concentration which gave a specific viscosity between 0.8 and 1.0. After cooling, the solution was centrifuged in order to get an even consistency. Then, a 10ml. quantity of the solution was placed into Ostwald's viscosimeter, fixed in a water bath at 25°C±0.1. After standing to equalize the temperature of test solution with that of water bath, 1ml. of the dextranase solution was pipetted into the viscosimeter and the measurement was started immediately after mixing the solution and continued at intervals of five minutes.
Inhibition of dextranase activity by mercuric chloride.

Mercuric chloride was dissolved into filtrate from the culture of *Penicillium funiculosum*, so as to give the concentration of $10^{-2}$M and the mixture was kept for 30 minutes at 30°C. A 1 ml. portion of the solution thus prepared was then added to 10 ml. of ‘Neto’ solution with specific viscosity of about 1.2. The specific viscosity was determined with respect to the lapse of time for two hours.

Calculation of intrinsic viscosity.

There has been established that the intrinsic viscosity of dextran depends largely upon its mean molecular weight, as pointed out by Kobayashi (1953). So, the value of intrinsic viscosity was computed from the figures of specific viscosity by the following formula,

$$[\gamma] = \lim_{c \to 0} \left[ \frac{\eta_{sp}}{C} \right]$$

where C is the concentration of test solution in terms of percentage of ‘Neto’.

Identification of isomaltose, an end product derived from dextran by the action of dextranase.

By the action of dextranase from *Penicillium funiculosum*, a remarkable reduction in viscosity of dextran can be induced, followed by an increase of a small amount of reducing sugar in the system. Kobayashi (1954) found out that isomaltose and isomaltotriose were positively present as end product of dextran. So, the detection of these compounds would be competent to identify dextran.

In the present work, a paper chromatographic isolation of isomaltose was carried out by a developing solvent, a mixture of butanol, pyridine and water with the following proportions; 3:2:1.5

A 10 ml. portion of ‘Neto’ solution was added with 1 ml. of the dextranase solution and five drops of a mixture of chloroform and toluene serving preventive measure of spoilage during the progress of enzymatic action. After standing for 24 hours at 40°C, 10 ml. of ethanol was added to the whole solution to remove the unattacked ‘Neto’ compound as precipitate. Then the filtrate obtained from the above mixture was concentrated to dryness on a water bath. The dried residue was next dissolved in 0.5 ml. water and about 25 gamma of the solution was placed on the one end of a paper strip and developed by the butanol-pyridine-water mixture.

After the development was completed, a detection specific for isomaltose was made by spraying aniline hydrogen phthalate, a mixture of 930 mg of aniline and 1.6 g of phthalic acid dissolved in 100 ml. of butanol saturated with water. The strip of paper was then heated for 5 minutes at 105°C. to develop the dark brown colored spot of isomaltose.
Results and Discussion

In Table 1, the values of intrinsic viscosity for 'Neto' samples and dextran samples are shown for the benefit of comparison. A sample of pure dextran particularly prepared for clinical purpose, a product of Meito Co., Inc., gave a value of 0.75, almost doubled the values for the sample under study. There were a slight difference in the intrinsic viscosity between the 'Neto' from commercial Kamaboko and that from the culture, in which Leuconostoc mesenteroides was inoculated in a sterilized Kamaboko medium. However, the value for the dextran, obtained from a pure culture of L. mesenteroides in a medium containing pepton, yeast extract, sucrose, K$_2$HPO$_4$, and NaCl, was found to be rather higher than those of 'Neto' sample.

Table 1. Intrinsic viscosities of pure clinical dextran, 'Neto' and the dextran prepared by pure culture of *L. mesenteroides* isolated from spoiled sugared Kamaboko.

<table>
<thead>
<tr>
<th>Variety of Sample</th>
<th>Intrinsic Viscosity [$\eta$]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Clinical dextran</td>
<td>0.75</td>
</tr>
<tr>
<td>2. 'Neto' *</td>
<td>0.33</td>
</tr>
<tr>
<td>3. Pure cultured dextran **</td>
<td>0.40</td>
</tr>
<tr>
<td>4. 'Neto' ***</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* Prepared from commercial sugared Kamaboko.
** Cultivation of *L. mesenteroides*, isolated from a spoiled sugared Kamamoko, was carried out in a medium containing pepton K$_2$HPO$_4$, yeast extract, NaCl and sucrose.
*** Cultivation of the above strain of *L. mesenteroides* was carried out in a sterilized sugared Kamaboko.

KOBAYASHI (1952) mentioned that the intrinsic viscosity of dextran gives a linear relation with its mean molecular weight, for instance, each value for 0.39, 0.48, 0.84 and 0.80 corresponds to 53,200, 78,000, 152,000 146,000 of mean molecular weight.

In Table 2, an example for no productivity of dextran was demonstrated in the medium in which yeast extract was entirely absent.

Table 2. Effect of omission of yeast extract on the acid and dextran production in sucrose medium performed by *L. mesenteroides*.

<table>
<thead>
<tr>
<th>Medium</th>
<th>PH</th>
<th>Sucrose g%</th>
<th>Reducing sugar g%</th>
<th>Dextran g%</th>
</tr>
</thead>
<tbody>
<tr>
<td>With yeast extract (Before Inoculation)</td>
<td>7.51</td>
<td>10</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>With yeast extract</td>
<td>4.88</td>
<td>0.35</td>
<td>4.45</td>
<td>2.27</td>
</tr>
<tr>
<td>Without yeast extract</td>
<td>7.44</td>
<td>8.12</td>
<td>0.83</td>
<td>0</td>
</tr>
</tbody>
</table>

The basal medium employed has the same composition as given in the remark under Table 1.
respectively. Consequently, the value, 0.75, for the clinical dextran should be equivalent to a molecular weight between 130,000 and 140,000. And similarly, the intrinsic viscosity of 0.33 found for ‘Neto’ can be interpreted as a mean molecular weight of 40,000.

The fact that the value for the dextran produced by a strain of Leuconostoc mesenteroides from spoiled Kamaboko is low might be a problem, however, it could be attributed to a characteristic of each strain of Leuconostoc. McClesky (1947) grouped Leuconostoc mesenteroides into four different types by distinction of the colonies. And chemical and physical properties of these four types, A, B, D and F, were distinguishable with each other, as reported by Jeanes (1956). Incidentally, the low level claimed for the intrinsic viscosity of ‘Neto’ from sugared Kamaboko seems to have some bearings with the type strain of Leuconostoc which dominantly develops on Kamaboko.

Dextran, once formed on sugared Kamaboko, is not likely decomposed by other kinds of microorganism which inhabit on the same stuff, because there is quite a similarity of the value of intrinsic viscosity between the ‘Neto’ from spoiled Kamaboko and dextran produced by Leuconostoc mesenteroides which has been isolated from the aforementioned sample, as shown in Table 1.

The breakdown of clinical dextran by dextranase from Penicillum funiculosum took place abruptly, immediately after addition of the enzyme to the sample. Figure 1 indicates the rapid decrease of specific viscosity happening in first ten minutes and a following slow diminution.

In case of Neto sample, the reduction in specific viscosity effected by that enzyme was observed as ranging from 1.0 to 0.4, in approximately 20 minutes standing, as seen in Figure 2. The difference in the extent of breakdown for both kinds of dextran, might be due to the possible prevalence in number of α-1, 6 glucosidic linkage of the clinical dextran over ‘Neto’ dextran.

Because dextranase is capable of hydrolyzing only α-1, 6 linkage of dextran, so, the percentage of content of the linkage other than α-1, 6-linkage would be particularly...
Fig. 2. Specific viscosity and reciprocal of specific viscosity of 'Neto' under the action of the dextranase isolated from a culture of Penicillium funiculosum.

Fig. 3. Inhibitory effect of HgCl₂ against the dextranase from Penicillium funiculosum. The upper curve indicates the slowing-down of viscosity fall with application of a HgCl₂-added dextranase.
large in the case of ‘Neto’ dextran.

However, pattern of the change in reciprocal of specific viscosity was found linear to the time of reaction for both types of dextran.

This may mean that splitting by dextranase happens at a particular position in the structure of dextran. Probably, linear relationship between the reciprocal of specific viscosity and the time of incubation will not be observed, if all the linkages involved in such a highly polymerized compound were broken at random.

It is already known that the activity of dextranase is remarkably inhibited by a few kinds of heavy metal salt, such as HgCl₂, CuSO₄ and AgNO₃. In Figure 3, HgCl₂ indicates an inhibitory effect on the dextranase solution prepared from Penicillium funiculosum at its concentration of 10⁻² M, after incubation for 30 minutes at 30°C in the enzyme solution.

By paper chromatographic isolation, presence of isomaltose was confirmed in the ‘Neto’ solution treated with dextranase. As shown in Table 3, Rf value of 0.3 obtained for the sample agreed well with that of pure isomaltose. There was found also another spot with Rf value 0.18, which is assumed as isomaltotriose because of its good conformity in Rf value, though a standard run with pure isomaltotriose was not carried out. From the finding above discussed, it will be certain that the slimy product, so-called ‘Neto’, from sugared Kamaboko chiefly consists of a kind of dextran with a comparatively small number of α-1, 6 linkage.

Table 3. Paper chromatographic identification of the end product of ‘Neto’ and pure dextran by means of a dextranase from Penicillium funiculosum.

<table>
<thead>
<tr>
<th>Sample used</th>
<th>Rf</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isomaltose</td>
<td>0.30</td>
<td>—</td>
</tr>
<tr>
<td>Dextran*</td>
<td>0.30</td>
<td>0.18</td>
</tr>
<tr>
<td>Dextran*</td>
<td>0.29</td>
<td>0.18</td>
</tr>
<tr>
<td>‘Neto’ *</td>
<td>0.29</td>
<td>0.18</td>
</tr>
</tbody>
</table>

* Clinical dextran.

Acknowledgements

The authors wish to thank Dr. Tsuneo Kobayashi of the Tokyo University for his encouragement and assistance. Also, the authors thank Prof. Yasuhiko Tsuchiya of Tohoku University for his effort in supplying us with isomaltose.

Summary

It became more certain that the ‘Neto’ product from sugared Kamaboko may be dextran by the following observations.

1. Viscosity of the ‘Neto’ solution as well as that of a clinical dextran solution decreased when subjected to hydrolysis by dextranase which was prepared from a culture of Penicillium funiculosum. And the rate of reduction in viscosity was greater for pure clinical dextran than for ‘Neto’ dextran. Therefore, the number of α-1, 6 linkage
in the structure of ‘Neto’ dextran would be smaller than that in the pure clinical dextran.

2. The activity of the dextranase from culture of *Penicillium funiculosum* was weakened by addition of mercuric chloride.

3. The intrinsic viscosity of ‘Neto’ dextran was smaller than that of pure clinical dextran. The difference would be due to the type of *Leuconostoc* strain which grows on Kamaboko product.

4. The presence of isomaltose in the hydrolysate resulted by dextranase action was ascertained by paper chromatographic identification. And, probably, isomaltooltriose could be formed as well under the same circumstance.

References

2) A. JEANES: Journal of Bacteriology, 64, 513-519, (1952).