HOKKAIDO UNIVERSITY

Title	BACTERIOLOGICAL STUDY OF SHIOKARA OR "SOUSED SQUID" GENERAL DISCUSSION
Author(s)	NAGAO, Kiyoshi
Citation	北海道大學水産學部研究彙報, 7(2), 113-118
Issue Date	1956-08
Doc URL	http://hdl.handle.net/2115/22956
Type	bulletin (article)
File Information	7(2)_P113-118.pdf

Instructions for use

Hokkaido University Collection of Scholarly and Academic Papers : HUSCAP

BACTERIOLOGICAL STUDY OF SHIOKARA OR "SOUSED SQUID" GENERAL DISCUSSION*

LATE Kiyoshi NAGAO
Faculty of Fisheries, Hokkaido University

The ripening of Shiokara has hitherto been ascribed to enzymatic and bacterial action. Some studies have been reported on the chemical changes during the ripening process of Shiokara, and on the presence of certain autolytic enzymes which are concerned in it, but no investigation has ever been made on the ripening of Shiokara from the view point of bacterial metabolism.

The manifold chemical activities of bacteria are catalysed by enzyme formed within the bacterial cells. Some bacterial species can exist under widely different chemical and physical environments; they require different types of enzymes in order to deal with the different external conditions, and they can maintain themselves in dynamic equilibrium with their environment. Shiokara contains much sodium chloride in solution.

In such an environment, bacteria require different types of enzymes in order to deal with such external conditions.

In this paper, the author wishes to throw light on the phenomena of ripening of Shiokara by studying it from the view point of bacterial metabolism.

1. In the early stage, the ripening of Shiokara was found to be mainly due to the autolyzing enzymes. The ripening by bacterial action was found to have begun after 20 days fermentation.

2. The Shiokara was extracted with water and then the extracted substance was separated into several kinds of nitrogen fraction. Each fraction of nitrogen was measured. The percentage of the nitrogen in the combined forms to the nitrogen in the extracted material was determined. Also the percentage of the different forms of nitrogen to the non-protein nitrogen was determined. The experimental results were as follows:

1) The percentage of the non-protein nitrogen to the total nitrogen in the extracted materials was, 55.8 per cent at the first day, 68.5 per cent at 9 days old. From 9 to 20 days later this percentage was almost constant. After 20 days of fermentation this percentage was increased and at 33 days later amounted to 79.3 per cent.

2) The percentage of amino-nitrogen in monoamino fraction to the total nitrogen in monoamino fraction was, 34.8 per cent at the first day. From 0 to 12 days later this percentage was increased logarithmically. At 33 days later this percentage amounted to 68.1 per cent. It is considered that monoamino acid is the primary chemical compoment related to the taste of Shiokara.

* The manuscripts left by the late Mr. K. Nagao are published in the form of this paper after arrangement by Mr. T. Kimura and revision by Dr. E. Tanikawa. (Editorial staff)

3. The phenomenon of ripening of Shiokara under several conditions was observed by determining the amount of amino-nitrogen and free amino acids. Experimental conditions were as follows:

Sample 1. Filled with H_2 an air-tight vessel, which had been half filled with Shiokara.

Sample 2. Filled with H_2 another air-tight vessel, which had been half filled with Shiokara, mixed with toluene.

Sample 3. Blow air into Shiokara, half filled in an open vessel.

Sample 4. Mixed toluene in Shiokara, which half filled a covered vessel.

Sample 5. Kept in natural condition in an open vessel (control sample).

From 0 to about 10 days later the amount of amino-nitrogen increased rapidly in every sample. This was mainly due to the action of autolyzing enzymes. After 20 days of fermentation the amount of amino-nitrogen increased again in Sample 3. This was mainly due to the action of bacterial enzymes.

In the early stage, free amino acids such as aspartic acid, glutamic acid, glycine, alanine, valine, leucine, phenylalanine, tyrosine, arginine, lysine, histidine, proline, serine, threonine and ornithine were detected in Shiokara by means of paper chromatographic method. Free tyrosine, arginine and lysine in Samples 1 and 2 disappear during ripening process. Free histidine in Samples 3, 4 and 5 disappear during its ripening process. Oxyproline was detected in every sample at the later stage of ripening.

4. Many halophilic or halotolerant bacteria were isolated aerobically from Shiokara. Eight species were identified as follows:

Bacillus subtilis, *Bacillus subtilis* sp. (*Bac. mesentericus fuscus*), *Bacillus cereus* var. *mycoides*, *Micrococcus flavus*, *Micrococcus perflavus*, *Micrococcus freudenreichii*, *Micrococcus subflavescens*, *Lactobacillus* sp.

Two species of facultative anaerobes were identified but none of strict anaerobe was isolated.

5. A study was made of the difference in the numbers of bacteria found in Shiokara which was manufactured by using hot air sterilized salt and non-sterilized salt. The results obtained were as follows:

1) The maximum number of colonies on the plate agar-agar containing 5, 10, 15 and 20 per cent of sodium chloride respectively at 35° and 25° was obtained after 3 days. This is supposed to be due to the use of vessel of 70% moisture in which 33% sulphuric acid was put, for the cultivation of bacteria in Shiokara.

2) The results of the comparison of use of hot air sterilized salt and non sterilized salt for the ripening of Shiokara, show that the number of bacilli present in raw material influenced the growth of bacilli in the Shiokara, although the initial growth of bacilli was slowed by the addition of the salt.

6. From the observations on the relation between ripening and the number of bacilli, the ripening by bacterial action was found to have taken place after 20 days of fermentation.

7. He prepared a proteolytic enzyme of *Bac. subtilis* which was isolated from Shiokara, by using either lysis, lyophilization, or treatment with acetone and ether. The enzyme showed optimal activity at pH 6.8 to 7.2 and temperature of 40°. It is of interest that the enzyme was not much affected by the presence of a high concentration of sodium chloride. The proteolytic activity of an enzyme prepared by acetone-ether treatment was nearly the same as that prepared by lyophilization, whereas that of an enzyme prepared by lysis was lower than the others.

8. A proteolytic enzyme of *Bac. subtilis* was prepared by treatment with acetone and ether.

1) This enzyme was activated by Mn^{++}, Co^{++} and Zn^{++}. It was not activated by Mg^{++}.

2) When Mn^{++}, Co^{++} or Zn^{++} was added at the start of hydrolysis, a definite lag phase was observed before the first order reaction took place. When the enzyme was treated, however, with Mn^{++}, Co^{++} or Zn^{++} at 37° for three hours before the substrate was added, the hydrolysis took place according to the first order reaction without any lag phase. The metal does not act simply as an activator of the enzyme but appears to be chemically combined with the protein. As to why a metal ion requires time to react with the protein, the author would suggest that in the enzyme-substrate complex the metal ion is linked to the polar groups of the enzyme on the one hand, and to polar groups of the peptide on the other. In this manner a chelate ring may be formed in which the electronic structure of the peptide bond is loosened to such an extent that hydrolysis takes place.

3) The Co^{++} enzyme was more active in the presence of phosphate buffer than veronal buffer.

4) The hydrolysis rate was proportional to the enzyme concentration.

5) Ca^{++} ion acts as an inhibitor of this enzyme in the absence of phosphate, but this inhibition does not occur with phosphate buffer apparently because of the removal of calcium.

6) In the case of cysteine, on the contrary, a higher activity was obtained when it was mixed simultaneously with the enzyme and substrate.

7) This enzyme was also activated by ascorbic acid and reductic acid.

9. An attempt was made to determine the mechanism of enzymatic protein breakdown. The proteolysis was compared to an explosive disintegration whereby the large molecule is split into many small products without an accumulation of intermediates. Such "all or none" splitting was observed when gelatin was exposed to the action of this enzyme.

10. Protein maintains dynamic equilibrium between decomposition and resynthesis *in vivo*. Accordingly, amino-acid metabolism is not characterized by the fact that it is

decomposed to carbon dioxide and water as are carbohydrate and fat. But it is characterized by the fact that the degradation of amino acid may lead to the resynthesis of the same or different type of protein constituent or other biological material being both quantitative and qualitative equilibrium in and out of bacterial cells.

Some bacteria use even amino acid as their energy source. The free energy liberated by an oxidation of L-amino acids transferred to another enzyme reaction, transamination, which requires free energy.

For life to exist, it is an absolute essential that some mechanism should be present for trapping some of the free energy of reaction, for storing it and transferring it so that it can be used for various vital processes.

The role of L-amino acid oxidase in metabolism may be characterized as not that of a simple ammonia liberation, but that of a conversion to the corresponding keto acid, which is then transformed to the L-amino acid by transamination. Thus a micro-organism maintains equilibrium both quantitative and qualitative in and out of cells.

11. The formation of an enzyme within a cell was conditioned by the chemical constitution of the medium, by the physicochemical conditions holding during growth and by the age of the culture.

1) The changes in the external pH during growth were followed by alteration in glutamic dehydrogenase content of the cells, but the pH optimum (pH 7.4) does not vary with the pH of medium during growth.

2) The variation of potential activity and effective activity of glutamic dehydrogenase in relation to changes in pH of medium during growth were studied. The potential activity was roughly constant whatever the pH in the medium. The effective activity was greatly affected by the pH of medium during growth. The potential activity is the activity estimated at the optimal pH of the enzyme and it represents the total formation of enzyme within the cell: the effective activity is the activity estimated at the pH of the environment in which the cell was grown.

3) The presence of sodium chloride in the substrate solution inhibits the activity of glutamic dehydrogenase.

4) The glutamic dehydrogenase was formed to a greater extent when growth occurs at 37° then when at 23°, 30° and 42°.

5) The glutamic dehydrogenase was formed during growth, reaching maximum at about the time of cessation of cell division. After the end of growth the activity declined.

12. Washed suspensions of freshly harvested cells of *Bac. subtilis*, *Bac. subtilis* sp., and *Esch. coli* oxidized common naturally occurring amino acids of L-series. But the rate at which different amino acids are oxidized by these bacterial cell suspensions shows considerable variations, and some amino acids are not all attacked. Aspartic acid, glutamic acid and alanine are oxidized more rapidly than most of the other amino acids.

13. Any of the enzymes which oxidize L-amino acids will be characterizd either on

the basis of their origin or on the basis of some particular substrate which they oxidized readily. The author does not have adequate data to decide precisely how many amino acid oxidases are concerned altogether in bacterial suspension. He undertook to clarify the substrate specificity by comparison of the effects of various inhibitors on the activity of L-amino acid oxidases and of the stability of these enzymes. The experimetal results were as follows:

1) Auto-respiration was promoted by 10^{-3}~10^{-5}M 2,4-dinitrophenol, but the activity of L-amino acid oxidases was inhibited by 10^{-3}M 2,4-dinitrophenol.

These enzymes were strongly inhibited by 10^{-2}M azide.

The auto-respiration and the activity of L-amino acid oxidases were strongly inhibited by 10^{-4}M crystal violet, 10^{-2}~10^{-4}M sodium dehydroacetate, 10^{-2}~10^{-4}M methylene blue and 10^{-3}M KCN.

Caprylic alcohol, even in minute traces in which it dissolves in water, completely inhibited the activity of the enzymes.

10^{-2}M Benzoic acid completely inhibits the activity of the enzymes.

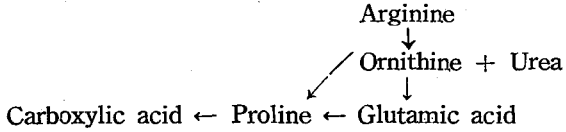
These experimental results are affected by the reaction of multi-enzyme systems, and therefore it was not possible to clarify the substrate specificity of these enzymes.

2) The presence of sodium chloride in the substrate solution inhibits the activity of these enzymes.

3) However, as the age of the bacterial suspension increased, the number of amino acids attacked dwindled. From this experimental result, it is assumed that several enzymes are involved in the oxidation of amino acids by fresh suspensions of bacteria.

14. The intact cells of *Bac. subtilis* were able to accumulate arginine, in the free form within the cells, and the concentration within the cells was higher than that existing in the external medium at equilibrium. The concentration of free arginine within the cells was determined by the balance between the rate at which the amino acid entered the cell and the rate at which it was being metabolized within the cell. A considerable portion of arginine that entered the cell appeared to be converted into cellular matter in subsequent reactions, a dynamic equilibrium existing between uptake and utilization.

The intact cells of *Bac. subtilis* yielded evidence consistent with the idea that the following metabolic pathway was involved.



Citrulline was not found as an intermediate and was not attacked and therefore was ruled out as a possible intermediate.

15. The occurrence and general importance of transaminases in the formation of amino acids has been variously discussed. The advent of the paper chromatographic method

has afforded an opportunity to clarify knowledge on the occurrence of transaminases and their possible relationship to amino acid formation. Studies on a series of transaminases present in *Bac. subtilis* were carried on as follows:

1) The enzyme was prepared by treatment of intact cells with lysozyme and then dialyzed for 20 hrs. at 0° to get a material free from free amino acid.

2) Glutamic-oxalacetic transaminases have been demonstrated which produce glutamic acid from α-ketoglutamic acid with the following amino donors: aspartic acid, alanine, tyrosine, methionine, phenylalanine, valine and histidine, and, to a lesser degree, with lysine, arginine and leucine.

3) Glutamic-pyruvic transaminases have been demonstrated which produce alanine from pyruvic acid with the following amino donors: aspartic acid and glutamic acid.

16. The author would focus attention on one aspect of the problem of enzyme formation by summarizing his present knowledge on the question of the precursor which is converted into active enzyme. Many possibilities can be entertained as to the origin and the nature of the precursor material as well as to the number and the extent of the reactions to transform it into junctional enzyme.

Experimental results obtained by the present writer may be summarized as follows:

Washed suspensions of freshly harvested cells of bacteria oxidized common naturally occurring L-amino acids. But the rate at which different amino acids are oxidized by these bacterial cell suspensions shows corsiderable variatiors. Aspartic acid, glutamic acid and alanine are oxidized more rapidly than most of the other amino acids.

The enzyme formation is higher in velocity and larger in amount when oxidizable amino acids are incubated with washed cell suspensions than when non-oxidizable amino acids are incubated with them.

Oxidizable amino acids are oxidized easily to the corresponding keto acid, and then the keto acid is transformed to the L-amino acid by transamination. Thus a large proportion of amino acid synthesized in endogenous reaction is incorporated into protein.

17. Adaptation involves a relation between the organism, the enzyme, and the substrate, but sometimes substances other than the specific substrate may play a part in the formation or activity of an enzyme. The most striking example of this is found when fermentable carbohydrate is added to the medium. No satisfactory explanation of this effect has yet been put forward. The inhibitory action of the presence of glucose during growth on the formation of L-amino acid oxidases could not be attributed to the production of acid and consequent alternation of the medium pH. It can be explained by the action or the behavior of the prosthetic group or the coenzyme of these enzymes.

18. When two different amino acids were added to the cell suspensions of *Bac. subtilis* simultaneously, no competitive interaction was observed between those two acids for the formation of an enzyme corresponding to each amino acid.

There are enzyme systems which are transformed mutually and those which can not be transformed.