Beta-Galactosidase Activity of the Cell-free Extract of Psychrotrophic Bacteria in Frozen Aqueous Solution Part II

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An alternative reaction procedure was devised for the confirmation of the previous finding that the cell-free extract of psychrotrophic bacteria showed β-galactosidase activity in a really frozen aqueous solution. Various freezing conditions suitable for the enzymic reaction were examined and though the state was not easy to be reproduced the reaction was noticed to proceed in a transparently frozen state.

Introduction

The finding reported in the previous paper[1] that the cell-free extract of psychrotrophic bacteria showed β-galactosidase activity in a frozen aqueous solution aroused some intractable questions. First, whether the reaction occurred in a really frozen aqueous solution or in a super-cooled solution just before freezing must be clarified since a compulsory freezing procedure practiced in the previous study could not exclude two minutes time-lag before freezing. Secondly, there are a variety of states of frozen water[2] and therefore which of them allowed the enzymic reaction should be defined. For the third, can enzymic reaction occur in a really frozen aqueous solution if experimentally any super-cooled and non-frozen zone is excluded from the reaction solution?

Furthermore, various questions such as Tyndall figures[3] or liquid-like layer on ice[4] as mentioned in the part of Discussion must be explained.

In order to clarify these questions an alternative reaction procedure was invented and after several experiments as described in the present paper some of these questions were experimentally answered.

Materials and Methods

The strain of bacteria used A Gram negative rod designated tentatively as P-15 was used throughout the present study. It produces constitutively an intra-cellular β-galactosidase.

The procedure of preparation of the bacterial cells and their cell-free extract The bacteria were sub-cultured for 4 days at 5°C and cultured for harvest for 4 more days at 5°C,

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both in the medium composed of 0.3% beef extract (Difco), 0.1% peptone (Nissui) and 0.025% NaCl with the pH of 7.0. The cells were collected by centrifugation of 10,000 rpm for 15 minutes and suspended in distilled water.

Fifty ml of cell suspension of DCW concentration of 14.8 mg/ml were ultrasonically disrupted by applying 4 times of 2.5 minutes oscillation of 20 KHz, 185 W below 10°C with 5 minutes interval between each oscillation. Cell-free extract was obtained by 15 minutes centrifugation of 5,000 rpm at 5°C. The crude protein concentration of the cell-free extract determined by Lowry method[5] was 6.3 mg/ml.

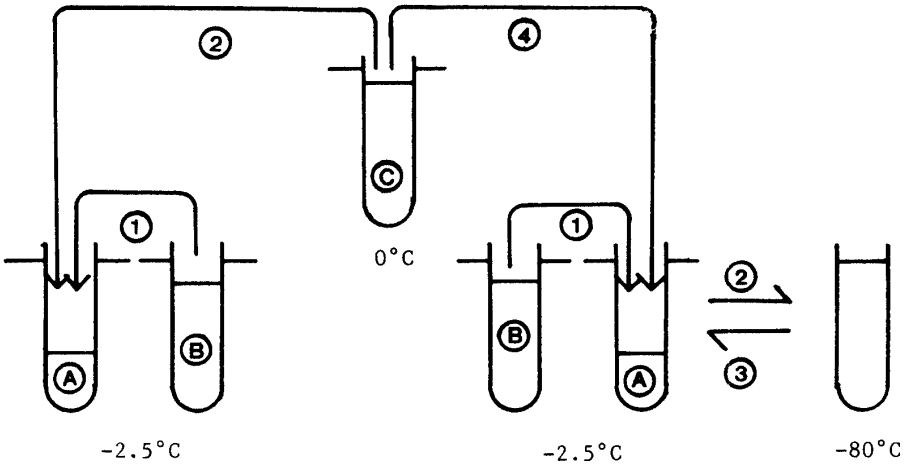
The procedure of the enzymic reaction In the experiments described in the previous paper[1] the reaction was carried out as follows. The mixture of 1.0 ml of 0.25 M phosphate buffer of pH 7.0 and 1.0 ml of 20 mM 2-nitrophenyl-β -D-galactopyranoside (abbreviated as ONPG) contained in a test tube was pre-cooled at minus 2.5°C for 15 minutes. The enzymic reaction was started by pouring this mixture into another pre-cooled test tube containing 0.5 ml of the cell-free extract having 1.5 mg of the crude protein and the test tube was rapidly transferred into a deep-freezer of minus 80°C. After 5 minutes to enforce freezing of the mixture, the test tube was taken back to the cool water bath of the temperatures designed. (Fig. 1)

In the present experiments, an alternative reaction procedure as following was adopted. The mixture of 1.0 ml of 0.25 M phosphate buffer of pH 7.0 and 1.0 ml of 20 mM ONPG was pre-cooled at minus 2.5°C or minus 5°C for 15 minutes. The enzymic reaction was started by pouring this mixture into another pre-cooled test tube containing 0.5 ml of the cell-free extract and either simultaneously or after some time incubation a small pre-cooled glass tube was inserted into the mixture to accelerate freezing with or without vigorous shaking. Then the test tube was taken back to the cool water bath of the temperatures designed. (Fig. 2)

The reaction was terminated by pouring 7.5 ml of ice-cold 0.1 M Na_2CO_3 into the frozen aqueous mixture. By pouring running tap water carefully around the test tube, the frozen mixture was thawed within 3 minutes. The rise of temperature during the thawing did not appreciably affect the amount of O-nitrophenol formed as to disturb the experiments. The amount of O-nitrophenol was determined by the absorbance at 420 nm in the same way as described in the previous paper[1].

Results

Enzymic reaction in a solution rapidly frozen at minus 5°C In the previous paper[1], the

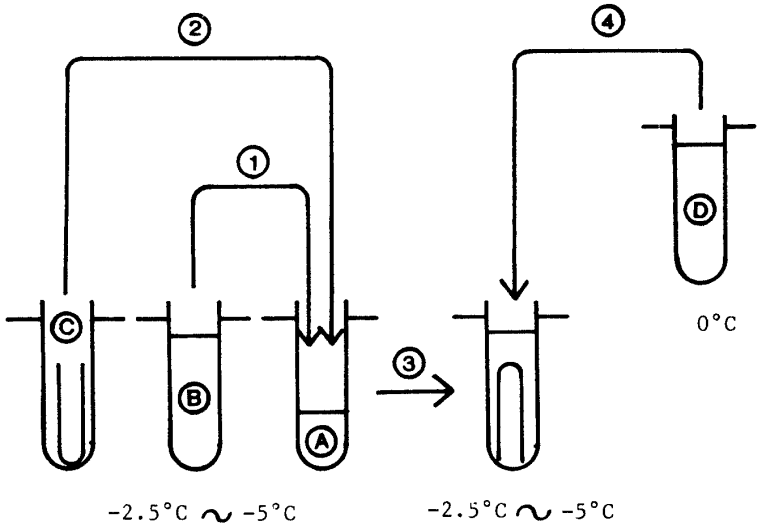


Ⓐ Cell-free extract

Ⓑ Buffer and ONPG

Ⓒ 0.1 M Na_2CO_3

Fig. 1. Enzymic reaction procedure A



Ⓐ Cell-free extract

Ⓑ Buffer and ONPG

Ⓒ A small glass tube

Ⓓ 0.1 M Na_2CO_3

Fig. 2. Enzymic reaction procedure B

super-cooled cell-free extract was shown to exhibit β-galactosidase activity at minus 5°C almost proportionally to time within 5 hours. In parallel with this experiment, the super-cooled reaction mixture was brought to a sudden freezing by inserting a pre-cooled small glass tube into the test tube from 0 to 5 hours. (Fig. 3) Though the amounts of O-nitrophenol formed varied rather accidentally, from the comparison between the previous experiment and the present one, the reaction mixtures frozen at the early stage seem to confirm the enzymic reaction in a frozen state. However, appearance of yellow color of O-nitro-

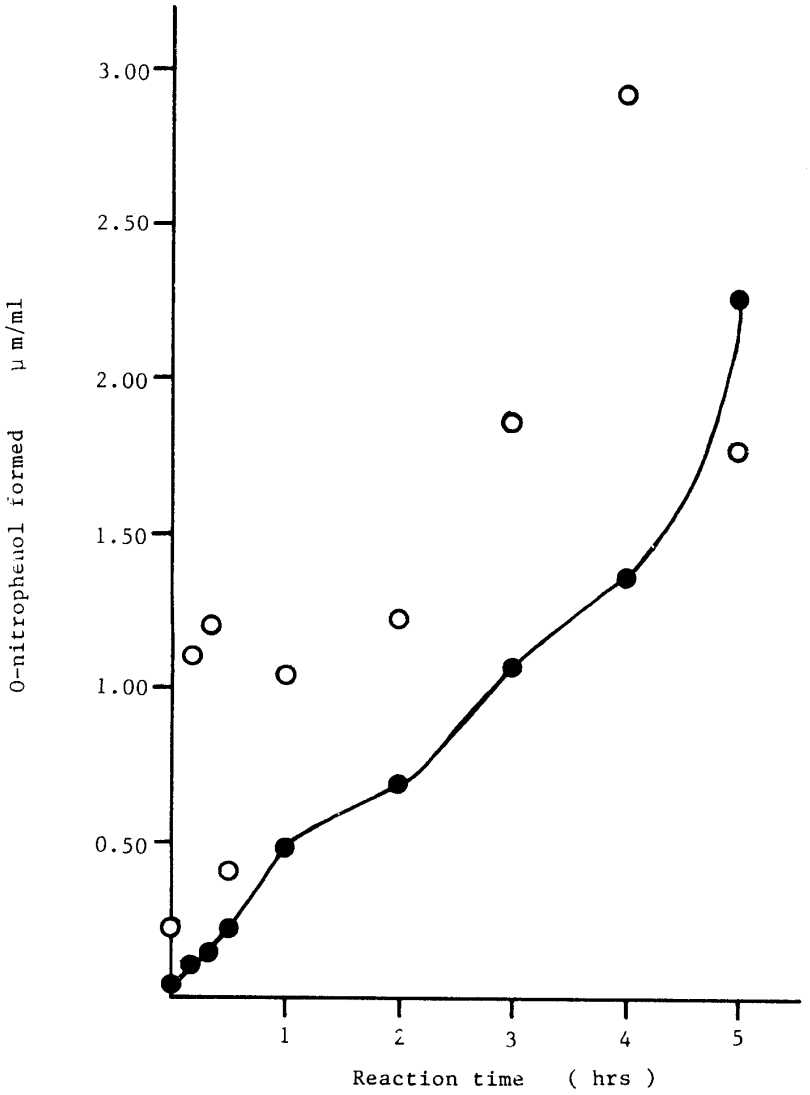


Fig. 3. O-nitrophenol formed with the cell-free extract at minus 5°C

● without freezing (the previous experiment)

○ The reaction mixture was kept at minus 5°C without freezing for the time indicated and then was instantaneously frozen by inserting a small glass tube and kept at minus 5°C until 5 hours lapsed.

phenol at the surface of the frozen reaction mixture observed around 2 hours keeps this confirmation still from the final.

Enzymic reaction in a solution rapidly frozen at minus 2.5°C and so maintained or transferred to minus 5°C After several experiments undisclosed here, the enzymic reaction seemed to be influenced by the frozen state of the reaction mixture. It proceeded in a transparently frozen state but least in an opaquely frozen state. Until now, however, the attempts to prepare reproducibly a transparently frozen state were all in vain. The results of the experiment in which a frozen mixture was maintained at minus 2.5°C or transferred to minus 5°C after freezing and standing for an hour at minus 2.5°C are shown in Table 1 and Table 2. Though the reaction fluctuated, a large amount of O-nitrophenol was found to be formed both at minus 2.5°C and at minus 5°C in a completely frozen state. The color development was observed evenly in the whole reaction mixture excluding suspicion of surface reaction.

Enzymic reaction in a solution slowly frozen at minus 2.5°C and transferred to minus 5°C In the experiments shown in Table 1 and Table 2, the reaction mixture was rapidly frozen at minus 2.5°C in the beginning of the reaction. In the following experiments summarized in Table 3 and Table 4, freezing was performed slowly at minus 2.5°C for 20 to 30 minutes and thereafter at minus 5°C. The results are compared with those frozen rapidly by vigorous shaking. In the reaction mixtures rapidly frozen no more than less amounts of O-nitrophenol were produced. Among the reaction mixtures slowly frozen, only in one case, an appreciable amount of O-nitrophenol was noticed to be formed.

Discussion

By introducing a revised procedure and examining the condition of freezing, the author succeeded to follow the proceeding of the enzymic reaction in a really frozen state.(Table 1 and Table 2) The enzymic reaction proceeded in a transparently frozen state but not in an opaquely frozen state. Nakaya mentioned about the two states of ice[6]. The one is a cluster of fine crystals and the other an ordinary ice. The former is opaque and the latter is transparent. Kanno described eight different states of ice[2]. At present, the author can not define the frozen state in which the enzymic reaction proceeds. The color development of O-nitrophenol occurred evenly in the whole frozen mixture as recognized in the process of thawing. Therefore, the possibility of light-induced dissolution inside the ice i.e. Tyndall figure phenomenon[3] can be denied.

Likewise, the attribution to liquid-like (transition) layer on ice[4] for the enzymic reaction is inappropriate because color development was not limited to the thin layer.

Table 1 O-nitrophenol formed in a solution rapidly frozen at minus 2.5°C and so maintained

Cell-free extract (crude protein mg/ml)	Time of complete freezing at -2.5°C	Reaction time and State -2.5°C	O-nitrophenol formed μm/ml
0.31	3 min	30 min (F)*	0.04
	3	1 hrs (F)	0.12
	3	2 (F)	0.07
	3	48 (F)	0.66
	3	48 (F)	3.82

Reaction proceeding:
Pre-cool -2.5°C (10 min), Mix and insert a small glass tube -2.5°C, Standing -2.5°C (30 min to 48 hrs)

* F stands for a frozen state.

Table 2 O-nitrophenol formed in a solution rapidly frozen at minus 2.5°C and transferred to minus 5°C

Cell-free extract (crude protein mg/ml)	Time of complete freezing at -2.5°C	Reaction time and State -2.5°C -5°C	O-nitrophenol formed μ m/ml
0.31	3 min	30 min(F)	0.05
	3	1 hr (F)	0.07
	3	1 (F) 1 hrs(F)	0.07
	3	1 (F) 2 (F)	0.09
	3	1 (F) 47 (F)	0.18
	3	1 (F) 47 (F)	3.02

Reaction proceeding:
Pre-cool -2.5°C (10 min), Mix and insert a small glass tube -2.5°C, Standing -2.5°C (30 min to 1 hr), Transferred to -5°C (1 hr to 47 hrs)

Table 3 O-nitrophenol formed in a solution slowly frozen at minus 2.5°C and transferred to minus 5°C after 20 min

Insertion of a small tube	Time of partial freezing	Time of complete freezing	Reaction time and State -2.5°C		-5°C		O-nitrophenol formed μm/ml
without shaking	-	-	20 min	(NF)*	48 hrs	(NF)	2.88
	-	37 min	20	(NF)	48	(F)	1.37
	-	20 min >	20	(F)	48	(F)	0.24
	5 sec	20 min >	20	(F)	48	(F)	0.42
	5	20 min >	20	(F)	48	(F)	0.23
with shaking	5 sec	3 min	20 min	(F)	48 hrs	(F)	0.22
	5	3	20	(F)	48	(F)	0.24
	5	3	20	(F)	48	(F)	0.32

Reaction proceeding:
Pre-cool -2.5°C (10 min), Mix -2.5°C, Standing -2.5°C (10 min), Insert a small glass tube -2.5°C, Standing -2.5°C (10 min),Transferred to -5°C (48 hrs)
Cell-free extract crude protein 0.31 mg/ml

* NF stands for a non-frozen state.

Table 4 O-nitrophenol formed in a solution slowly frozen at minus 2.5°C and transferred to minus 5°C after 30 min

Insertion of a small tube	Time of partial freezing	Time of complete freezing	Reaction time and State -2.5°C		-5°C		O-nitrophenol formed μm/ml
without shaking	-	-	30 min	(NF)	48 hrs	(NF)	4.68
	-	30 min >	30	(F)	48	(F)	0.24
	5 sec	30 min >	30	(F)	48	(F)	0.23
	5	30 min >	30	(F)	48	(F)	0.28
	5	30 min >	30	(F)	48	(F)	0.25
with shaking	5 sec	3 min	30 min	(F)	48 hrs	(F)	0.26
	5	3	30	(F)	48	(F)	0.25
	5	3	30	(F)	48	(F)	0.25
	5	3	30	(F)	48	(F)	0.25

Reaction proceeding:
Pre-cool -2.5°C (10 min), Mix -2.5°C, Standing -2.5°C (10 min), Insert a small glass tube -2.5°C, Standing -2.5°C (20 min),Transferred to -5°C (48 hrs)

Cell-free extract crude protein 0.3i mg/ml

Finally, a question remains whether chemical or enzymic reaction can occur in a really frozen solution. Theoretically the molecules can move but the range for their movement must be very limited, particularly in a crystal lattice. Then, how broad is this range in a well mixed frozen solution ? Evidences of chemical reactions inside crystals reported by Toda[7] and formation of oxide fibers by unidirectional freezing of gel reported by Maki, Kokubo and Sakka[8] are very encouraging for the concept of enzymic reaction in a frozen aqueous solution reported here.

The result of the present study was partially presented at the annual meeting of Hokuriku Branch of The Japanese Biochemical Society on May 22, 1993.

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