Beta-Galactosidase Activity of Psychrophic Bacteria at the Sub-zero Degree Temperature

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For the confirmation of β-galactosidase activity of psychrotrophic bacteria at the sub-zero degree temperature, the reaction procedure was improved so that it could ensure the enzymic reaction to start and stop according to the program. In this revised procedure, ethylene glycol, glycerol, D-sorbitol and D-mannitol were found more or less to accelerate the enzymic reaction at 5°C. Beta-galactosidase activity was detected also at the temperatures of plus 2.5°C, 0°C and minus 2.5°C. In the latter case (minus 2.5°C), β-galactosidase activity was shown not only in the presence of anti-freezing agent, glycerol, but also in its absence, therefore, in a frozen condition.

Introduction

Psychrophilic and psychrotrophic microorganisms attract much interest both scientifically and technologically despite of the hither-to scanty number of reports about them.[1,2] Particularly, the behavior of their enzymes which work at the sub-zero degree temperature remains as the target for discussion. Almost none of these reports describe the data of enzymic activity at the sub-zero degree temperature[2,3,4] and only a few mention about it, though assayed in a super-cooled liquid solution or in the presence of anti-freezing agent.[1,5,6]

The present authors reported about the β-galactosidase activity of psychrotrophic bacteria which worked in so wide a range of temperature as from minus 7.8°C to plus 60°C.[7] However, the activity detected at the sub-zero degree temperature needed further confirmation owing to the insufficient procedure. The present study deals with the improved procedure of enzymic reaction in which β-galactosidase activity was unequivocally shown at the sub-zero degree temperature both in the presence of glycerol and in its absence, consequently, in a frozen state.

Materials and Methods

The strain of bacteria used for the experiment A Gram negative rod designated tentatively as P 15 was used throughout the present study. This strain was obtained from dairy product by incubating it at 5°C.[7]

The procedure of preparation of the bacterial cells used as the enzyme The bacteria were sub-cultured with shaking of 130 rpm for 2 days at 30°C in a 30 ml medium dispensed in a 300 ml flask which was composed of 0.3 % beef extract (Difco), 0.1 % peptone (Nissui) and 0.025 % NaCl with the pH of 7.0. They were transferred into the 300 ml harvestmedium contained in a 500 ml flask of the same composition as that of the sub-culture and cultured with the similar shaking for 4 days at 30°C. The cells were collected by centrifugation of 10,000 rpm for 15 minutes and suspended in 10 ml of distilled water. The cell concentration was 73 mg dry weight per ml of water. The dry cell weight was determined by drying the collected cells for 6 hours at 110°C.

The improved procedure of the enzymic reaction For the assay of β -galactosidase activity, the reaction mixture was composed of 1.0 ml of 0.25 M phosphate buffer of pH 7.0, unless otherwise specified, 1.0 ml of 20 mM 2-nitrophenyl- β -D-galactopyranoside (abbreviated as ONPG) in distilled water and 0.5 ml of cell suspension containing 36.5 mg cells, making up the total volume of 2.5 ml. For the assay of the enzymic activity at the sub-zero degree temperature, both phosphate buffer and ONPG solution were so prepared as to contain 20 % (V/V) of glycerol. The phosphate buffer and the ONPG solution were dispensed together in a test tube of the inner diameter of 18 mm and of the height of 180 mm and pre-incubated for 15 minutes at the temperature designed in a water bath containing a non-freezing liquid Nybrine (Nissoh Maruzen Chemical). The cell suspension was dispensed in a Eppendorf tube of 1.5 ml capacity whose lid was taken off, inserted into the test tube described above and pre-incubated for the same period side by side with the other components. The enzymic reaction started with the addition of the cell suspension into the mixture of the other components by vigorous shaking in a vertical direction. During the enzymic reaction a mild shaking in a horizontal direction (70 times reciprocal shaking per minute)continued.

The enzymic reaction was stopped in a way chosen as adequate and appropriate from the result described later. This procedure was as follows. Either the reaction mixture was instantaneously poured into 7.5 ml of 0.1 M Na_2CO_3 solution dispensed in another test tube pre-cooled in an ice water or just the opposite way, namely, the Na_2CO_3 solution into the reaction mixture.

The assay of the amount of O-nitrophenol After the addition of the Na_2CO_3 solution and thawing the mixture when necessary, within 5 minutes around 0°C, the 10 ml mixture was centrifuged (5,000 rpm, 10 minutes) and the supernatant was assayed for the amount of O-nitrophenol by its absorbance at 420 nm.

The determination of the amount of O- nitrophenol The amount of O-nitrophenol formed in the reaction mixture was calculated according to the following equation in which 0.782 is the experimentally determined mili-molar absorbance at 420 nm of O-nitrophenol.

$$\mu \text{ moles / ml of the reaction mixture} = \frac{OD_{420}}{0.782}$$

The chemical reagents used Twelve compounds tested as the additives to the enzymic reaction mixture were purchased as follows. Ethylene glycol, glycerol, sodium L-glutamate monohydrate, L-alanine, glucose, sucrose, galactose and sodium chloride were the special grade products, D-sorbitol and D-mannitol were the first grade and olive oil was a practical grade of Wako Pure Chemical Industries, Ltd.. L-lysine was the extra pure grade product of Tokyo Kasei Kogyo Co., Ltd..

Results

Choice of a method to stop the enzymic reaction at arbitrary times It is a very annoying problem how to stop the enzymic reaction which might have been carried out at the sub-zero degree temperature. For one thing, it must be avoided to promote any significant degree of the enzymic reaction by the shift of temperature during the termination procedure. With that consideration in mind, two procedures were examined. In one procedure, the 2.5 ml reaction mixture were poured instantaneously into 7.5 ml of 0.1 M Na_2CO_3 solution kept at the room temperature 22°C. In the other procedure, the reaction mixture was poured into a similar Na_2CO_3 solution which was pre-heated at 90°C.

As shown in Fig.1, the enzymic reaction conducted at 30°C seemed to have been stopped elegantly by the former procedure while in the latter procedure, color development was so drastically enhanced by the hot Na_2CO_3 solution as to disappoint its adoption.

In the following, three more procedures were examined (Fig.2). In these procedures, 2.5 ml of the reaction mixture were poured instantaneously into 7.5 ml of pre-heated distilled water (pH 7.2) of 83°C, or into 7.5 ml of 40 % urea solution (pH 7.5) kept at the room temperature 22°C or into 7.5 ml of 0.1 M Na_2CO_3 (pH 10.5) pre-cooled in ice water, respectively. Among these three procedures and the one in the previous examination, 0.1 M Na_2CO_3 solution pre-cooled in ice water was chosen as the terminating agent in the following experiments because this procedure was simpler than others and the color development was much clear and distinct.

Effect of various compounds on the β-galactosidase reaction It seems highly probable that

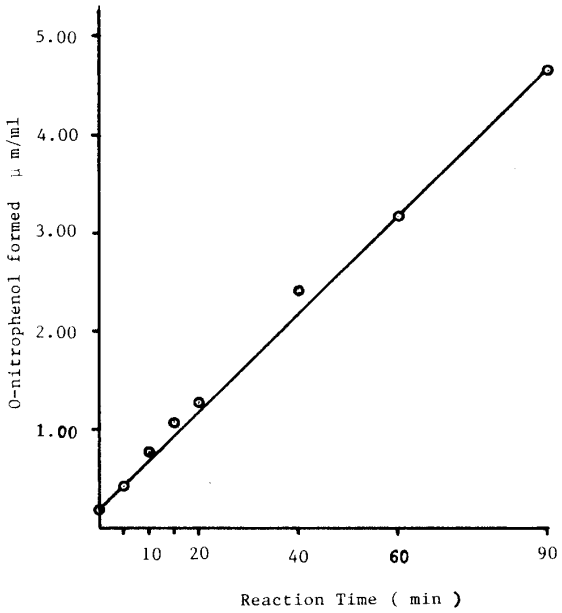

Fig.1. O-nitrophenol formed by the reaction at 30°C

Termination by 0.1 M Na_2CO_3(22°C)

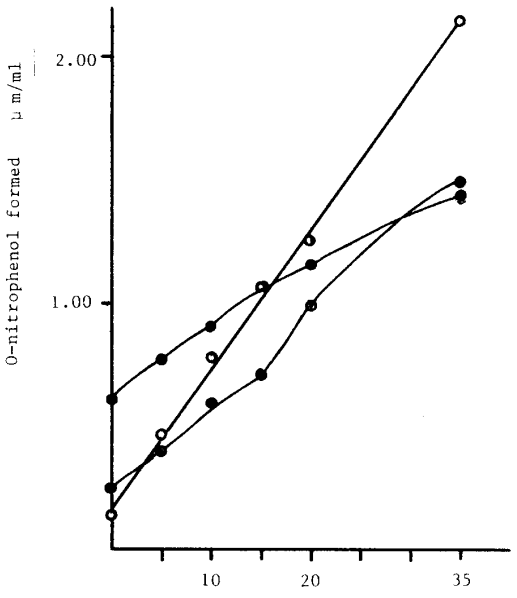

Fig.2. O-nitrophenol formed by the reaction at 30°C

Termination by hot water (83°C) (⊜)
40 % urea (●)
cold 0.1 M Na_2CO_3 (o)

the enzymes having a potential activity at very low temperatures might be influenced by the presence of some protective compounds.[8,9] Twelve compounds were tested tentatively in one chosen condition at two temperatures. As shown in Table 1, ethylene glycol, glycerol, D-sorbitol and D-mannitol, each in the concentration of 5 %, were found more or less to accelerate the enzymic reaction at 5°C but no such acceleration was observed at minus 5°C. In the latter case (minus 5°C), all components of the reaction mixture, namely, 1.0 ml of 0.25 M phosphate buffer, 1.0 ml of 20 mM ONPG solution as well as 0.5 ml of cell suspension were so prepared as to contain 20 % (V/V) of glycerol. Twelve compounds were dissolved in the phosphate buffers in the concentration of 12.5 %. The suppressive effect of L-lysine can not be properly appreciated because the pH of the reaction mixture widely deviated.

Beta-galactosidase activity in the vicinity of 0°C The time courses of the enzymic reaction were pursued at these two temperatures and a sharp difference of the enzymic behavior was noticed between above and beneath 0°C (Fig.3).

Disappointment in the attempt to find some protective effect of the compounds at the sub-zero degree temperature together with failure to follow the proceeding of the enzymic reaction prompted the authors to pursue the reaction more decimally around 0°C.

The experiments were carried out at the temperatures of plus 2.5°C, 0°C and minus 2.5°C (Fig. 4). Two kinds of the reaction mixture were arranged for the test at minus 2.5°C.

Table 1. Effect of various compounds on the formation of O-nitrophenol

Compounds added 5 % (W/V)	at plus 5°C			at minus 5°C		
	Time (min)	pH	O-nitrophenol formed(μm/ml)	Time (min)	pH	O-nitrophenol formed(μ m/ml)
ethylene glycol	90	7.0	0.50	120	7.0	0.00
glycerol	90	7.0	0.79	120	7.0	0.00
olive oil	90	7.0	0.42	120	7.0	0.02
monosodium L-glutamate	90	7.0	0.43	120	7.0	0.02
L-lysine	90	11.0	0.29	120	11.0	0.20
L-alanine	90	7.0	0.44	120	7.0	0.18
glucose	90	7.0	0.27	120	7.0	0.00
sucrose	90	7.0	0.35	120	7.0	0.00
galactose	90	7.0	0.32	120	7.0	0.00
D-sorbitol	90	7.0	0.50	120	7.0	0.03
D-mannitol	90	7.0	0.62	120	7.0	0.22
NaCl	90	7.0	0.40	120	7.0	0.01

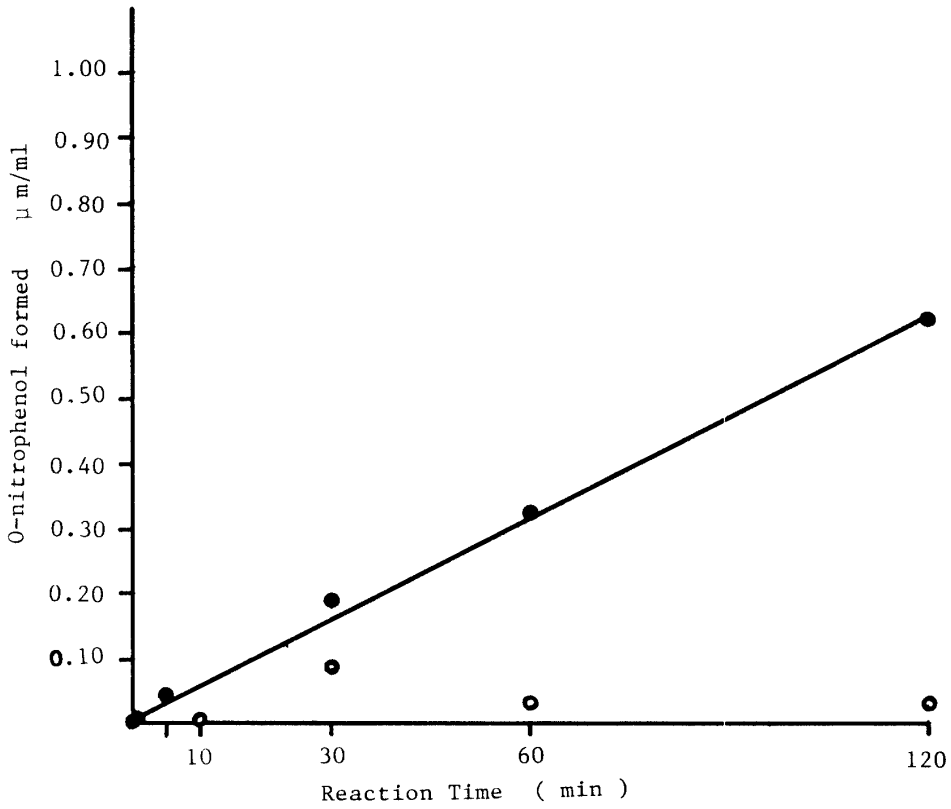

Fig.3. O-nitrophenol formed by the reaction at plus 5°C (●) and at minus 5°C (○)

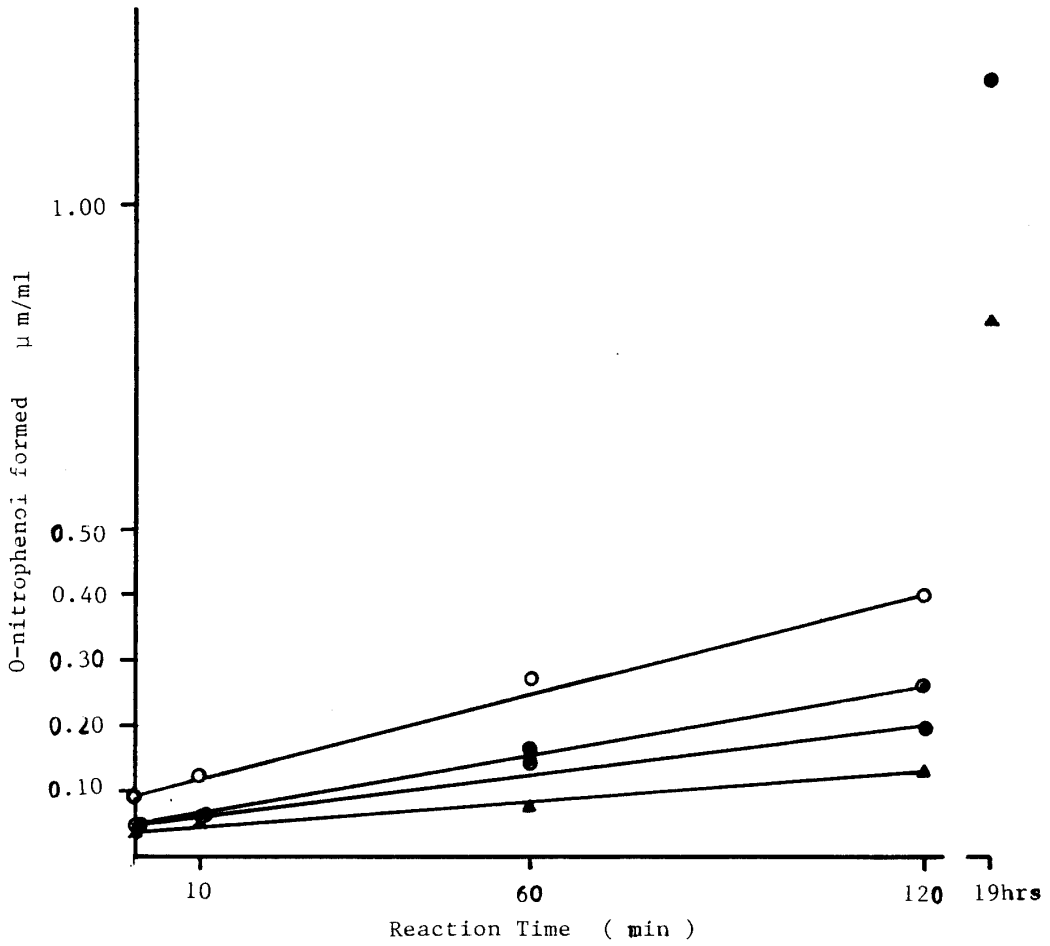

Fig.4. O-nitrophenol formed by the reaction
at plus 2.5°C (o)
0°C (⊙)
minus 2.5°C (▲) with glycerol
minus 2.5°C (●) without glycerol

One was prepared in the presence of 20 % (V/V) glycerol and the other in its absence since it had been noticed that the reaction mixture could be super-cooled without freezing around this temperature for several hours. This expectation was upset when the pre-cooled cell suspension was poured into the reaction mixture and the instantaneous freezing resulted in. However, with the lapse of time over an hour or two, the color development of O-nitrophenol in a frozen state was noticed and finally confirmed unequivocally in 19 hours reaction at minus 2.5°C.

Discussion

In the previous report,[7] β -galactosidase activity of the psychrotrophic bacteria designated as P 15 was shown over the wide range of temperature from minus 7.8°C to plus 60°C. However, the confirmation of the activity at the sub-zero degree temperature was needed. In the present study, by setting an appropriate procedure for the enzymic reaction and for its termination the activity at the sub-zero degree temperature was definitely confirmed

in the presence of glycerol as well as unexpectedly in a frozen state.

These results suggest a possibility that the enzymic reaction of psychrotrophic enzymes might not follow solely the conventional theory of temperature relationship of chemical reactions.

At high temperatures enzymic activity usually decreases due to the deterioration of enzyme molecules. On the contrary, at low temperatures the behavior of enzymes, particularly, those of psychrotrophic organisms presents interesting problems both kinetically and structurally. The experiments of the present study were conducted with using the cells grown at 30°C. So in the near future much work is to be done with using the solubilized enzyme.

In the meantime, the authors feel that the present enzyme could be called the enzyme looking forward to thawing in the spring.

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