Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/gmcl19

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Version of record first published: 04 Oct 2006.

To cite this article: Kazuo Kimura (1996): Enzymic Hydrolysis of Beta-Galactoside in a Frozen Aqueous Solution, Molecular Crystals and Liquid Crystals Science and Technology. Section A. Molecular Crystals and Liquid Crystals, 277:1, 107-112

To link to this article: http://dx.doi.org/10.1080/10587259608046010

Mol. Cryst. Liq. Cryst., 1996, Vol. 277, pp. 107–112
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ENZYMIC HYDROLYSIS OF BETA-GALACTOSIDE IN A FROZEN AQUEOUS SOLUTION

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Abstract Enzymic hydrolysis of 2-nitrophenyl ß-D-galactopyranoside was found to proceed in a frozen aqueous solution.

INTRODUCTION

Beta-galactosidase is essential for hydrolysis of lactose (ß-D-galactopyranosyl-(1→4)-D-glucose) in mammals and its inductive formation in an enterobacterium, Escherichia coli hatched Monod's operator gene theory in 1961. Studies of ß-galactosidase of a psychrotrophic bacterium with respect to the reaction temperature revealed accidentally an evidence that 2-nitrophenyl-ß-D-galactopyranoside was hydrolyzed in a frozen aqueous solution.

TEMPERATURE PROFILE OF BETA-GALACTOSIDASE OF A PSYCHROTROPHIC BACTERIUM

The cells of a psychrotrophic bacterium isolated from a dairy plant showed intracellular ß-galactosidase activity in a wide range of temperature between 0°C and 60°C. Furthermore, the ultrasonically ruptured cell-free extract exhibited the enzymic activity even at the sub-zero temperature as low as minus 18°C in a frozen aqueous solution.(Table I)

SETTING OF REACTION CONDITION AND REACTION TIME

For the confirmation of the enzymic reaction in a frozen aqueous solution, appropriate reaction conditions were required to be set so that the reaction in a frozen state could start and stop at the designed times. It was difficult to realize this. However, each of the following two procedures (A and B) could meet almost practical needs.

In either procedure, the reaction mixture was composed of 1.0 ml of 0.25 M phosphate buffer of pH 7.0, 1.0 ml of 20 mM 2-nitrophenyl-ß-

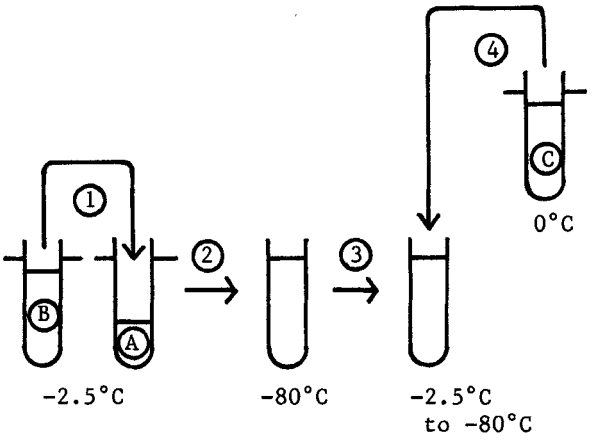
D-galactopyranoside(abbreviated as ONPG) in distilled water and 0.5 ml of the cell-free extract containing 3.2 mg or less of the crude protein. The mixture of 1.0 ml of phosphate buffer and 1.0 ml of 20 mM ONPG was pre-cooled at minus 2.5°C for 15 minutes. Then, the mixture was poured into another test tube containing 0.5 ml of the cell-free extract. In procedure A, the complete reaction mixture was immediately transferred into a deep-freezer of minus 80°C. After 5 minutes, the test tube was taken back to the cool water bath of the temperatures designed.(Figure 1) In procedure B, a small pre-cooled glass tube was inserted into the reaction mixture to accelerate freezing with or without vigorous shaking. Then, the test tube was transferred to the cool water bath of the temperatures designed.(Figure 2)

TABLE I o-Nitrophenol formed with the cell-free extract at various sub-zero temperatures

Cell-free extract (Crude protein mg/ml)	Reaction temperature	Reaction time and state	o-Nitrophenol formed (μm/ml)
0.1	-2.5°C	0 hrs	0.02
		1 (F)*	0.18
		2 (F)	0.68
		24 (F)	2.68
1.3	-2.5°C	0	0.23
		1 (F)	4.81
		2 (F)	3.77
		24 (F)	4.82
1.3	-15°C to -18°C	1 (F)	4.90
		2 (F)	2.70
		24 (F)	4.98
1.3	-80°C	1 (F)	0.75
		2 (F)	1.30
		24 (F)	0.91

Reaction proceeding: procedure A as described in Figure 1.

(F)* stands for a frozen state.

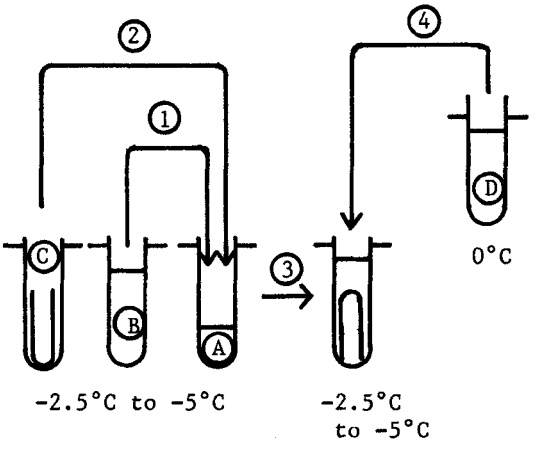


(A) Cell-free extract

(B) Buffer and ONPG

(C) 0.1 M Na_2CO_3

FIGURE 1 Enzymic reaction in procedure A.



(A) Cell-free extract

(B) Buffer and ONPG

(C) A small glass tube

(D) 0.1 M Na_2CO_3

FIGURE 2 Enzymic reaction in procedure B.

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 experiment. The amount of o-nitrophenol was determined by the absorbance at 420 nm.

ENZYMIC REACTION IN A SOLUTION RAPIDLY FROZEN AT MINUS 5°C

Two series of the enzymic reaction at minus 5°C were conducted. The one was performed without freezing and the other was carried out by freezing at the times indicated and kept at minus 5°C until 5 hours lapsed. (Figure 3) The cause of fluctuation in the results can not be defined at present but might be owing to the difference of the crystal states of ice.[1,2]

ATTEMPT TO EXCLUDE ENVIRONMENTAL AND CHEMICAL FACTORS AROUND THE ENZYME MOLECULES

Enzymic reactions at the sub-zero temperature in a frozen aqueous solution are often believed to proceed in the presence of some anti-freezing substance, mostly the lipids containing unsaturated fatty acids.[3] To eliminate this possibility, purification of the enzyme was attempted though at present partially, by removing nucleic acids with streptomycin, ammonium sulfate precipitation, DEAE-cellulose and CM-cellulose column chromatography. The hydrolysis of ONPG proceeded slowly but substantially in a frozen aqueous solution at minus 2.5°C.(Table II)

ASSUMPTIVE REACTION MECHANISM

Enzymic reaction in a frozen aqueous solution was hardly believed to occur. But the possibility of the contribution of liquid-like layer[4] and light-induced dissolution of ice, i.e. Tyndall figure phenomenon[5] can be denied from the calculation of the surface area of the reaction mixture and the observation of evenly-colored ice on its thawing. The influence of anti-freezing lipids can be excluded by their removal, though at present being partial, from the cell-free extract. Existence of minor quantity of not-frozen water in protein molecules described by Uedaira[6] might be contributive. On the other side, the concept of the

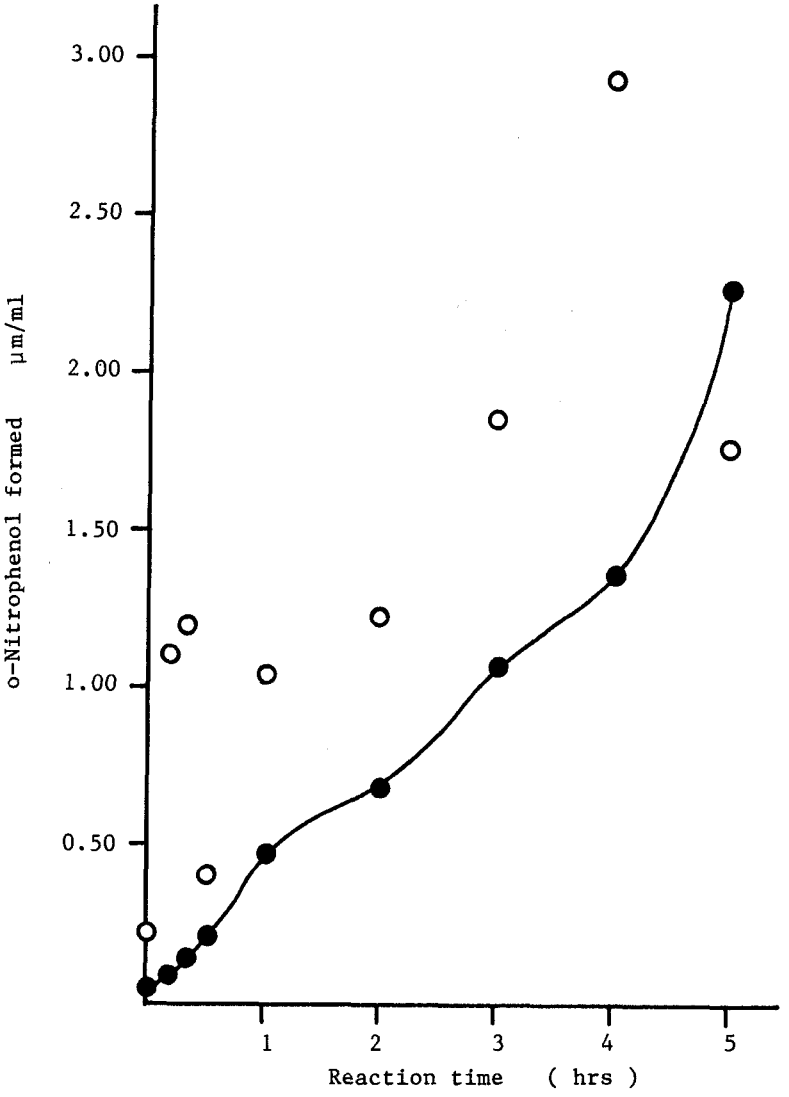


FIGURE 3 o-Nitrophenol formed with the cell-free extract at minus 5°C.

● Without freezing.

○ 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 as described in Figure 2.

TABLE II Enzymic activity of a partially purified enzyme in a frozen aqueous solution at minus 2.5°C

Enzyme	Enzyme solution used (ml)	Time of complete freezing at -2.5°C	Reaction time at -2.5°C	o-Nitrophenol formed (μ m/ml)
CM-cellulose Effluent	0.5	0 min	20 min	0.25
DEAE-cellulose Eluate	1.5	1 min	24 hrs	0.29

Reaction mixture: Enzyme solution 0.5 to 1.5 ml, 20 mM ONPG in 0.25 M phosphate buffer (pH 7.0) 1.0 ml, filled with distilled water to the total volume 2.5 ml.

movement of organic molecules in solid crystals demonstrated by Toda[7] is an encouraging support to the phenomenon described in the present study.

Under these circumstances, the author is expecting a possibility of erosion of water molecules from ice for proceeding of the enzymic hydrolysis of ß-galactoside in a frozen aqueous solution.

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