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ORIGINAL CONTRIBUTION

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Hydrolysis of starch or pullulan by glucoamylase or pullulanase immobilized on poly(*N*-isopropylacrylamide) gel

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Abstract Starch or pullulan was hydrolyzed using glucoamylase or pullulanase immobilized on *N*-isopropylacrylamide gel. The gel used is temperature sensitive; its mesh size becomes smaller at higher temperatures (30 °C) and larger at lower temperatures (20 °C). The molecular weight distribution of starch is wide and it consists of high-molecular-weight amylopectin, amylose and glucose. From the change in the chromatograms for the substrate and products, it was found that the hydrolysis rate at 30 °C was faster than that at 20 °C for amylose, though it was the reverse for amylopectin. This finding suggests that the smaller molecular sized amylose can enter the gel phase at both temperature, while the larger molecular sized amylopectin can hardly do so; only the end group, which can partly enter the gel phase at 20 °C (larger mesh size), was hydrolyzed. Further, several molecular weight pullulans (monodisperse) were hydrolyzed and the experimental chromatograms for substrate and products confirm the hydrolysis mechanism estimated.

Key words Polymeric gel – Mesh size – Immobilized enzyme – Starch – Pullulan

Nomenclature

D = diameter of the gel at swelling equilibrium [m]
D_0 = diameter of the gel at gel preparation [m]
M = molecular weight $\left(=\sqrt{M_n M_w}\right)$ [–]
M_n = number-average molecular weight [–]
M_w = weight-average molecular weight [–]
r = radius of solute [m]
V = volume of the gel at swelling equilibrium [m^3]
V_0 = volume of the gel at gel preparation [m^3]
w_1^G = weight fraction of solute inside the gel [–]
w_1^S = weight fraction of solute outside the gel [–]
θ = reaction ratio [–]
ξ = mesh size of gel network [m]

Introduction

It has been shown that some polymeric gels undergo continuous or discontinuous volume changes depending on external conditions, e.g., temperature, pH and solvent concentration [1, 2]. Therefore, the gels are expected to be applied as a size-selective extraction solvent and other functional materials [3]. As an interesting application of the gels, immobilized enzyme reactions have been considered. It has been shown that microencapsulated enzymes, while prevented from leaking out to give rise to hypersensitivity or immunological reactions, can act on external substrates dialyzing across semipermeable membranes [4]. Recently, it has been

reported that the *N*-isopropylacrylamide (NIPA) gel with an entrapped enzyme shows a discontinuous volume change, according to both substrate and product composition changes within the gel phase [5]. In immobilized enzyme reactions, the mass transfer rates of substrate and product within the pores of the gel may be changed by the mesh sizes of the gel. Therefore, where the gel is used as an immobilizer of enzymes, selective reaction of the substrate can be expected.

In this work, we studied hydrolysis as a reaction model and NIPA gel with immobilized enzyme as a reaction field. We used soluble starch or monodispersed pullulan as substrate, and glucoamylase or pullulanase as enzyme, and the possibility of selective reaction of the substrate is discussed. As the starch used consists of amylopectin, amylase (both high-molecular-weight substances) and glucose (low-molecular-weight substance), it is rather complex to discuss the hydrolysis mechanism in terms of the molecular size of the substrate and the mesh size of the gel. Therefore, monodispersed pullulan was adopted.

Experimental

Materials

NIPA (main-chain monomer), purchased from Eastman Kodak, was recrystallized from a benzene-hexane mixture (27 vol% of benzene) at room temperature and dried under vacuum at room temperature for 1 day. *N*, *N*′-Methylenebisacrylamide (BIS; cross-linker), ammonium peroxodisulfate (AP; initiator) and *N*,*N*,*N*′,*N*′-tetramethylenediamine (TEMED; accelerator) were purchased from Nakarai. Starch was purchased from Nakarai and was the same sample used in a previous paper [6]; the weight-average molecular weight is 30000. Starch was dried at 105 °C for 4 h before use. Monodispersed pullulan (Lot No. 61101) was purchased from Showa Denko. Table 1 shows the molecular weights and molecular sizes of pullulans calculated by the equation proposed by Kremer et al. [7]. Glucoamylase (*Rhizopus niveus*; Lot No. 135290) and pullulanase (*Aerobacter aerogenes*; Lot No.307161) were purchased from Funakoshi and Hayashibara Biochemical Laboratory, respectively.

Table 1 Molecular weight and size of pullulan

$M_w \times 10^{-4}$	M_w/M_n	$M \times 10^{-4}$ [a]	$2r$ [nm]
78.8	1.23	71.1	57.4
40.4	1.13	38.0	41.6
21.2	1.13	19.9	29.8
11.2	1.12	10.6	21.5
4.73	1.06	4.59	13.9
2.28	1.07	2.20	9.23
1.18	1.10	1.13	6.75
0.59	1.09	0.57	4.74

[a] $M = \sqrt{M_n M_w}$

Immobilization of enzyme

NIPA gels were prepared by free radical polymerization in water at 0 °C. NIPA (150 mmol), BIS (1.5 mmol) and glucoamylase (9 μg/g solution) or pullulanase (9, 4.5 or 2.1 μg/g solution) were dissolved in 125 ml water, while AP (0.8 mmol) was dissolved in another 125 ml water. The two solutions were cooled to 0 °C and then mixed. Furthermore, TEMED (0.2 ml) was added to the mixture. After 30 min, this mixture was transferred to glass tubes of 0.85 mm internal diameter and 50 mm length for measurement of gel volume, and plastic tubes of 10 mm internal diameter and 120 mm length for measurement of concentration inside the gel. After 1 day, the cylindrical gels were taken out of the tubes. The swollen gel was cut into about 5-mm lengths. These chips were soaked in excess pure water (about 500 ml).

Measurement of gel volume

The NIPA gel samples (chips about 5 mm in length) were immersed in test tubes filled with pure water. The test tubes were then set in a temperature-controlled water-bath (20–40 °C) for at least 4 h. The diameter of the gels, D, was measured using a calibrated microscope under swelling equilibrium conditions. By assuming that the gel swells isotropically, the swelling ratio of the gel was calculated as $V/V_0 = (D/D_0)^3$, where V and V_0 are the volumes of gel under equilibrium and initial conditions, respectively, and D_0 is the diameter of gel under initial conditions ($D_0 = 0.85$ mm).

Measurement of concentration inside the gel for starch and pullulan

Measurement of the concentration inside the gel for starch was reported in our previous paper [6]. For pullulan, in the present study, gel samples were immersed in vials (10 ml) filled with a pullulan-water mixture. The vials were then set in a temperature-controlled water-bath at 25 °C for 2 weeks under an ultraviolet lamp. Then the gels were removed from the vials and placed in centrifuge tubes with a hydrophobic filter, to remove surface solvent. The tubes were centrifuged for 3 min at 500 rpm. The amount of water inside the gel was determined by evaporation, and the amount of pullulan remaining inside the gel was weighed by a balance.

Hydrolysis of starch

NIPA gel with immobilized glucoamylase of 9 μg/g solution (about 5 g at swelling equilibrium) was immersed in vials (100 ml) filled with 0.1 wt% starch-water mixture (about 20 g). The vials were then set in a temperature-controlled water-bath (20 and 30 °C). After reaction, the solution outside the gel was analyzed by gel permeation chromatography. Further, hydrolysis of starch by glucoamylase aqueous solution was carried out in the same way, for comparison.

Hydrolysis of pullulan

NIPA gel with immobilized pullulanase of 4.5 μg/g solution (about 8 g at swelling equilibrium) was immersed in vials (100 ml) filled with 0.1 wt% pullulan-water mixture (about 32 g). The vials were then set in a temperature-controlled water-bath at 25 °C. After reaction, the solution outside the gel was analyzed by gel permeation chromatography. Further, hydrolysis of pullulan by pullulanase aqueous solution was performed using the same procedure. However, the weight of the pullulan-water mixture used in the immobilized system was 100 times that of the pullulanase aqueous solution to adjust the hydrolysis reaction rate, because the hydrolysis rate in immobilized systems is much smaller than that in aqueous systems.

Results and discussion

Swelling behavior of gel

The temperature dependence of the swelling ratio of NIPA gel in pure water is shown in Fig. 1. The volume of NIPA gel, which immobilizes enzyme, decreases with increasing temperature. The phase transition is also observed. Although the amount of immobilized enzyme (9 μg/g solution) is the same, immobilization of pullulanase considerably affects the swelling behavior of the NIPA gel, though glucoamylase shows little effect. However, pullulanase shows little effect on the swelling behavior of the NIPA gel when the amount of immobilized pullulanase is less than 4.5 μg/g solution. It can be considered that the number of cross-linkages increases with a decreasing quantity of enzymes inside the gel.

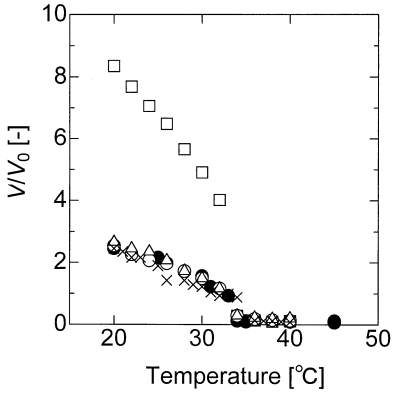

Fig. 1 Swelling ratio V/V_0 of *N*-isopropylacrylamide (NIPA) gel in pure water. △, ○, □ Immobilized pullulanase of 2.1, 4.5 and 9 μg/g solution, respectively; × immobilized glucoamylase of 9 μg/g solution; ● enzyme free

Concentration of starch and pullulan inside the gel

It was found in our previous paper [6] that the NIPA gel can absorb low molecular weight glucose, but exclude high molecular weight starch even though it is in a swollen state. Figure 2 shows the relationships between pullulan concentration inside the NIPA gel, w_1^G, and the molecular weight of pullulan. For a comparison, the same plots are shown for PEG [8]. The pullulan concentration inside the NIPA gel decreases as the molecular weight of pullulan increases, because higher molecular weight pullulan has difficulty entering the gel. Pullulan ($2r$ = 9.2 nm), with a molecular size a little larger than the mesh size of the gel (ξ = 7.4 nm), entered the gel because the molecule was soft. The mesh size of the gel was estimated using the equation proposed by Peppas et al. [9]. Pullulans with a molecular size higher than 13.9 nm were excluded by the gel.

Hydrolysis of starch

Figure 3 shows changes in the chromatogram for the hydrolysis of starch by immobilized glucoamylase. The

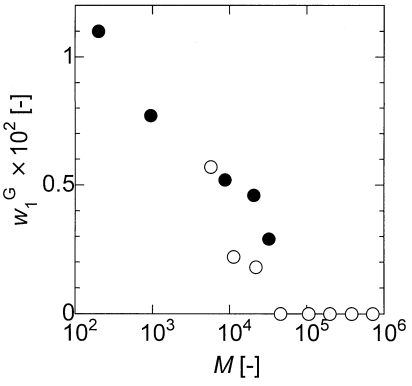

Fig. 2 Relationships between concentration inside NIPA gel and molecular weight at 25 °C and $w_1^s = 0.01$. ○ Pullulan, ● PEG [8]

molecular weight of starch is distributed, and it consists of amylopectin, amylose (both high molecular weight) and glucose (low molecular weight). Starch was hydrolyzed by immobilized glucoamylase and glucose was produced. It is shown that the hydrolysis rate of amylopectin at 20 °C was faster than that at 30 °C, though that of amylose was the reverse. The mesh size of the gel is smaller at higher temperatures and larger at lower temperatures and the molecular size of amylopectin is larger than that of amylose. Therefore, it seems that amylopectin can partly enter the gel at 20 °C while amylose can enter the gel at both temperatures.

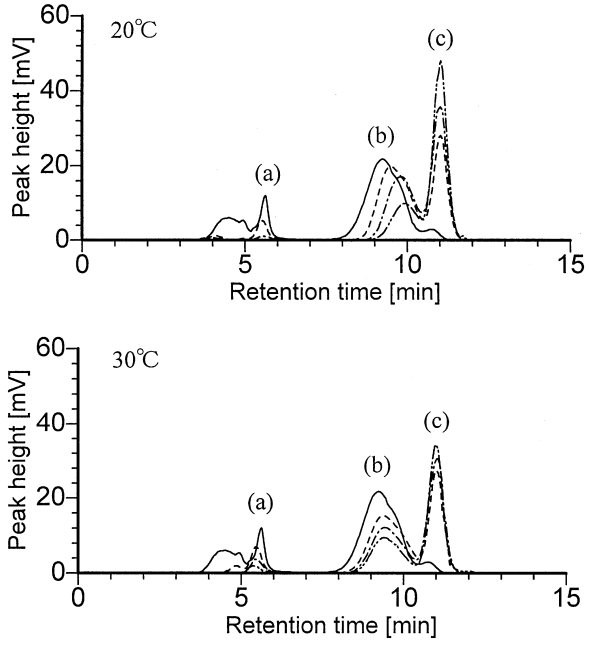

Fig. 3 Hydrolysis of starch by glucoamylase immobilized on NIPA gel. *a* Amylopectin, *b* amylose, *c* glucose; — initial condition; - - -, —·—, —··— after 2, 4 and 7 days, respectively

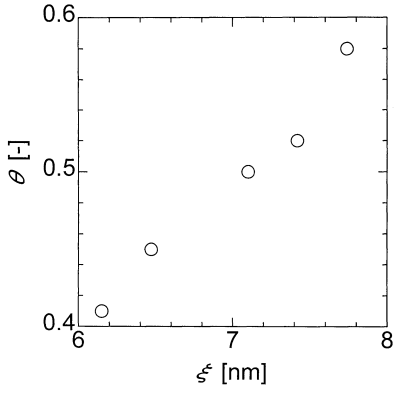

Fig. 4 Relationship between mesh size ξ and reaction ratio θ (after 7 days)

Figure 4 shows the relationship between mesh size ξ and reaction ratio θ, which is defined by the following equation.

$$\theta = \frac{\text{Amount of glucose produced by glucoamylase immobilized on NIPA gel}}{\text{Amount of glucose produced by glucoamylase aqueous solution}} \quad (1)$$

Equation (1) refers to the reduced glucose production rate under the same conditions except enzyme activity. As shown in Fig. 4, the hydrolysis rate of the immobilized system increases with increasing mesh size of the gel (decreasing temperature). In general, the enzyme activity increases with increasing temperature; however, the hydrolysis rate of immobilized enzymes can be controlled by the mesh size of the gel.

Hydrolysis of pullulan

Figures 5 and 6 show the change in maltotriose amount in the hydrolysis of pullulan by pullulanase aqueous solution, or by pullulanase immobilized on NIPA gel. In

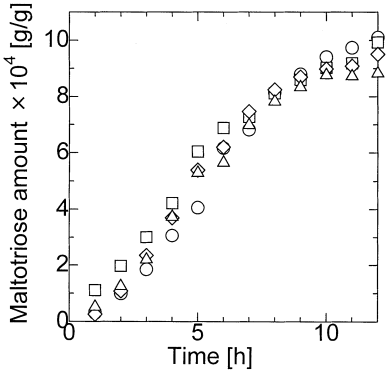

Fig. 5 Change of maltotriose amount in hydrolysis of pullulan by pullulanase aqueous solution at 25 °C. □ M = 5900, △ M = 11 300, ◇ M = 45 900, ○ M = 199 000

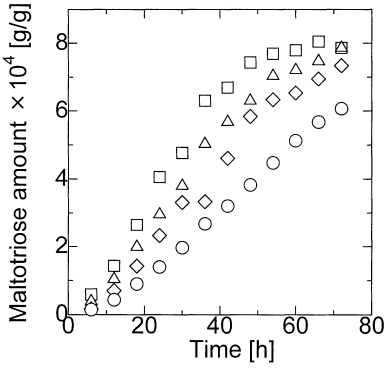

Fig. 6 Change of maltotriose amount in hydrolysis of pullulan by pullulanase immobilized on NIPA gel at 25 °C. □ M = 5 900, △ M = 11 300, ◇ M = 45 900, ○ M = 199 000

the case of hydrolysis by pullulanase aqueous solution, as shown in Fig. 5, the hydrolysis rate of pullulan was almost the same for each molecular weight (molecular size) of pullulan. However, as shown in Fig. 6, the hydrolysis rate of pullulan by immobilized pullulanase decreased with increasing molecular weight (molecular size) of pullulan.

Figures 7 and 8 show changes in the chromatogram for hydrolysis of pullulan by pullulanase aqueous solution, or by pullulanase immobilized on NIPA gel. In pullulanase aqueous solutions, as shown in Fig. 7, the peak of pullulan shifts to the right as time proceeds. This suggests that pullulan is decomposed randomly. The hydrolysis of pullulan appears to occur in almost the same manner at each molecular weight (molecular size) of pullulan. However, for immobilized pullulanase, the peak of pullulan ($M = 199\,000$), which cannot enter the gel (see Fig. 2), became low, and that of maltotriose high, as time proceeded (Fig. 8). It is considered that pullulan ($M = 199\,000$) is hydrolyzed from the end groups which can enter the gel network. However, the change in the peak of pullulan ($M = 11\,300$), which can enter the gel (see Fig. 2), was almost similar to that by pullulanase aqueous solutions. It is estimated that pullulan ($M = 11\,300$) is hydrolyzed randomly as in pullulanase aqueous solutions.

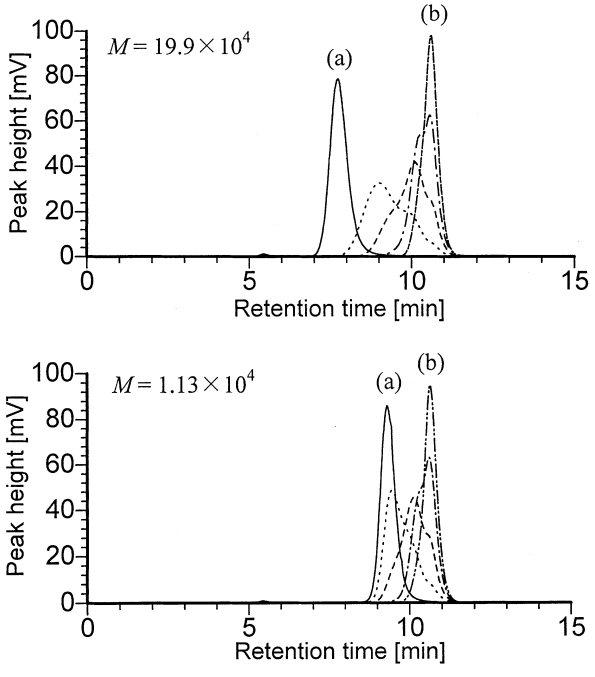

Fig. 7 Hydrolysis of pullulan by pullulanase aqueous solution at 25 °C. *a* Pullulan, *b* maltotriose; — initial condition; ····· , - - -, –·–,–··– after 1, 3, 6 and 10 h, respectively

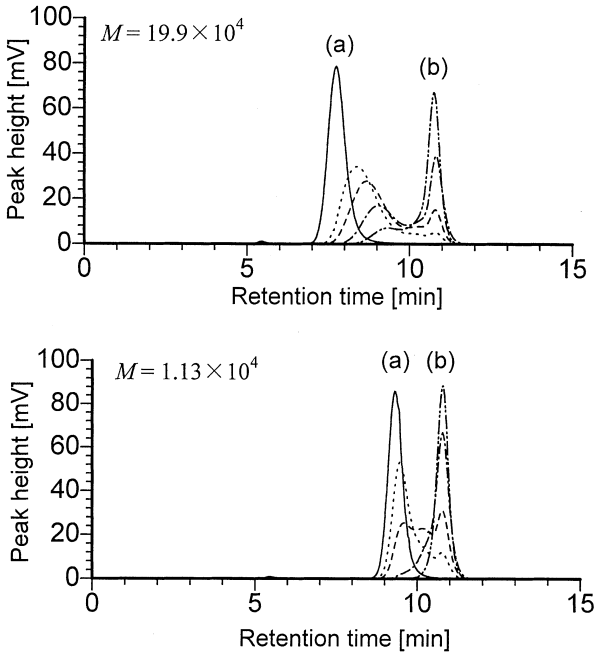

Fig. 8 Hydrolysis of pullulan by pullulanase immobilized on NIPA gel at 25 °C. *a* Pullulan, *b* maltotriose; — initial condition; ····· after 12 h; - - -, –·–,–··– after 1, 2 and 3 days, respectively

Conclusion

Pullulan, which can enter the gel, is hydrolyzed randomly. However, pullulan, which cannot enter the gel, is hydrolyzed from the end groups, which can partly enter the gel. For starch, the molecular weight is distributed widely, and it consists of substances with high (amylopectin and amylose) and low (glucose) molecular weights. Because the mesh size of the gel is small at higher temperatures and large at lower temperatures, and the molecular weight (molecular size) of amylopectin is larger than that of amylose, the substance which can enter the gel is regulated by the mesh size. From the experimental results for pullulan, it is estimated that amylopectin and amylose with a higher molecular weight may be hydrolyzed from the end group, while amylose with a lower molecular weight may be hydrolyzed randomly in the gel phase. Therefore, a substrate which can enter the gel can be screened by mesh sizes of the gel and a selective reaction of substrate may be possible based on molecular size.

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