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Immobilization and Properties of β-Galactosidases from *Macrophomina phaseoli* and *Sclerotium tuliparum**

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β-Galactosidases from *Macrophomina phaseoli* and *Sclerotium tuliparum* were immobilized on porous glass beads. The properties such as optimum temperature, pH-stability and behavior towards various metal ions and reagents except for *N*-bromosuccinimide of the immobilized enzymes were similar to those of the native enzymes, although *Km* values of the immobilized enzymes were 2–5 fold higher than those of the native enzymes. The substrate specificities of the immobilized enzymes were similar to those of the native enzymes, but the immobilized enzyme from *M. phaseoli* showed higher relative activity towards whey, lactose, and skim milk than the native enzyme did. Immobilized enzymes from *M. phaseoli* and *S. tuliparum* retained 100% and 78% of the original activities, respectively, after being kept for two months at pH 5.0 and 37°C, whereas the native enzymes from *M. phaseoli* and *S. tuliparum* showed 62% and 6% of the original activities, respectively. Both immobilized enzymes could be used repeatedly 10 times without any activity loss. These results suggest that the immobilized enzyme from *M. phaseoli* is suitable for hydrolytic cleavage of lactose in dairy products.

An immobilized β-galactosidase may be useful to hydrolyze lactose contained in dairy products, because lactose-free milk is an important nutrient source for infants suffering from lactose intolerance. Immobilization of β-galactosidase was carried out by using porous cellulose sheet,[1] cellophane sheet,[2] polyacrylamide,[3] fibre,[4] and glass beads.[5~7] Previously, we showed that β-galactosidases from *Macrophomina phaseoli* and *Sclerotium tuliparum* can be utilized as therepeutic agents for lactose intolerance.[8] In an attempt to apply them to the hydrolysis of lactose in dairy products, the enzymes from *M. phaseoli* and *S. tuliparum* were immobilized on porous glass. Porous glass was selected as a solid support, because it is resistant to attack by microorganisms and has enough mechanical durability, appropriate pore size and a relatively big surface area, and also a high flow rate can be obtained in the column reaction. Some properties, action patterns and continuous enzyme reaction of the immobilized enzymes are described in this paper.

Materials and Methods

Enzymes and reagents β-Galactosidase from *M. phaseoli* and β-galactosidase I from *S. tuliparum* were purified to a homogeneous state.[9,10] *o*-Nitrophenyl β-D-galactopyranoside (ONPG), *p*-nitrophenyl β-D-galactopyranoside (PNPG), and lactose were purchased from Nakarai Chemical Co. (Kyoto). Glass beads (particle size 100–200 mesh, exclusion limit 500Å, internal porous volume 35–40%) were obtained from Bio-Rad Laboratories (Calif., USA). γ-Aminopropyl triethoxysilane and milk were products of Tokyo Kasei Co. Ltd. and of Meiji Nyugyo Co. Ltd. (Tokyo), respectively. Skim milk and whey were prepared according to the methods of Sukegawa.[11] The lactose content of them was adjusted to 4%, and they were used after sterilization at 70°C for 10 min.

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Enzyme assay

1) *Activity towards ONPG* Native β-galactosidase activity was determined by the methods described previously.[8] Immobilized enzyme activity was determined as follows: A reaction mixture containing 1.0 ml of 5 mM ONPG, 1.0 ml of 0.1 M HCl-Na-acetate buffer (pH 2.0) for the *Sclerotium* enzyme or 0.1 M acetate buffer (pH 5.0) for the *Macrophomina* enzyme, 0.5 ml of water, and a suitable amount of immobilized enzyme (usually 5 mg) was incubated at 37°C for 10 min with mechanical shaking.

2) *Activity towards lactose, skim milk, and whey* Activity of the native enzyme was determined according to the methods described previously.[9] Activity of the immobilized enzyme was measured as follows: A reaction mixture containing 2.0 ml of 4% lactose, skim milk, or whey, 2.0 ml of the same buffer described above, 1.0 ml of water, and immobilized enzyme (usually 20 mg) was incubated for 10 min at 37°C with mechanical shaking. Then 0.2 ml of the reaction mixture was incubated with 5.0 ml of Blood Sugar Color Test Solution (TC-III, Boehringer, Germany) at 25°C for 1 hr and the absorbance at 430 nm measured. One unit of the enzyme activity was defined as the amount of enzyme which liberated 1 μmole of *o*-nitrophenol and D-glucose per minute. D-Glucose obtained from the substrates by the column reaction was also determined with the Blood Sugar Color Test reagent.

Preparation of immobilized enzyme Immobilized enzyme was prepared according to the methods of Robinson[12] with minor modifications. Porous glass beads were dried by heating at 500°C for 2 hr. The glass was refluxed in 2% γ-aminopropyl triethoxysilane in acetone at 45°C for 24 hr. The aminoalkylsilane glass (1 g) thus obtained was placed in a cold 1% aqueous solution of glutaraldehyde and shaken for 24 hr at 4C°. The derivative was rinsed and suspended in 5 ml of 0.05 M phosphate buffer (pH 7.0) containing an appropriate amount of the enzyme (2–20 mg). After shaking for 24 hr at 4°C, the beads were washed thoroughly with 0.5 M NaCl and then with 0.05 M acetate buffer (pH 5.0) until activity was no longer detectable in recovered solutions by washing. The immobilized enzymes were kept in 0.05 M acetate buffer (pH 5.0) at 4°C. The calculation of the amount of the enzyme bound to the glass was based on the difference in protein content between the protein added and that in recovered solutions by washing. Protein was determined by the method of Lowry.[13]

Results

Amount of bound enzyme and activity The relationship of the activities of added enzyme and bound enzyme was determined (Fig. 1). The amount of protein bound to glass increased with increasing amounts of added enzyme. When 20 mg of the enzyme

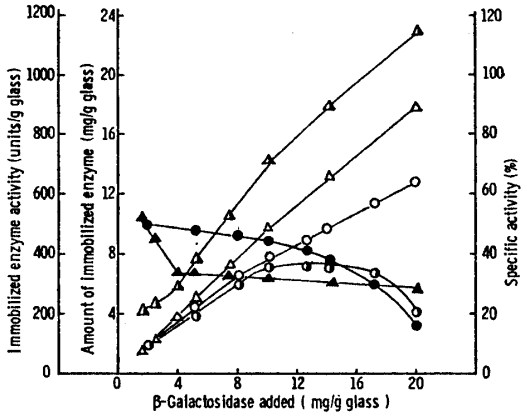

Fig. 1. Activity, amount and specific activity of immobilized β-galactosidases from *M. phaseoli* and *S. tuliparum*.
Specific activity of the immobilized enzymes is expressed as the percentage of that of the native enzymes. Activity was measured by using ONPG under standard conditions. Specific activity of the native enzymes was 100 units/mg for *M. phaseoli* and 230 units/mg for *S. tuliparum*.
Activity of immobilized enzyme, —◑—, *M. phaseoli*; —▲—, *S. tuliparum*
Amount of immobilized enzyme, —○—, *M. phaseoli*; —△—, *S. tuliparum*
Specific activity, —●—, *M. phaseoli*; —▲ , *S. tuliparum*.

was added to 1 g of glass, the amount of bound protein was 12.7 mg (63%) for the *Macrophomina* β-galactosidase and 17.7 mg (88%) for the *Sclerotium* enzyme. However, specific activities of the bound enzymes decreased from 52% to 16% for *M. phaseoli* and from 48% to 28% for *S. tuliparum*, when compared with the native enzymes, according to the increase of the amount of the enzymes added. With regard to the immobilized enzyme from *M. phaseoli*, the maximum activity (355 units/g glass) was obtained with the addition of 12–16 mg of the enzyme. This phenomenon may be due to the diffusional limitation of the substrate and/or the product, conformational changes, and denaturation of the enzyme.

Properties of the immobilized enzymes Properties of the immobilized enzymes were compared with those of the native enzymes, and the results are presented in Table 1. The optimum pH of the immobilized enzyme from *M. phaseoli* was pH 5.0, but that of the bound enzyme from *S. tuliparum* was pH 2.0, the same as the native enzyme. The optimum temperatures of these two immobilized enzymes were the same as those of the respective native enzymes, *i.e.* 60°C for *M. phaseoli* and 53°C for *S. tuliparum*. The stable pH range of the immobilized enzyme from *M. phaseoli* was almost the same as that of the native enzyme. The bound enzyme from *S. tuliparum* was stable in a pH range of 2.5 to 6.0, showing a slightly wider pH range than the native enzyme. The thermal stability of the bound enzyme from *M. phaseoli* was lower than that of its native enzyme, while the immobilized enzyme from *S. tuliparum* showed the same thermal stability as its native enzyme. Differences of the behavior to metal ions and reagents of the enzymes were not seen before or after immobilization, except for *N*-bromosuccinimide (NBS). The effect of NBS on these two immobilized enzymes was weaker than that on the native enzymes.

K_m and V_m of the immobilized enzymes The K_m and V_m values of the native and immobilized enzymes were determined and the results are shown in Table 2. For ONPG, the K_m values of the immobilized enzymes from *M. phaseoli* and *S. tuliparum* were 2–4 fold higher than those of the native enzymes. The K_m values for lactose were also 3–5 fold higher those of the native enzymes. The V_m values of the immobilized enzyme from *M. phaseoli* were 56% for ONPG and 65% for lactose, and the V_m values of the bound

Table 1. Properties of immobilized β-galactosidases from *M. phaseoli* and *S. tuliparum*.

Property	*Macrophomina* enzyme		*Sclerotium* enzyme	
	native	immobilized	native	immobilized
Optimum pH[a]	5.0	4.0	2.0	2.0
Optimum temperature[b]	60°C	60°C	53°C	53°C
pH-stability[c]	4.0~8.0	4.0~8.0	3.0~6.0	2.5~6.0
Thermal stability[d]	55°C	45°C	55°C	55°C
Inhibitor (1 mM)[e]				
metal ion (Hg^{++})	70%	60%	—	—
reagent (NBS)	0%	61%	0%	10%
(I_2)	70%	70%	—	—

a) Enzyme assay method a) except for various pHs of Britton-Robinson buffer.
b) Enzyme assay method a) except various temperatures and 15 min reaction.
c) Britton-Robinson buffer, 37°C, 3 hr.
d) The enzyme was kept in 0.05 M acetate buffer (pH 5.0) for 30 min at various temperatures and showed more than 90% of the original activity at the indicated temperature.
e) The enzyme was incubated with 1 mM metal ions or reagent (pH 5.0, 0.05 M acetate buffer) for 30 min at 37°C and the remaining activity was measured by using ONPG.

Table 2. K_m and V_m of immobilized β-galactosidases from *M. phaseoli* and *S. tuliparum*.

Substrate		K_m (mM)		V_m (units/mg enzyme)	
		Macrophomina	*Sclerotium*	*Macrophomina*	*Sclerotium*
ONPG	native	0.52	1.4	125	428
	immobilized	1.0	6.7	70(0.56)	469(1.10)
Lactose	native	15	20	105	103
	immobilized	56	74	68(0.65)	78(0.76)

Data in parentheses are V_m (immobilized enzyme)/V_m (native enzyme).
Activity was measured with a concentration of 0.2–4 mM ONPG or 3.3–100 mM lactose by the methods described in the text. Immobilized enzymes used contained 6.4 mg protein/g glass for *M. phaseoli* and 7.7 mg protein for *S. tuliparum*.

enzyme from *S. tuliparum* were 110% for ONPG and 76% for lactose when compared with the V_m values of the native enzymes.

Activity of immobilized β-galactosidases towards various substrates To determine whether the substrate specificity was changed by immobilization, activities of native and immobilized enzymes towards various substrates were examined. As shown in Table 3, immobilized β-galactosidase from *M. phaseoli* showed slightly higher activity towards whey, skim milk, and lactose than the native enzyme did. On the other hand, the bound enzyme from *S. tuliparum* hydrolyzed whey at a lower rate than the native enzyme, while PNPG, skim milk and lactose were hydrolyzed at a higher or the same rate.

Stability of immobilized enzymes after repeated use To investigate the outcome of repeated use of glass-bound enzymes in a batch system, the stability of the enzymes was examined. A mixture of 0.1 M HCl-Na-acetate buffer (pH 2.0) for the *Sclerotium* enzyme and 0.1 M acetate buffer (pH 5.0) for the *Macrophomina* enzyme, 22.5 mg of ONPG and the immobilized enzymes (10 mg), in a total volume of 15 ml, was incubated for 10 min at 37°C with shaking and then filtered. The residual immobilized enzymes were washed with water, and again incubated under the same conditions as above and the interval between each use was 15 min. The results indicate that the immobilized enzymes were very stable after the 10 th repeated use, showing the original activity, and that they

Table 3. Activity of immobilized β-galactosidases from *M. phaseoli* and *S. tuliparum* towards various substrates.

Substrate (Conc.)	Activity**			
	Macrophomina enzyme		*Sclerotium* enzyme	
	native	immobilized	native	immobilized
ONPG (2 mM)	100(100)*	100(45)*	100(230)*	100(76)*
PNPG (2 mM)	86	85	3	6
Lactose (1.6%)	73	78	31	35
Whey (1.6%)	84	93	28	16
Skim milk (1.6%)	33	37	11	11

* Specific activity (units/mg protein).
** Activity towards ONPG was taken as 100%.
Immobilized enzymes used contained 6.4 mg protein/g glass for *M. phaseoli* and 7.7 mg protein/g glass for *S. tuliparum*.

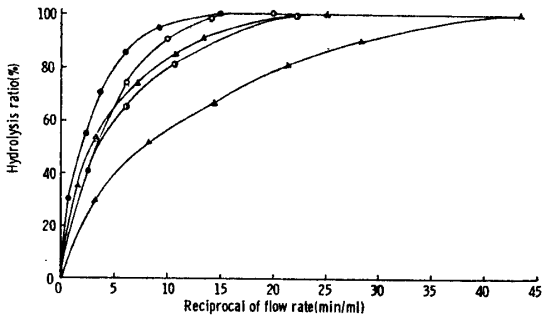

Fig. 2. Relation between flow rate and β-galactosidase activity.
Immobilized enzymes (2 mg protein/g glass) were packed in jacketed columns (1.5 × 1 cm) and maintained at 37°C. Lactose, whey, and skim milk solution (1.6% in 0.05 M acetate buffer, pH 5.0 for the *Macrophomina* enzyme, or in 0.05 M HCl-AcONa buffer, pH 3.0 for the *Sclerotium* enzyme) were passed through the column. D-Glucose formed by the column reaction was determined with Blood Sugar Color Test Reagent.
M. phaseoli enzyme: —○—, Lactose; —●—, Whey; —◑—, Skim milk,
S. tuliparum enzyme: —△—, Lactose; —▲—, Whey.

are therefore suitable for repeated use.

Hydrolysis of lactose, whey, and skim milk with immobilized enzyme in a column The immobilized enzyme was packed into a jacketed column and maintained at 37°C. The substrate solution was then passed through the column at various flow rates. As shown in Fig. 2, the *Macrophomina* enzyme hydrolyzed whey most rapidly, but lactose was hydrolyzed faster than whey by the *Sclerotium* enzyme. In the column reaction, the *Macrophomina* enzyme was found to be more suitable than the *Sclerotium* enzyme. These results are comparable with the results shown in Table 3.

A continuous enzyme reaction was examined with immobilized enzyme from *M. phaseoli*. The immobilized enzyme (200 mg; 60 units) was packed into a jacketed column (1.5 × 1.6 cm) and maintained at 37°C. 40% whey solution bufferized with 0.05 M acetate buffer (pH 5.0) was passed through the column at a flow rate of 4 ml/hr. The enzyme column was very stable even after 10 days, retaining the activity observed at the beginning.

Stability of immobilized enzymes with time With regard to the continuous enzyme reaction in a column, the stability of the immobilized enzymes during a period of two months at 37°C was also examined. The results are shown in Fig. 3. The immobilized enzyme was more stable than its native enzyme. The bound enzyme from *M. phaseoli* retained the original activity even after two months.

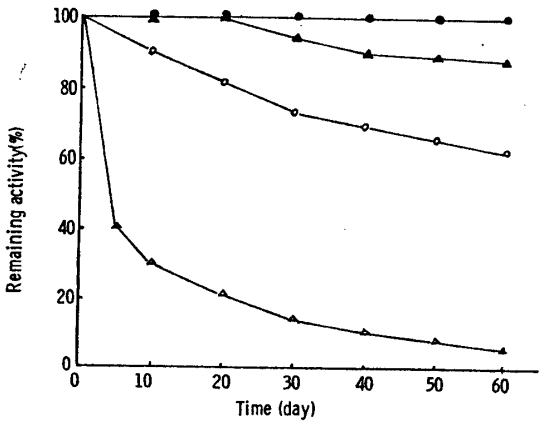

Fig. 3. Stability of immobilized β-galactosidases from *M. phaseoli* and *S. tuliparum*.
The immobilized enzymes were kept in 0.05 M acetate buffer (pH 5.0) at 37°C.
M. phaseoli enzyme:
—○—, Native; —●—, Immobilized.
S. tuliparum enzyme:
—△—, Native; —▲—, Immobilized.

Discussion

When 20 mg of the enzymes were added to 1 g of glutaraldehyde glass, the amount of the enzymes bound to the glass was 12.7 mg for the *Macroephomina* enzyme and 17.7 mg for the *Sclerotium* enzyme. These values are similar to the value for the *Escherichia coli* enzyme[12] and higher than the binding value of 3–7 mg in *Aspergillus niger*.[5,9] The specific activity of the bound enzymes decreased with increasing amounts of the added enzymes. The K_m values of the bound enzyme from *M. phaseoli* were 2–4 fold higher of those of the native enzyme and V_m values were 56–65% compared with the native enzyme. On the other hand, the immobilized enzyme from *S. tuliparum* showed 2–5 fold higher K_m values than the native enzyme and V_m values were 110% for ONPG and 76% for lactose. These results suggest the possibility of diffusional limitation, conformational changes and denaturation of the enzymes caused by immobilization. The K_m values of immobilized enzymes were 2–5 fold higher than the native enzymes, but it is reported that the K_m value of the enzyme from *A. niger* attached to porous glass did not vary before or after immobilization.[5] Action patterns of both the enzymes were slightly affected by the immobilization. In case of the *Macrophomina* enzyme, immobilization led to a slight increase in the relative hydrolysis rate for whey, lactose, and skim milk and these results differed from the results that using immobilized β-galactosidase made it hard to hydrolyze whey and skim milk because of interference by solid materials in them.[11,14]

In conclusion, the properties of β-galactosidases from *M. phaseoli* and *S. tuliparum* did not vary to a great extent by immobilization, although the specific activity was lowered and the K_m value increased. Stability of the enzymes at 37°C increased after fixation. The immobilized enzyme from *M. phaseoli* effectively hydrolyzed lactose, whey and skim milk. These results suggest that the immobilized β-galactosidase from *M. phaseoli* is useful for hydrolytic cleavage of lactose in dairy products.

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