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Pharmaceutical Studies on β-Galactosidases from *Macrophomina phaseoli* and *Sclerotium tuliparum*[1]

MAMORU SUGIURA, MUTSUKO SUZUKI, TOKIKO SHIMOMURA, and MASANORI SASAKI

Tokyo College of Pharmacy[2]

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The enzymatic properties of crude β-galactosidases from *M. phaseoli* and *S. tuliparum* were studied from the standpoint on the pharmaceutical aspects in comparison with the properties of the enzyme from *Aspergillus oryzae* which is available on the market as a therapeutic agent for lactose intolerance. Optimum pH of *Macrophomina* and *Sclerotium* enzymes were 4.5 and 1.5, respectively. Stable pH ranges of the enzymes were 4 to 8.5 for *Macrophomina* and 3 to 6 for *Sclerotium* enzyme, and both enzymes were stable up to 60° and 55° for 30 min, respectively. These enzymes were not affected by addition of metal ions or reagents tested (excepting N-bromosuccinimide). The enzymes hydrolyzed lactose, and lactose in milk and dry milk with the same rate as *Aspergillus* enzyme. In a powder state, both enzymes were more stable than *Aspergillus* enzyme on standing at relative humidity (RH) of 92% and 30° and also stable to human gastric and intestinal juices in the presence of substrates. The enzymes were not affected by diluents tested. Furthermore, *Macrophomina* enzyme was stable for binders and disintegrators, whereas *Sclerotium* enzyme was completely inactivated by aerozol and sodium dodecyl sulfate (SDS) among the agents tested. Against wetting agents tested, *Macrophomina* enzyme was stable, but *Sclerotium* enzyme was stable to isopropanol and low concentration of ethanol and acetone. Both enzymes were more stable than *Aspergillus* enzyme under the pressure range of 0.5 to 2.0 ton.

From these results, it is suggested that β-galactosidases from *M. phaseoli* and *S. tuliparum* can be effectively utilized as a therapeutic agent for lactose intolerance.

Keywords——lactose intolerance; β-galactosidase; lactase; therapy of lactose intolerance; pharmaceutical aspects of lactase; *Macrophomina phaseoli*; *Sclerotium tuliparum*; milk; dry milk

Since Durand[3] and Holzel, *et al.*[4] reported on hereditary lactose intolerance in child, many investigators have been engaged in the studies on lactose intolerance, and primary and secondary lactose deficiency.[5-10] It has been clarified that diarrhea due to lactose

1) This work is a part CXXXIII of "Studies on Enzymes" by M. Sugiura.
2) Location: *1432-1, Horinouchi, Hachioji, Tokyo, 192-03, Japan.*
3) P. Durand, *Minerva Pediat.*, **10**, 706 (1958).
4) A. Holzel, V. Schwarz, and K.W. Sutcliefe, *Lancet*, **1**, 1126 (1959).
5) P. Sunshine and N. Kretchmer, *Pediatrics*, **7**, 38 (1964).
6) H.A. Weijers, J.H. Van de Kamer, W.K. Dicke, and J. Ijsseling, *Acta Paediat.*, **50**, 55 (1961).
7) H.A. Weijers and J.H. Van de Kamer, *Acta Paediat.*, **51**, 371 (1962).
8) J. Lloyd-Still, *Acta Paediat. Scand.*, **58**, 147 (1969).
9) A. Holzel, *Pediat. Clin. N. Amer.*, **12**, 635 (1965).
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malabsorption was caused by deficiency of lactase, or by low lactase activity in small intestine, followed by extraordinal bacterial fermentation in large intestine. The therapy for lactose intolerance in child is to keep anyone free from ingestion of lactose, or to give lactase (β-galactosidase [EC 3.2.1. 23]) together with milk or dry milk. Since lactose is one of the nourishing sources for an infant and galactose is an important nutrient for brain, it seems unsuitable to give lactose-free diet for the therapy of lactose intolerance. Weijers[11] has proposed that administration of lactase might be effective for the therapy of lactose intolerance.

Among many β-galactosidases, the enzyme obtained from *Aspergillus oryzae* has been the only enzyme available on the markets as therapeutic agent for lactose intolerance untill now. However, the enzyme is stable to relatively low temperature and in a narrow pH range. The authors have found that β-galactosidases from *Macrophomina phaseoli* and *Sclerotium tuliparum* are very stable.[12] In this report, to know whether or not these enzymes are suitable as a therapeutic agent for lactose intolerance, the properties of the enzymes are studied from a pharmaceutical standpoint in comparison with those of *Aspergillus* enzyme.

Materials and Methods

Enzymes and Substrates——Enzyme preparations employed were ethanol precipitates from the culture filtrates of *M. phaseoli*[13] and *S. tuliparum*.[14] β-Galactosidase from *Aspergillus oryzae* "Galactase" was purchased from Tokyo Tanabe Pharm. Co. Ltd. (Tokyo). *o*-Nitrophenyl β-D-galactopyranoside (ONPG) was obtained from Nakarai Chemicals Co. (Kyoto), lactose was a product of Wako pure Chemicals Ind. Co. Ltd. (Osaka), milk was from Seikatsu Kyodo Kumiai (Tokyo), and dry milk was from Yukijirushi Nyugyo Co. Ltd. (Sapporo).

Enzyme Assay——a) Activity towards ONPG was measured by the methods described previously.[12]

b) Activity towards lactose, milk and dry milk; A reaction mixture containing 2.0 ml of 5% lactose (or 50% milk and 15% dry milk) and 2.0 ml of 0.1 M HCl–AcONa buffer (pH 2.0) for *Sclerotium* enzyme or 0.1 M acetate buffer (pH 4.5) for *Macrophomina* and *Aspergillus* enzyme, and 1.0 ml of enzyme solution were incubated at 37° for 10 min, and then 0.5 ml of the reaction mixture was added to 5.0 ml of 0.33 M perchloric acid. After keeping the above mixture at 20° for 20 min, a produced precipitate was filtered off on a Toyo filter paper No. 131. To 0.2 ml of the filtrate was added 3.0 ml of Galactose UV-Test (Boehringer, Germany) solution I, 0.1 ml of solution II and then 0.02 ml of solution III. After standing the mixture at 20° for 40 min, absorbance at 340 nm was measured.

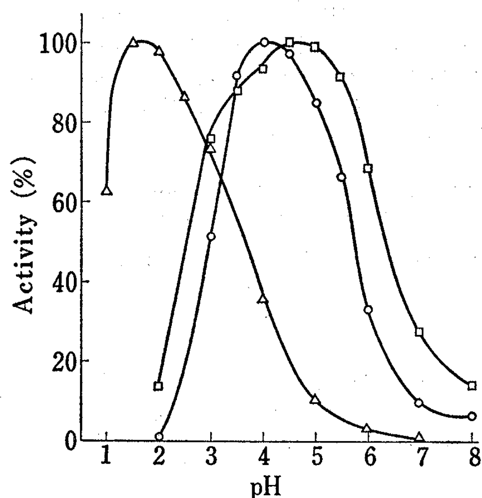

Fig. 1. Effect of pH on Activity of β-Galactosidases from *Asp. oryzae* (○), *M. phaseoli* (□) and *S. tuliparum* (△)

Results

Optimum pH

The optimum pH of the enzymes was determined in Britton–Robinson buffer by use of ONPG as substrate at 37° for 10 min. As shown in Fig. 1, the optimum pH of the enzymes from *A. oryzae*, *M. phaseoli* and *S. tuliparum* was 4.0, 4.5 and 1.5, respectively.

pH and Thermal Stability

pH-Stability of these enzymes was examined at 37° for 3 hr. As shown in Fig. 2, stable pH range of *Macrophomina*, *Sclerotium* and *Aspergillus* enzymes was 4 to 8.5, 3 to 6 and 5 to 7, respectively. The thermal stability of these enzymes was also examined at pH 5.0 for 30 min. The

11) H.A. Weijers, *Mod. Treat.*, **2**, 378 (1965).
12) M. Sugiura, M. Suzuki, M. Sasaki, and T. Shimomura, *Chem. Pharm. Bull.* (Tokyo), **24**, 788 (1976); *idem, ibid.*, **24**, 794 (1976).
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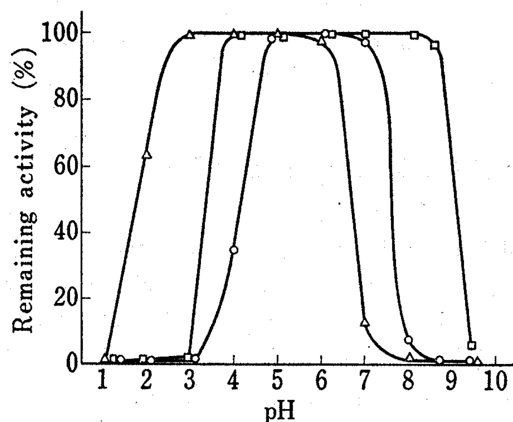

Fig. 2. Effect of pH on Stability of β-Galactosidases

The enzyme preparation (1—2 mg) was incubated in various pH's Britton-Robinson buffer at 37° for 3 hr, and then the solution was diluted to 20-fold volume with the optimum pH's buffer and the remaining activity was measured under standard conditions.

—○—, *Asp. oryzae*; —□—, *M. phaseoli*; —△—, *S. tuliparum*.

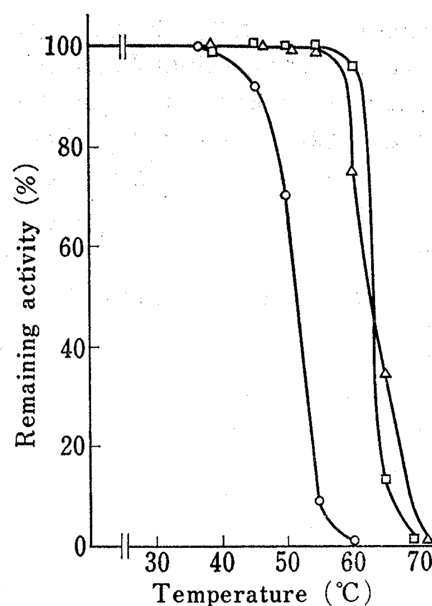

Fig. 3. Effect of Temperature on Stability of β-Galactosidases

The enzyme preparation (1—2 mg) was incubated in 0.05 M acetate buffer (pH 5.0) at various temperatures for 30 min and then the solution was diluted to 20-fold volume with the optimum pH's buffer and the remaining activity was measured with enzyme assay method (a).

—○—, *Asp. oryzae*; —□—, *M. phaseoli*; —△—, *S. tuliparum*.

TABLE I. Effect of Various Metal Ions and Reagents on the Activity of β-Galactosidases

Metal ion and reagent (1 mM)	Remaining activity (%)		
	Aspergillus	*Macrophomina*	*Sclerotium*
None	100	100	100
Hg^{2+}	37	100	94
Ca^{2+}	100	101	102
Mg^{2+}	100	102	102
Zn^{2+}	100	102	100
Co^{2+}	100	102	103
Mn^{2+}	103	102	108
Cu^{2+}	103	102	100
Fe^{3+}	99	101	103
Na^{+}	103	102	100
K^{+}	103	101	104
EDTA	102	102	101
o-Phenanthroline	101	101	102
Cysteine	104	102	106
2-Mercaptoethanol	101	102	106
Iodine	82	95	91
p-Chloromercuribenzoate[a)]	103	102	100
Diisopropyl fluorophosphate	103	102	104
Monoiodoacetic acid	103	103	103
N-Bromosuccinimide	2	50	3

The enzyme preparation (1—2 mg) dissolved in 0.05 M acetate buffer (pH 5.0) was incubated with an equal volume of a metal ion or a reagent solution (2 mM, pH 5.0) at 37° for 30 min. The mixture was diluted to 20-fold volume with optimum pH's buffer and the remaining activity was determined with enzyme assay method (a).

a) 0.1 mM.

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results are shown in Fig. 3. *Aspergillus* β-galactosidase was stable up to 37° and maintained 70% of the original activity at 50°, but completely inactivated at 60°. On the other hand, *Macrophomina* and *Sclerotium* enzymes were stable up to 60° and 55°, respectively.

Effect of Some Metal Ions and Reagents

Effect of metal ions and reagents on the activity of the enzymes was studied in the presence of 1 mM of the effectors at pH 5.0 and 37°. As shown in Table I, *Aspergillus* enzyme was inactivated only by Hg^{2+}, but the other enzymes were inactivated only by N-bromosuccinimide among various reagents tested.

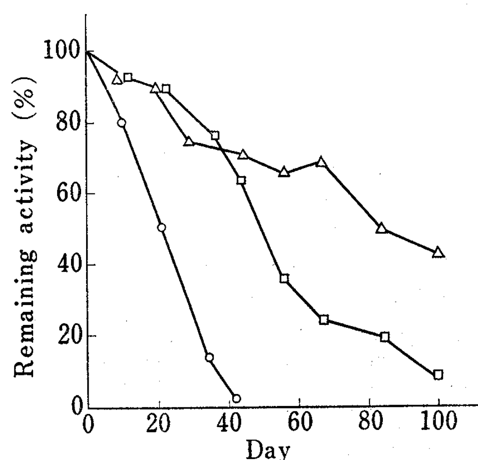

Fig. 4. Loss of the Activity of β-Galactosidases on Standing under the Condition of Relative Humidity of 92% at 30°

—○—, *Asp. oryzae*; —□—, *M. phaseoli*; —△—, *S tuliparum*.

Stability of Crude Enzyme Powder

Effects of humidity and temperature on stability of the crude enzyme powder were determined under the following conditions described by Kubo[15]: relative humidity (RH) 92% and 30°; RH 75% and 20°; and RH 52% and 5° for 100 days. All the enzymes were stable under both conditions of RH 52% and 5° and RH 75% and 20° after 100 days. As shown in Fig. 4, however, at RH 92% and 30°, the enzymes gradually lost their activities. Fifty percent of the original activity was lost after 20 days for *Aspergillus* enzyme, 50 days for *Macrophomina* enzyme and 85 days for *Sclerotium* enzyme, showing the order of the stability among these enzymes.

Stability of the Enzymes in the Presence of Human Gastric and Intestinal Juices

Stability of the enzymes in the presence of human gastric and intestinal juices was examined by incubation of the enzymes at 37° for 3 hr in the presence and absence of milk or dry milk and by assaying the remaining activity with use of ONPG as substrate. When a small amount of human gastric juice was mixed, *Aspergillus* enzyme immediately lost the activity in the absence of milk or dry milk, but *Macrophomina* and *Sclerotium* enzymes were quite stable under the conditions tested as shown in Fig. 5a. When a large amount of the gastric juice was added, *Sclerotium* enzyme was stable in the presence of milk or dry milk, and *Macrophomina* enzyme was also stable in the presence of milk. However, *Aspergillus* enzyme was unstable in the presence of milk or dry milk as shown in Fig. 5b.

When human intestinal juice was added, all the enzymes were stable in the presence of milk or dry milk as shown in Fig. 6a and 6b. However, in the absence of the substrate only *Macrophomina* enzyme was stable, whereas *Sclerotium* and *Aspergillus* enzymes were inactivated by the intestinal juice.

Substrate Specificity of the Enzymes

Hydrolysis rates of the enzymes for lactose, milk and dry milk were examined in relation to that for ONPG, and the results are shown in Table II. All the enzymes showed a similar hydrolysis rate for lactose, milk and dry milk.

Effect of Diluents on the Enzymes

An equal amount of the enzyme powder (20 mg) and a diluent was mixed, and after incubating at 40° or 60° for 30 min, 60 min and 120 min the mixture was dissolved in 400 ml

15) F. Kubo, T. Ueno, M. Horioka, B. Kobo, and T. Ishihara, *Yakuzaigaku*, **19**, 276 (1959).

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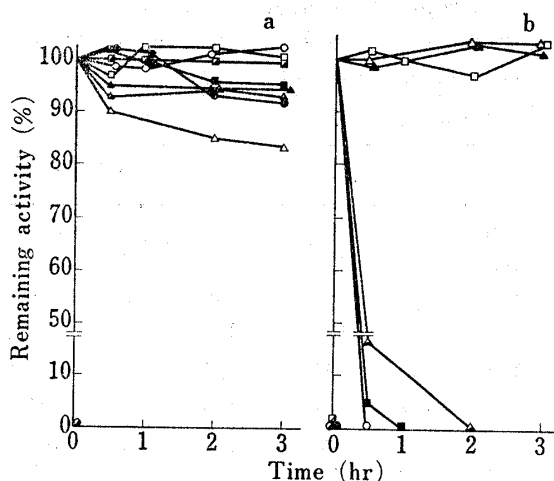

Fig. 5. Stability of β-Galactosidases in the Presence of Human Gastric Juice

Incubation at 37°; reaction mixture composed of following ratios
a: substrate:gastric juice:saline solution:β-galactosidase (5 ml : 1 ml : 4 ml : 0.5 ml).
b: substrate:gastric juice:β-galactosidase (5 ml : 5 ml : 0.5 ml).
Substrate: milk, 15% dry milk solution (or water); enzyme preparation; 4—8 mg 1 ml aliquot of the solution was withdrawn at indicated period and diluted 10-fold volume with the optimum pH's buffer and the remaining activity was determined with enzyme assay method (a).
—◑—, —○—, —●—, (substrate; none, milk, dry milk), *Asp. oryzae*, —◪—, —□—, —■—, *M. phaseoli*; —◭—, —△—, —▲—, *S. tuliparum*

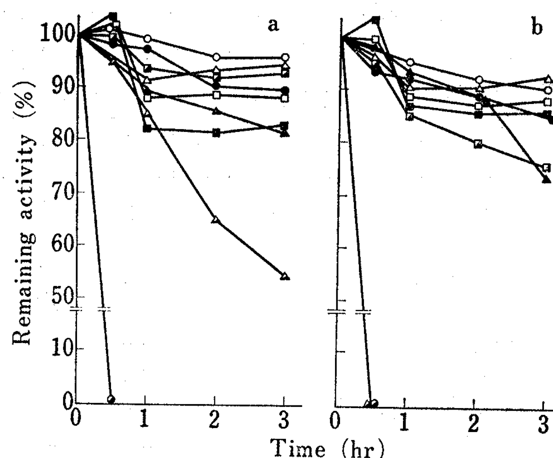

Fig. 6. Stability of β-Galactosidases in the Presence of Human Intestinal Juice

Conditions and methods were the same as decribed in Fig. 5, but human intestinal juice was used instead of human gastric juice in the experiment.
—◑—, —○—, —●—, (substrate; none, milk, dry milk), *Asp. oryzae*, —◪—, —□—, —■—, *M. phaseoli*, —◭—, —△—, —▲—, *S. tuliparum*.

Table II. Relative Rate of Hydrolysis towards Various Substrates

Substrate	Percent of relative hydrolysis rate (unit/ONPG unit)		
	Aspergillus	*Macrophomina*	*Sclerotium*
ONPG	100	100	100
Lactose	21	26	24
Dry milk	16	21	24
Milk	16	19	28

Activity towards ONPG was measured with enzyme assay (a) and that towards lactose, dry milk and milk was determined with enzyme assay (b). One unit of the enzyme activity was defined as the amount of the enzyme which liberated 1 μ mol of *o*-nitrophenol or D-glucose per minute under the reaction conditions, and the relative hydrolysis rate was expressed as percent of the activity towards ONPG.

of cold water and the remaining activity was determined by using ONPG. As shown in Table III, none of the enzymes was affected by all diluents tested.

Effects of Binders and Disintegrators on the Enzymes

An equal volume (1 ml) of 1% solution of a binder or disintegrator was mixed with 1% solution of the enzymes adjusted to pH 5.0 with 0.1 M acetate buffer (pH 5.0) and then incubated at 40° for 15 min, 30 min and 60 min. After the incubation the mixture was diluted to 100 fold volume with the cold 0.1 M HCl–AcONa buffer (pH 2.0) for *Sclerotium* enzyme and 0.1 M acetate buffer (pH 4.5) for *Macrophomina* and *Aspergillus* enzymes, and the remaining

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Table III. Effect of Diluents on Activity of β-Galactosidases

Diluent	Temp. (C°)	Remaining activity (%)								
		Aspergillus			*Macrophomina*			*Sclerotium*		
	Time (min)	30	60	120	30	60	120	30	60	120
Lactose	40	90	92	106	96	96	98	90	94	93
	60	98	93	93	102	103	103	100	98	100
Sucrose	40	101	91	102	100	95	98	87	92	91
	60	96	92	90	107	102	104	103	103	104
Starch	40	99	96	100	103	100	103	93	87	93
	60	101	95	96	103	104	104	102	69	98
Alumin. Silic.	40	103	102	114	97	101	103	100	99	102
	60	100	99	97	101	99	96	108	108	105
Kaolin	40	105	100	106	103	105	105	97	117	118
	60	94	91	90	88	100	81	90	96	92
$CaCO_3$	40	108	106	105	98	98	98	84	86	100
	60	96	95	99	105	99	97	86	105	119

activity was measured by using ONPG as substrate. As shown in Table IV, the activity of *Aspergillus* enzyme was lost immediately with aerozol, sodium dodecyl sulfate (SDS) and lowered to 50% of the original activity by incubation with polyvinyl pyrrolidone for 60 min. *Sclerotium* enzyme was also completely inactivated by incubation with aerozol and SDS. But *Macrophomina* enzyme was little affected by all the agents tested.

Table IV. Effect of Binders and Disintegrators on Activity of β-Galactosidases

Binder	Remaining activity (%)								
	Aspergillus			*Macrophomina*			*Sclerotium*		
Time (min)	15	30	60	15	30	60	15	30	60
Aerozol	0	0	0	105	97	100	0	0	0
Acacia	115	109	104	102	94	94	107	111	107
Gelatin	117	107	107	99	92	94	82	88	88
CMC	110	109	104	101	96	97	83	76	87
MC	111	97	94	97	90	92	84	102	79
SDS	11	0	0	97	92	91	0	0	0
PVP	85	65	48	98	95	92	93	97	97
PVA	108	101	100	101	93	92	102	113	112

CMC : carboxy methyl cellulose.
MC : methyl cellulose.
SDS : sodium dodecyl sulfate.
PVP : polyvinyl pyrrolidone.
PVA : polyvinyl alcohol.

Effect of Wetting Agents on the Enzymes

One ml of various wetting agent solution (30, 60 and 90%) was mixed with enzyme powder and incubated at 40° for 30, 60 and 120 min and then the agents were evaporated to dryness under a reduced pressure. After the enzyme powders were dissolved in 100 fold volume of cold water, the remaining activity was determined under usual conditions. The results are shown in Table V. *Aspergillus* enzyme was stable in high concentration but partially inactivated in low concentration of acetone and isopropanol, and markedly inactivated in all the concentration of ethanol tested. *Macrophomina* β-galactosidase was stable for all

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Table V. Effect of Wetting Agents on Activity of β-Galactosidases

Wetting agent	Conc. (%)	Remaining activity (%)								
		Aspergillus			*Macrophomina*			*Sclerotium*		
	Time (min)	30	60	120	30	60	120	30	60	120
Acetone	30	75	63	48	98	95	96	90	87	93
	60	74	57	28	92	80	76	83	69	64
	90	96	91	86	97	95	97	92	84	88
Ethanol	30	43	22	6	99	97	99	93	89	101
	60	16	9	0	71	54	35	70	70	81
	90	34	28	0	99	89	88	54	53	54
Isopropanol	30	84	68	66	98	93	90	89	94	91
	60	65	58	66	94	84	90	92	96	106
	90	99	101	89	93	87	90	104	98	109

the wetting agents tested, but lost its activity on standing in 50% ethanol with a longer period of incubation. *Sclerotium* enzyme was stable in the presence of isopropanol, 30% and 90% acetone and 90% ethanol.

Effect of Pressure on the Enzymes

One hundred mg of the enzyme powder was directly compressed with a single-stroke tabletting machine (Kimura K-2 type) with a 8 mm dies, at pressures from 0.5 to 2.0 tons. Pressure was measured with a strain gauge.[16] A compressed tablet was crushed into powder and the remaining activity was determined. As shown in Fig. 7, *Aspergillus* enzyme lost about 30% of the original activity at the pressure of 0.5 ton. *Macrophomina* and *Sclerotium* enzymes were, however, stable at 0.5 ton, although 25% of the original activity was lost at 1.0 ton.

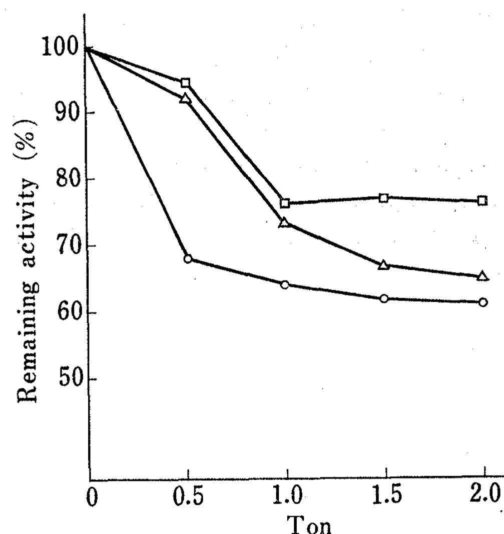

Fig. 7. Effect of Pressure on Activity of β-Galactosidases

—○—, *Asp. oryzae*; —□—, *M. phaseoli*; —△—, *S. tuliparum*.

Discussion

To utilize β-galactosidases from *M. phaseoli* and *S. tuliparum* as a therapeutic agent for lactose intolerance, the enzymatic properties were investigated from the viewpoint of pharmaceutical aspects in comparison with those of commercial β-galactosidase from *Asp. oryzae*. *Aspergillus* enzyme was stable in the pH range from 5 to 7, while *Sclerotium* enzyme in the pH range from 3 to 6 and *Macrophomina* enzyme in the pH range from 4 to 8.5. A stable pH range of *Macrophomina* enzyme was wider than the pH ranges of *Sclerotium* and *Aspergillus* enzymes. When a mixture of *Macrophomina* and *Sclerotium* enzymes is used as a therapeutic agent for lactose intolerance, ingested lactose may be more easily hydrolyzed in the stomach and intestine because of different optimum pH's and of wider stable pH range.

16) Y. Kanaya, Y. Imai, and K. Asahina, *Yakuzaigaku*, **32**, 31 (1972).

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Macrophomina and *Sclerotium* β-galactosidases were stable up to 60° and 55°, respectively. *Macrophomina* and *Sclerotium* enzymes were little affected by metal ions, but *Aspergillus* enzyme was partially inactivated by Hg^{2+}. All the reagents tested except N-bromosuccinimide did not affect these enzyme activities.

Enzymes from *M. phaseoli* and *S. tuliparum* were more stable than the enzyme from *Asp. oryzae* at RH 92% and 30°, but none of the enzymes showed loss of the activity under the conditions of RH 75% and 20°, and RH 52% and 4°. Moreover, *Macrophomina* and *Sclerotium* enzymes were also more stable than *Aspergillus* enzyme in the human gastric and intestinal juices. Hydrolysis rates of various substrates by *Macrophomina* and *Sclerotium* enzymes were similar to the hydrolysis rate of *Aspergillus* enzyme. These enzymes were not inactivated by any diluents. Among the binders tested, *Aspergillus* and *Sclerotium* enzymes were completely inactivated by aerozol and SDS, and the former was also partially inactivated by polyvinyl pyrrolidone. However, *Macrophomina* enzyme was quite stable to the binders tested. *Macrophomina* enzyme was also stable to all the wetting agents tested under the various conditions, but *Sclerotium* enzyme maintained the activity in 30% ethanol, 30% and 60% acetone and isopropanol, and *Aspergillus* enzyme completely lost the activity by ethanol and was partially inactivated by 30% and 60% acetone and 30% and 60% isopropanol. *Macrophomina* and *Sclerotium* enzymes were also stable in the pressure at 0.5 ton, whereas *Aspergillus* enzyme lost 30% of the original activity at 0.5 ton.

In summary, *Macrophomina* and *Sclerotium* enzyme preparations were more stable at temperature and pH, and also to metal ions, reagents, human gastric and intestinal juices, and various pharmaceutical factors than *Aspergillus* enzyme. Both enzymes hydrolyzed lactose and also lactose in milk or dry milk with the same rate as *Aspergillus* enzyme. From these results, it is suggested that the *Macrophomina* and *Sclerotium* β-galactosidase can be effectively utilized as a therapeutic agent for lactose intolerance.

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