KOREAN J. FOOD SCI. TECHNOL.
Vol. 12, *No.* 1 (1980)

Hydrolysis of Lactose in Milk by Microencapsulated β-Galactosidase

Ok Ryun Baik, Rosa Uy* and Si Myung Byun
Department of Biological Science and Engineering
The Korea Advanced Institute of Science
(Received November 20, 1979)

Microencapsulated β-Galactosidase에 의한 우유 유당의 가수 분해

백 옥 련 · 로-사 위* · 변 시 명
한국 과학원, 생물 공학과
(1979년 11월 20일 수리)

Abstract

The nylon (poly 6, 10) microcapsules containing β-galactosidase were obtained by the interfacial polymerization of 1, 6-diaminohexane and sebacoyl chloride with β-galactosidase from *Escherichia coli*. They were generally spherical and had a mean diameter of 80 μ with 45 % of the activity recovery. In particular, there was no transport hamper of lactose through the membrane of microcapsules.

The characteristics of the microencapsulated enzyme were similar to those of soluble enzyme: optimal pHs, 7.0~7.2 for the soluble and 7.3~7.5 for the microencapsulated; optimal temperatures, 50°C for both; apparent K_m, 3.33×10^{-4}(on ONPG), 2.86×10^{-3} *M*(on lactose) for the soluble and 5.28×10^{-4} (on ONPG), 4.25×10^{-3} *M* (on lactose) for the microencapsulated; activation energies, 8.94 for the soluble and 9.78 Kcal/*mole* for the microencapsulated enzyme.

Using this microencapsulated β-galactosidase, hydrolyses of lactose and milk lactose were carried out and 80 % of 5 % lactose solution and 70 % of lactose in skim milk were hydrolyzed in 40 hr at 27°C. The reusability and operational stability showed that the remaining activity was 50 % of the original activity after 5 runs and 120 hr of total operating time at 27°C.

Introduction

Interests in immobilized β-galactosidase has been generated by nutritional and sensory qualities of dairy products or by-products. Many non-Caucasians show an intolerlance to lactose[1]. In addition, milk system offers ideal media for studying the use of immobilized enzyme in food processing.

Although the immobilization of β-galactosidase

* *3M Company Ltd., St. Paul, Minnesota, U.S.A.*

was studied by many investigators[2~8], application of the immobilized enzyme for lactose hydrolysis in milk system has been limited[3,4,6] and their products might be affected by the inhibition of milk constituents, such as galactose, galactosamine and glycopeptides[9].

Microencapsulation technique is also applied for enzyme which can be used in food industry. Immobilization of enzyme using a proper microencapsulation technique gives not only higher yield of enzyme activity, but also highly semipermeable membrane product[10]. The membrane does not allow the enzyme to leak out but allows the entry of substrate to be acted on by the closed enzyme. The product formed can leave the microcapsule, if they are small enough to compare with pore size of the membrane.

In this research, the immobilization techniques of β-galactosidase by microencapsulation in nylon membrane were developed and the microencapsulated enzyme was characterized with the comparison of soluble β-galactosidase. Lactose in milk was hydrolyzed by using this microencapsulated β-galactosidase.

Materials and Methods

Materials

The enzyme, β-galactosidase was purified from *E. coli* YUFE-B-7 by the procedure of affinity chromatography which will be presented elsewhere. Sebacoyl chloride, 1, 6-diaminohexane and terephthaloyl chloride were purchased from Aldrich (Milwaukee, WI) ; glutaraldehyde from Eastman Kodak Co. (Rochester, NY) ; Glucostat and hemoglobin from Worthington Biochem. Corp. (Freehold, NJ) ; o-nitrophenyl-β-*D*-galactopyranoside (ONPG) from Sigma Chem. Co. (St, Louis, MO) ; Tween 20 and Span 85 from ICI U.S. Inc. (Wilmington, DL). All other chemicals used in this work were analytical grades from Wako Pure Chem. Industry (Tokyo).

Microencapsulation

The preparation of nylon (poly 6, 10) microcapsule containing β-galactosidase was carried out according to the method of Chang *et al.*[10,11]. To the mixed solution containing 2. 2*ml* of 10 % hemoglobin solution, 0. 3 *ml* of β-galactosidase (2. 5 *mg* protein/*ml*) and 2. 5 *ml* of 0. 4 *M* 1, 6-diaminohexane in 0. 45 *M* sodium bicarbonate buffer, pH 9. 8, was added 25 *ml* of the mixed solvent of chloroform and cyclohexane (1 : 4, v/v) containing 1 % Span 85. The mixed solution was then mechanically emulsified at 4°C for 1 min. With stirring, 25 *ml* of the mixed solvent containing 0. 018 *M* of sebacoyl chloride was added and stirred for 3 min. The resulting microcapsules were collected by centrifugation, then washed with 25 *ml* of Tween 20 (50 %, v/v in water) and 0. 9 % NaCl solution several times. Fig. 1 shows the preparation of microencapsulated β-galactosidase.

2. 2 *ml* of 10 % Hb solution
0. 3 *ml* of β-galactosidase (2. 5 *mg* protein/*ml*)
2. 5 *ml* of 0. 4 *M* 1, 6-diaminohexane in 0. 45 *M* sodium bicarbonate buffer, pH 9. 8
↓ Shake for 10 sec at ice bath in 250 *ml* beaker gently
25 *ml* of "mixed solvent", chloroform : cyclohexane (1 : 4) containing 1 % Span 85 added
↓ Stir for 1 min at speed setting 65 of Pyro-Magnester (Lab-Line) with 5 *cm* bar
25 *ml* of 0. 018 *M* sebacoyl chloride solution (0. 1 *ml* in 25*ml* of mixed solvent) added
↓ Continue stirring for 3 min at speed setting 65
Stop reaction by adding 50 *ml* of mixed solvent
↓ Centrifuge at 350×g for 15 sec and remove supernatant
PPT saved
↓ Add 25 *ml* of Tween 20 (50 %, in water) Stir for 30 sec at speed setting 65
Decrease speed to 30 and 50 *ml* of water added
Stir further 30 sec
Centrifuge at 350×g for 1 min
Remove supernatant
PPT saved
↓ Wash with 0. 9 % cold NaCl several times
Suspend in 20 *ml* of 0. 9 % NaCl solution and store at 4°C

Fig. 1. Preparation of microencapsulated β-galactosidase

Stabilization and cross-linking of microencapsulated enzyme

The microcapslued enzymes were crosslinked by the method of Chang[10]. To a 20 *ml* suspension of microcapsules, 100 *ml* of the mixture of 0. 1 *M* sodium metaborate and 0. 001 *M* benzamidine HCl and 200 *μl*

of 50 % glutaraldehyde were added. The suspension was kept slightly agitated at 20°C for 1 hr and treated with 100 *ml* of 0.05 *M* sodium borohydride. After suspension was left at 4°C for 20 min, the supernatant was centrifuged off and the microcapsules were washed twice with 200 *ml* of 0.9 % saline solution.

Enzyme assay and kinetic analysis

The activity of β-galactosidase was assayed with a modification of the procedure of Worthington manual[12], in which colorless substrate ONPG is hydrolyzed to form galactose and yellow o-nitrophenol. The reaction volume was 3.0 *ml*, which contained 0.5 *ml* of 0.014 *M* ONPG in 0.01 *M* Tris-acetate buffer, pH 7.5 containing 0.01 *M* $MgCl_2$, 1.0 *ml* of 0.3 *M* sodium phosphate buffer, pH 7.5 containing 0.03 *M* $MgCl_2$, 0.3 *ml* of 1.0 *M* mercaptoethanol, 1.1 *ml* of distilled water and 0.1 *ml* of enzyme solution. The reaction mixture was incubated for 5 min at 37°C and the reaction was stopped by adding 1.0 *ml* of 1 *M* sodium carbonate to the reaction mixture. The absorbancy was measured at 405 nm against to a reagent blank.

With lactose as substrate, the glucose liberated during hydrolysis was estimated with Glucostat according to the Worthington manual[12].

For the microencapsulated β-galactosidase, the reaction mixture was stirred with a submergible magnetic stirrer and filtered with Whatman No. 1 filter paper. The resulting products were determined by the same procedure described in the soluble β-galactosidase.

The kinetic studies were carried out by the method of Byun *et al.*[13] with the soluble and microencapsulated enzymes using comparable amounts of activity as determined under the standard conditions.

The hydrolysis of standard lactose and lactose in skim milk

In order to hydrolyze lactose in milk by microencapsulated enzyme, skim milk was used as substrate and compared with the hydrolysis of lactose solution. Twenty two *g* of skim milk was dissolved in 200 *ml* of 0.05 *M* sodium phosphate buffer, pH 7.5 and the microcapsule (10 units of activity on lactose at 37°C) were added. The reaction mixture was slightly agitated at 27°C. At given times, each of 4 *ml* aliquots of hydrolyzate was taken and the reaction was stopped and milk protein was coagulated by adding 1 *ml* of 4.2 % perchloric acid. The slurry was filtered and glucose produced by the enzyme was determined by using Glucostat. Hydrolysis of lactose solution was performed at the same condition except for the use of 5 % lactose solution instead of skim milk.

Operational stability of microencapsulated β-galactosidase

The microencapsulated β-galactosidase in buffered 5 % lactose solution was incubated at 27°C for 24 hr. After incubation, microcapsules were recovered by filtration and washed with 0.9 % saline solution thoroughly. The remaining β-galactosidase activity was determined using ONPG as substrate at the standard assay condition described in the previous assay procedure. Recovered microcapsules were treated as above procedure repeatedly for several times.

Results

Microencapsulation

Fig. 2 shows the microencapsulated enzyme which was observed by phase microscope and Cambridge electron microscope. Microcapsules, prepared by the method described, had a mean diameter of 80 μ and a membrane thickness of approximately 0.02 μ. The equivalent pore diameter in the membrane of such microcapsules was approximately 34 Å. The resulting microcapsules were observed as very smooth round spheres in aqueous solution or in 0.9 % saline solution under the microscope (Fig. 2-A). Acetone treated or high salt-treated capsules, however, were shrinked the surface of membrane (Fig. 2-B), which were recovered again as smooth round forms, when they were suspended in water. The size of spheres was relatively homogeneous. It became smaller, however, when the capsules were prepared by higher concentration of emulsifying agent, Span 85 and by faster mechanical stirrer (speed of magnetic stirrer).

The recovered activity as the microencapsulated enzyme was 45 % of the starting enzyme activity used in the preparation. The permeability to protein

of the nylon microcapsule membrane was almost negligible. For the periods of storage, up to a month or so, leakage of enclosed hemolyzate and enzyme from microcapsules stored in 0.9% saline solution were essentially negligible.

It has been reported that enzyme stability is greatly increased when urease, asparaginase and catalase are microencapsulated with a high concentration of hemoglobin[10]. For β-galactosidase, however, no advantages of storage stability were observed. The microcapsule which was prepared with terephthaloyl chloride showed no differences from that with sebacoyl chloride.

The microencapsulated enzyme which was treated with glutaraldehyde had 10% of the β-galactosidase activity of the untreated microencapsulated enzyme and storage stability at 4°C was slightly improved. However, glutaraldehyde-treated microcapsule containing catalase shows great improvement of storage stability and recovered activity is one third of untreated microcapsule[11].

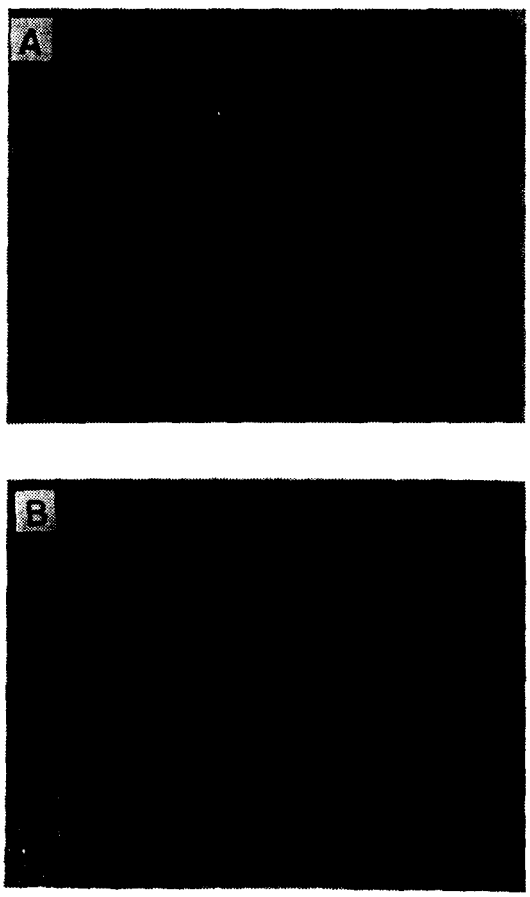

Fig. 2. A : Phase microscopic photograph of nylon (poly 6, 10) microcapsules containing β-galactosidase
B : Electron microscopic photograph of nylon (poly 6, 10) microcapsule containing β-galactosidase

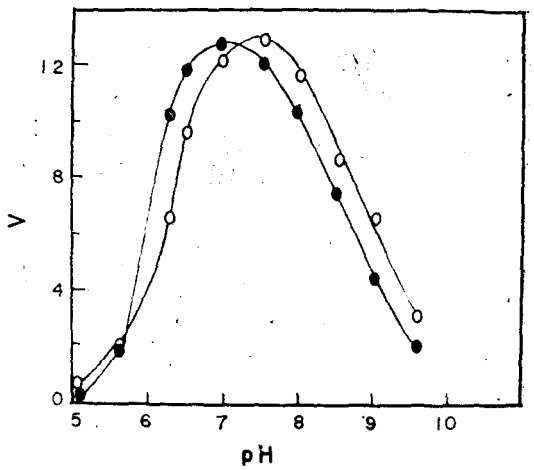

Fig. 3. pH profile of soluble and microencapsulated β-galactosidases on *o*-nitrophenyl-β-*D*-galactopyranoside (OPNG)
●——● : Soluble β-galactosidase
○——○ : Microencapsulated β-galactosidase

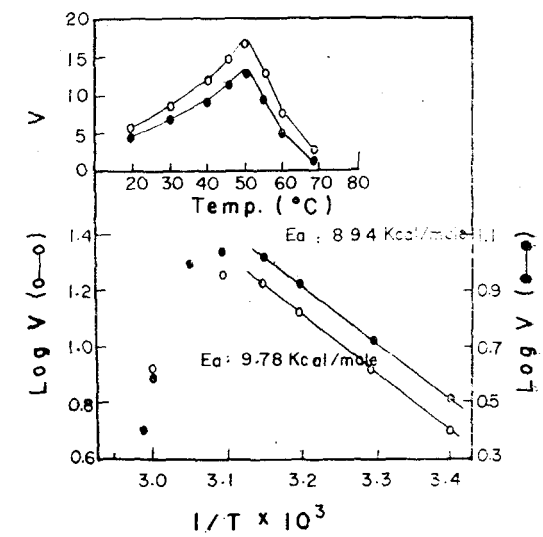

Fig. 4. The effects of temperature on ONPG hydrolysis by soluble and microencapsulated β-galactosidases
●——● : Soluble β-galactosidase
○——○ : Microencapsulated β-galactosidase

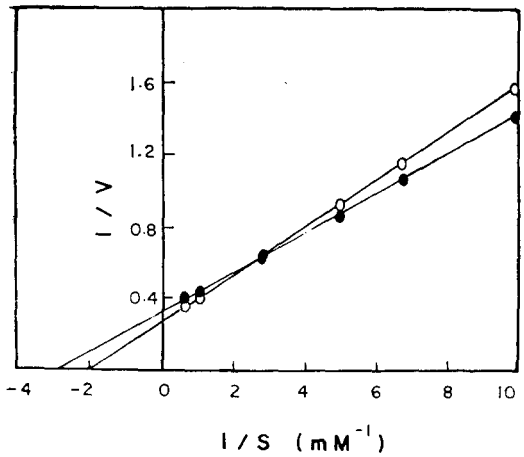

Fig. 5. Lineweaver-Burk plots for hydrolysis of ONPG by soluble and microencapsulated β-galactosidases
●——● : Soluble β-galactosidase
○——○ : Microencapsulated β-galactosidase

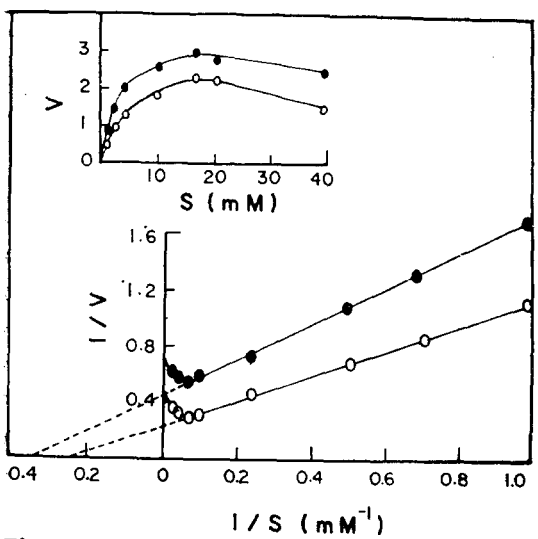

Fig. 6. Effects of substrate concentrations on lactose hydrolysis
●——● : Soluble β-galactosidase
○——○ : Microencapsulated β-galactosidase

Kinetic analysis

Fig. 3 shows the pH dependence of the relative rate of hydrolysis of ONPG with the microencapsulated and soluble β-galactosidases. There is a small shift in the optimum pH for reaction from 7.0~7.2 for the free enzyme to about 7.3~7.5 for the microencapsulated enzyme.

Fig. 4 shows the temperature effect on the micro-

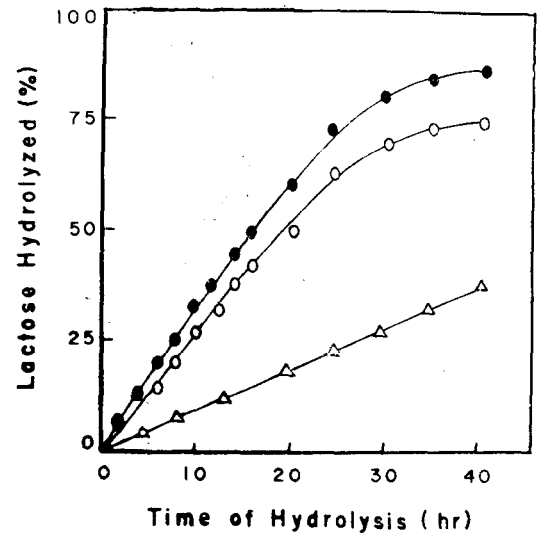

Fig. 7. Hydrolyses of 5% lactose solution and lactose in skim milk at 27°C, pH 6.5
●—●—● : 10 units of microencapsulated β-galactosidase in 5% lactose solution
△—△—△ : 4 units of microencapsulated β-galactosidase in 5% lactose solution
○—○—○ : 10 units of microencapsulated β-galactosidase in skim milk solution

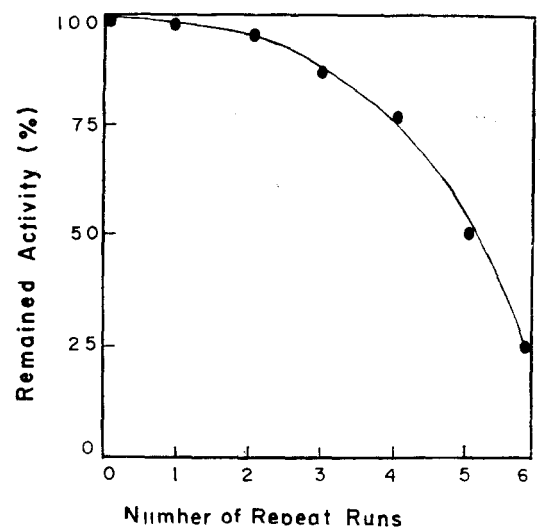

Fig. 8. Reusability of microencapsulated β-galactosidase in batch reactor containing 5% lactose solution at 27°C
One batch was continued for 24 hr

Table 1. Kinetic results of soluble and microencapsulated β-galactosidases

Enzyme	Substrate	Optimum pH	Optimum temperature (°C)	Activation energy (Kcal/*mole*)	K_m (M)
Soluble	ONPG	7.0~7.2	50	8.94	3.33×10^{-4}
	Lactose				*2.86×10^{-3}
Microencapsulated	ONPG	7.3~7.5	50	9.78	5.28×10^{-4}
	Lactose				*4.25×10^{-3}

* Modified Michaelis constant, K'_m

encapsulated and soluble β-galactosidases. The results indicated that the optimum temperatures for both microencapsulated and soluble β-galactosidases were 50°C. The apparent activation energies (*Ea*) were calculated to be 8.94 Kcal/*mole* for the soluble enzyme and 9.78 Kcal/*mole* for the microencapsulated enzyme.

The effects of substrate concentration on the enzyme activity were investigated. As shown in Fig. 5, the ONPG hydrolysis by the soluble and microencapsulated enzymes showed a Michaelis-Menten type kinetics. *Km's* on ONPG which were calculated from Lineweaver-Burk plots for the soluble and microencapsulated β-galactosidases were 3.33×10^{-4} and 5.28×10^{-4} *M*, respectively.

For lactose hydrolysis reaction, however, the hyperbolic law was not obeyed and this type was caused by substrate inhibition[14]. Modified Michaelis constants (K'_m) on lactose calculated from Lineweaver-Burk plots (Fig. 6), for the soluble and microencapsulated enzymes were 2.86×10^{-3} and 4.25×10^{-3} *M*, respectively.

Kinetic properties of the soluble and microencapsulated β-galactosidases are summarized in Table 1.

Hydrolysis of standard lactose and lactose in skim milk

One of the major considerations in evaluating feasibility of using β-galactosidase in hydrolyzing lactose is the rate and extent to which the disaccharide is cleaved. Fig. 7 shows the rate and limiting of hydrolysis of lactose solution and skim milk by the microencapsulated β-galactosidase. The result showed that 80 % of lactose present in a buffered 5 % lactose solution was hydrolyzed within 40 hr by 10 units of the microencapsulated enzyme at 27°C. In skim milk, 70 % of the lactose was hydrolyzed in 40 hr at 27°C.

Operational stability of microencapsulated β-galactosidase

In practical use, operational stability and reusability of a microencapsulated enzyme are more important than storage stability. The reusability is shown in Fig. 8. The remained activity was 50 % of initial activity after 5 runs. Giacin *et al.*[2] reported that the activity of collagen immobilized lactase was equivalent to 24 % of the initial activity after 5 runs.

Discussion

Since Chang *et al.*[10,11] have developed the microencapsultation procedure, varieties of membranes, techniques and uses have been studied. Especially nylon membrane by interfacial polymerization has aroused many worker's interest, which has good properties of strong mechanical strength and ultrathin membrane and the large surface to volume relationship.

In microencapsulation, emulsifying agents or detergents are very important. The detergent used in the preliminary emulsification stage and the final transfer stage are the oil soluble type (Span 85) and water soluble type (Tween 20), respectively. These nonionic materials appear to cause little damage to the dissolved proteins.

The droplet size in mechanically prepared emulsion is never uniform and the diameter of the microcapsules in any batch varies over a fairly wide range. With the ingredients and procedures, the diameter is determined mainly by the speed of the mechanical emulsifier and by the concentration of the emulsi-

fying agent[11]. At the high speeds of mechanical emulsifier, diameters tended to be smaller and more uniform. To increase the concentration of emulsifying agent (Span 85) to 5 %, decreased the diameters of microcapsules, but above 5 % had little effect on microcapsule size.

According to Østergaard *et al.*[15], it is possible to the produce both stable and active microcapsules by using the more reactive terephthaloyl chloride instead of sebacoyl chloride. Because we thought that the high pH of alkaline diamine solution may inactivate β-galactosidase, we prepared the microcapsules using terephthaloyl chloride at lower pH than 11. As results, however, terephthaloyl chloride was not more effective than sebacoyl chloride on stability of microcapsule and recovery of activity.

Glutaraldehyde, bifunctional reagent, reacts with α-amino group of amino acids, the N-terminal amino group of some peptide and ε-amino group of lysine. The stability of microencapsulated enzyme can be increased by high concentration of protein and crosslinking with glutaraldehyde after microencapsulation. But in this experiment, crosslinked microcapsule by glutaraldehyde had only 10 % of original activity of microcapsule, which is very low in compared to catalase or asparaginase (1/3 of original activity). This low yield seemed to be caused by the fact that sulfhydryl, phenolic, imidazole groups are partially reactive with glutaraldehyde[16] and those sulfhydryl and imidazole group in active site reacted with glutaraldehyde.

In hydrolysis of lactose, both lactose solution and milk (or whey) can be treated with the microencapsulated β-galactosidase effectively. Fig. 6 shows that 70 % of lactose in milk can be hydrolyzed with 87.5 % efficiency when hydrolysis degree of lactose is assumed as 100. The efficiency of 87.5 % by the microcapsuled enzyme can be compared to that of between 50~60 % with collagen bound enzyme[17]. A comparison of the activity of the collagen bound β-galactosidase showed partial, irreversible inhibition in whey as compared to lactose. The inhibitory effect of milk constituents has been reported by Harper *et al.*[9]. The inhibitors, galactose, galactosamine, glycopeptide and milk salts are found in whey. Glycopeptide and milk salts are not galactoside but inhibit the activity of immobilized β-galactosidase non-reversibly[2]. The microencapsulated β-galactosidases are affected by galactosamine, galactoses and milk salts, but small pore size of membrane of the capsule prevents passing the large glycopeptide. The cause of the inhibition, reversible or irreversible, is not readily apparent and further studies are required to characterize the whey constituent and/or constituents responsible for the inhibition. The differences in inhibitory action can also be studied to help elucidate the operation of enzymes in the immobilized state.

요 약

*E. coli*로 부터 분리 정제한 β-galactosidase를 1,6-diaminohexane과 sebacoyl chloride를 사용하여 계면 중합 반응에 의하여 나일론 막에 microencapsulation 시켜 고정화 시켰다. 얻어진 microcapsule은 구형이었고 평균 직경이 80 μ이었으며 이 방법에 의하여 microencapsulation 시킨 β-galactosidase의 효소 역가 효율은 45 %이었다. 유당의 막 투과는 거의 완전하게 이루어졌다.

Microencapsulation 시킨 β-galactosidase의 성질은 가용성 효소와 거의 비슷하였고 최적 pH는 7.0~7.2에서 7.3~7.5로 약간 이동 하였으며, 최적 온도는 50°C, K_m 값은 *o*-nitrophenyl-β-*D*-galactopyranoside(ONPG)와 유당에 대하여 가용성 효소는 각각 3.33×10^{-4} 및 $2.86\times10^{-3}M$이었고 고정화 효소는 5.28×10^{-4} 및 $4.25\times10^{-3}M$이었다. 활성화 에너지는 가용성 효소는 8.94와 고정화 효소는 9.78 Kcal/*mole*이었다.

이 고정화 효소를 사용하여 5 % 표준 유당 용액과 탈지 우유에 존재하는 유당을 가수 분해한 결과 40시간 안에 각각 80 및 70 %씩 가수 분해 하였다. 또한 공정중의 고정화 효소의 안정성을 살펴 본 결과 27°C에서 한번에 24 시간씩 5번 사용 후 남아 있는 역가는 50 %로서 실제 이용상 긍정적인 결과를 나타내었다.

Acknowledgement

Authors are thankful for a help of Dr. F. Wold, Department of Biochemistry, University of Minnesota, St. Paul, Minnesota 55104, to take picture of electron microscope of the microencapsulated β-galacto-

sidase.

References

1. Huang, S. S. and Bayless, T. M. : *Science*, **160**, 83 (1968)
2. Giacin, J. R., Jakubowski, J., Leeder, J. C., Gilbert, S. G. and Kleyn, D. H. : *J. Food Sci.*, **39**, 751 (1974)
3. Woychik, J. H. and Wondolowski, M. V. : *Biochem. Biophys. Acta*, **289**, 347 (1972)
4. Woychik, J. H. and Wondolowski, M. V. : *J. Milk Food Technol.*, **36**, 31 (1973)
5. Dahlqvist, A., Mattiason, B. and Mosbach, K. : *Biotech. Bioeng.*, **15**, 395 (1973)
6. Olson, A. C. and Stanley, W. L. : *J. Agr. Food Chem.*, **21**, 440 (1973)
7. Okos, E. S. and Harper, W. J. : *J. Food Sci.*, **39**, 88 (1974)
8. Vorsilak, P. and McCoy, B. J. : *J. Food Sci.*, **40**, 131 (1975)
9. Harper, W. J., Okos, E. and Baisdell, J. L. : *Enzyme Engineering*, Vol. 2, Pye, E. K. and Wingard, Jr., L. B. Ed., Plenum Press, p. 289 (1974)
10. Chang, T. M. S. : *Biochem. Biophys. Res. Comm.*, **44**, 1501 (1971)
11. Chang, T. M. S., MaCintosh, F. C. and Mason, S. G. : *Can. J. Physiol. Pharmacol.*, **44**, 115 (1964)
12. Worthington Biochem. Crop. : *Worthington Enzymes, Enzyme Reagents* p. 9 and p. 106, Worthington Biochem. Corp., NJ (1972)
13. Byun, S. M. and Wold, F.: *J. Korean Food Sci. Technol.*, **5**, 253 (1976)
14. Laidler, K. J. and Bunting, P. S. : *The Chemical Kinetics of Enzyme Action*, Clarendon Press, p. 84 (1973)
15. Østergaard, J. C. W. and Marling S. C. : *Biotech. Bioeng.*, **15**, 561 (1973)
16. Habeeb, A. F. S. A. and Hiramoto, R. : *Arch. Biochem. Biophys.*, **126**, 16 (1968)
17. Jakubowski, J., Giacin, J. R., Kleyn, D. H., Gilbert, S. G. and Leeder, J. G. : *J. Food Sci.*, **40**, 467 (1975)