0273-2289/97/6601-0057$10.75

Immobilization of Isoamylase on Carboxymethyl-Cellulose and Chitin

HWAI-SHEN LIU,* WEI-HSU CHEN, AND JINN-TSYY LAI

Department of Chemical Engineering, National Taiwan University, Taipei, Taiwan, ROC

Received September 11, 1995; Accepted February 22, 1996

ABSTRACT

Isoamylase, a starch debranching enzyme capable of hydrolyzing α-1,6-glucosidic linkage, was immobilized on CM-cellulose and chitin. The immobilization on chemically modified CM-cellulose (CM-cellulose azide) resulted in a specific activity of 1422 U/g-CMCI (CM-cellulose-isoamylase), 24% activity retention, and an optimal pH of 4.0. The immobilization of isoamylase on glutaraldehyde treated chitin gave 1638 U/g-CI (chitin-isoamylase), 46% activity retention, and an optimal pH of 2.4. The kinetic data (K_m) indicated that CI (0.69 g/L) has similar mass transfer resistance to free enzyme (0.67 g/L), whereas CMCI (3.57 g/L) has much greater transport resistance.

Index Entries: Isoamylase; CM-cellulose; chitin; immobilization; kinetics.

INTRODUCTION

Immobilization of enzymes has been recognized for its advantages, such as the reuse of expensive catalyst, simplification of downstream process, and the easy adaptation for continuous operation. Sometimes, immobilization can also provide better microenvironment for enzymes activity enhancing better thermal and operational stability *(1)*. Generally, there are several ways to immobilize enzymes on various supports. These includes physical adsorption, chemical attachment, and entrapment within a matrix and membrane. Chemical attachment is noted for its strong binding force at the expense of relatively high cost, compared with physical adsorption. Enzymes often bind to the support through the func-

*Author to whom all correspondence and reprint requests should be addressed.

tional groups of amino carboxyl, amino, and hydroxyl groups whereas the surfaces of supports frequently need to be activated by chemical agents, such as glutaraldehyde and cyanogen bromide.

Isoamylase (EC 3.2.1.68, glycogen 6-glucanohydrolase) is a debranching enzyme that catalyzes the hydrolysis of α-1-6-glucosidic linkages in amylopectin and glycogen. It has been produced by *Pseudomonas, Cytophaga, Bacillus* species, and so on. Isoamylase was applied to analyze the structure of glycogen and amylopectin *(2)* cyclomalto-oligosaccharides *(3)*, and the surfaces of starch granules *(4)* Compared with the other debranching enzyme, pullulanase, both have similar catalytic capability in hydrolysis of amylopectin, whereas isoamylase have better conversion when glycogen and β-limit dextrin are used as substrates. This is perhaps because that isoamylase could hydrolyzes both inner and outer branching points, whereas pullulanase cannot hydrolyze inner branching points *(5.)* In addition, isoamylase has higher activity, irreversible catalytic reaction, and is not inhibited by maltose. Because of these unique properties, isoamylase is useful to enhance the yield of glucose and maltose from starch *(6)* in practical industrial application.

Cellulose and its derivatives, the most abundant biopolymer on the earth, are widely used as carriers for immobilization because of its stability and inertness. They can be used directly as a physical adsorbent as well as modified for chemical binding. In many cases, the hydroxyl groups of cellulose or the carboxyl groups of CM-cellulose are activated for chemical attachment. The binding force of chemical covalent bond is strong, though the loss of enzymatic activity may be a concern. On the other hand, the second most abundant biopolymer, chitin, is a homopolymer of *N*-acetyl-glucosamine linked by β-(1–4) bonds. It is chemically similar to cellulose, which is a polymer of glucose linked also by β-(1–4) bonds. However, chitin is thought to be more biocompatible than cellulose, because of its structural difference in carbon-(2), which make it more suitable for enzyme immobilization. It was also reported *(7)* that a low pressure drop associated with chitin packed bed would be an advantage for continuous operation.

In this work, the immobilization of crude isoamylase from *Pseudomonas amyloderamosa* on chemically modified CM-cellulose (i.e., CM-cellulose azide) and glutaraldehyde-activated chitin were investigated. The motivation derives from the high cost and the commercial importance *(8)* of isoamylase as well as there has been no information concerning the immobilization of isoamylase available so far.

MATERIALS AND METHODS

Microorganism

Pseudomonas amyloderamosa WU7211-2 *(9)* (Culture Collection and Research Center, HsinChu, Taiwan) from JD210 by UV and NTG mutage-

nesis was cultivated to produce isoamylase in the medium containing maltose (2.5%), Proteimax HE90 (0.8%), $MgSO_4$ $7H_2O$ (0.05%), KH_2PO_4 (0.2%), and Glucose (0.5%). This strain can secret isoamylase into the culture medium extracellularly, and no other amylase can be detected in the culture supernant. Therefore, the fermentation broth after cultivation for 48 h was centrifuged (4000*g* for 20 min) to remove cells and the crude isoamylase was obtained for immobilization studies.

Preparation of CM-Cellulose-Isoamylase (CMCI)

The CM-cellulose (Sigma, St. Louis, MO) was modified to CM-cellulose azide for immobilization according to Mitz and Summaria *(10.)* Crude isoamylase is then added with freshly made CM-cellulose azide in a buffer stirred at 340 rpm to prepare CMCI. CMCI is then filtered off and washed several times with deionized water. The pH, temperature, reaction time of this immobilization step, binding between isoamylase and CM-cellulose azide, were subjected to investigation.

Preparation of Chitin-Isoamylase (CI)

Chitin (Sigma, 20–50 mesh) was pretreated to remove minerals, proteins, and pigment according to Stanley et al. *(11).* Pretreated chitin was activated by glutaraldehyde with acetic acid as a catalyst. Because glutaraldehyde may deactivate the enzyme, immobilization studies were carried out in two ways: by adding glutaraldehyde or isoamylase together (one-step) and separately (two-step). For the two-step method, chitin and glutaraldehyde were shaken at 500 rpm, room temperature overnight. The glutaraldehyde treated chitin was then washed and then mixed with isoamylase at 4°C overnight. The one-step method involved mixing chitin, glutaraldehyde, and isoamylase at the same time also at 4°C overnight. The concentration of glutaraldehyde and pH were the variables subjected to investigation.

Isoamylase Activity

The isoamylase activity (free enzyme) was assayed according to Harada et al. *(12)* with a slight modification. The reaction mixture consisted of 350 µL of 0.5% corn amylopectin (Sigma), 100 µL 0.1*M* acetate-HCl buffer (pH 3.5), and 50 µL enzyme solution. After incubation at 40°C for 10 min, the reaction was terminated by the addition of 500 µL 0.01*N* I_2-KI solution in 0.1 N HCl and then the reaction volume was diluted to 10 mL with deionized water. The optical density at 610 nm was then measured against a blank containing 350 µL of 0.5% corn amylopectin and 150 µL buffer. One unit (U) of isoamylase activity is expressed as the amount of enzyme causing an 0.01 increment in absorbance per minute. For the specific activity of immobilized isoamylase (both CMCI and CI), 140 mL of 0.5% corn amylopectin and 60 mL buffer was mixed. Then, the proper amount of immobi-

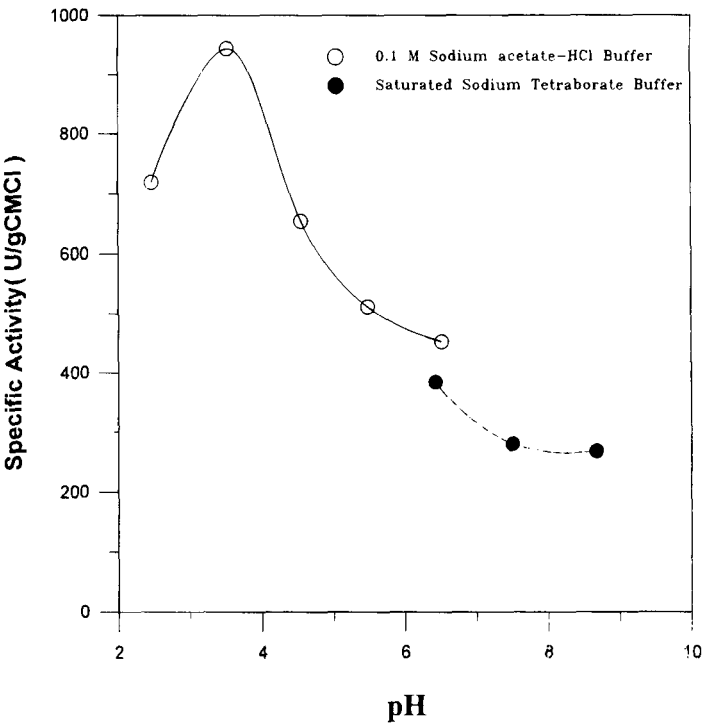

Fig. 1. The pH effect on covalent immobilization of isoamylase on CM-cellulose azide.

lized isoamylase was added and kept at 40°C, 175 rpm for 10 min. The suspension was filtered (Whatman, #1001055) and 500 µL of filtrate was stained with 500 µL 0.01*N* I_2-KI solution in 0.1 N HCl. The solution of iodine-amylopectin complex was then diluted to a total volume of 10 mL for optical density measurement at 610 nm. The disturbance caused by filtration was predetermined and corrected.

RESULTS AND DISCUSSION

Immobilization of Isoamylase with CM-Cellulose Azide

Figure 1 shows the effect of pH on the "effective" chemical binding between CM-cellulose azide and isoamylase, expressed as the specific activity of CMCI. The discontinuity of the curve is because of the different buffer systems in acidic and alkaline regions. It can be seen that the optimal pH for immobilization reaction is about 3.5. Temperature effect on the binding efficiency is also shown in Fig. 2. It can be seen that the specific activity of CMCI falls sharply between 5 and 10°C. The preference to low temperature for immobilization with CM-cellulose azide was frequently noted in literature *(13,14)*. Figure 3 shows the specific activity of CMCI for various time periods during the immobilization at pH 3.5 and 0°C. The specific activity of CMCI seems to reach a saturated value around 80 min

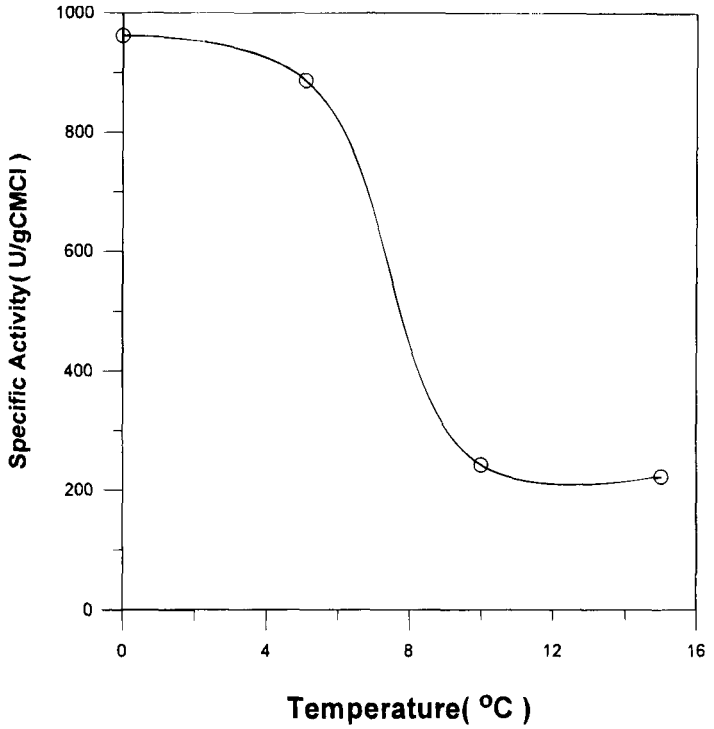

Fig. 2. The temperature effect of covalent immobilization of isoamylase on CM-cellulose azide.

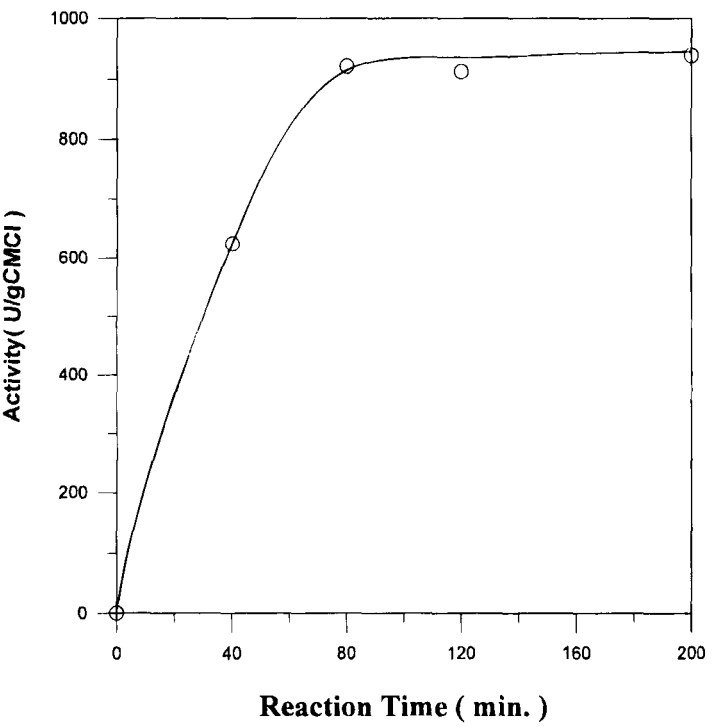

Fig. 3. The covalent immobilization of isoamylase on CM-cellulose azide for various time periods.

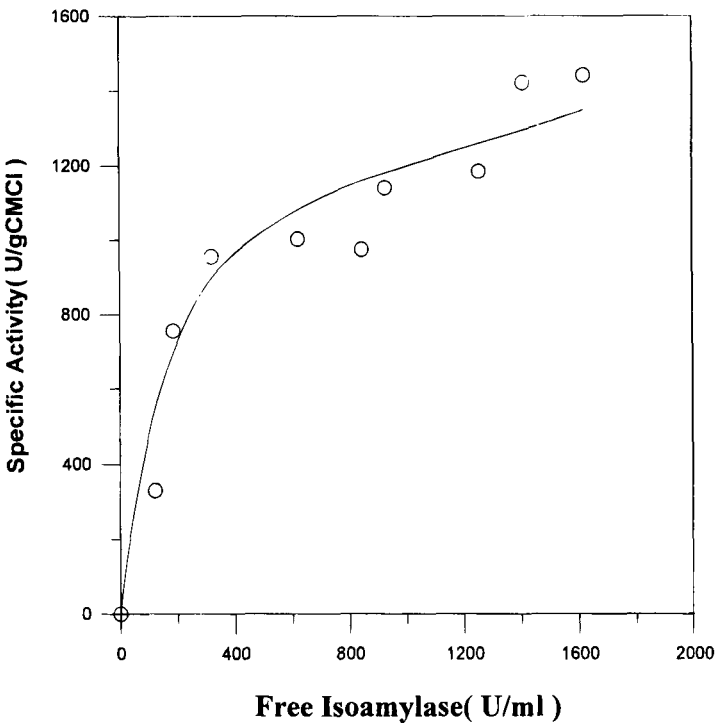

Fig. 4. The specific activity of CMCI as a function of free isoamylase concentration.

and remained constant up to 200 min. With the aforementioned result, the optimal conditions for isoamylase immobilization on CM-cellulose azide can be set at 0°C, 90 min and pH 3.5. The relationship between the specific activity of CMCI and free isoamylase activity is presented in Fig. 4. It indicated that the specific activity of CMCI increases with the activity of free enzyme. The maximum specific activity of CMCI obtained in this study was 1422 U/g—CMCI with about activity retention of 24%, implying a 76% of activity loss by the immobilized enzyme. The loss of activity retention may result from conformational change during the coupling between CM-cellulose azide and isoamylase; the steric hindrance between immobilized isoamylase and substrate; and the mass transfer resistance between carrier and substrate. Compared with the activity retention of other enzymes immobilized on CM-cellulose azide, e.g., diastase (2.4%) *(15)*, deoxyribonuclease (40%) *(15)*, creatine kinase (9%) *(15)*, ficin (8–12%) *(16)*, bromelain (52%) *(17)*, α-amylase (4.3%) *(18)*, ribonuclease (42%) *(13)*, the retention of isoamylase on this carrier is moderate.

Immobilization of Isoamylase with Chitin

For the immobilization of CI, Fig. 5 shows a comparison of the specific activity and activity retention of CI by the two methods. In the one-step method, enzyme was added to the chitin suspension simultaneously with glutaraldehyde, whereas the two-step method involved addition of

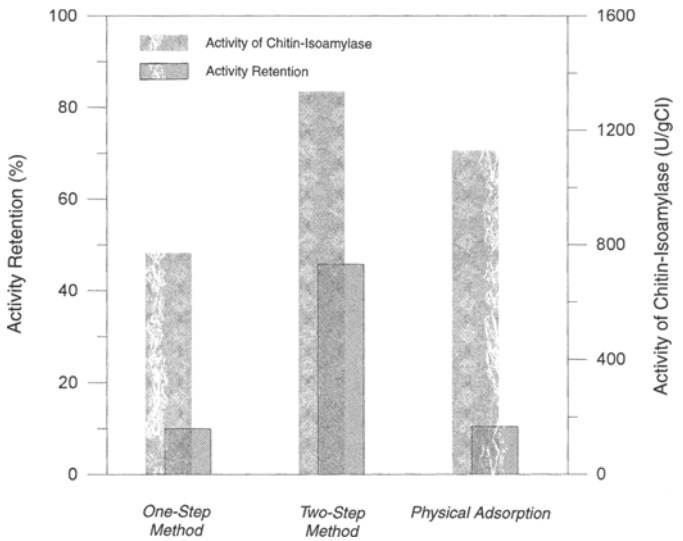

Fig. 5. The activity retention and specific activity of CI for three immobilization methods.

enzyme to the chitin suspension previously treated with glutaraldehyde. The result of immobilization by physical adsorption is also presented in this figure. Two-step method is preferred because of the highest specific activity and activity retention according to Fig. 5. The effect of glutaraldehyde concentration in the first step (activation of chitin) of the two-step method is presented in Fig. 6. There was no significant change in terms of the specific CI activity when the glutaraldehyde concentration was more than 2%. The effect of free isoamylase concentration on the specific activity of CI is shown in Fig. 7, which is very much different from that of CMCI in Fig. 4. The average activity retention of CI by the two-step method is about 46% with the specific activity of 1638 U/g-CI. Other activity retention's reported using chitin as the support are lactase (60%) *(11)* glucoamylase (from 16.4%, *(19)* to 66.7%) *(7)*, glucose isomerase (48%) *(20)*, urease (20–30%) *(21)*, inulinase (37%) *(22)*, acid phosphatase (20%) *(23)*, and α-chymotrpsin (14%) *(23)*. It could be seen that chitin seems to be a better support for immobilization owing to higher activity retention than those for CM-cellulose azide, based on both this study and previous literature.

Properties of CMCI and CI

The pH effect on the activities of free isoamylase, CMCI, and CI is shown in Fig. 8. The shift of optimal pH from 3.5 (free isoamylase) to 4.0 (CMCI) may be attributed to negatively charged carrier *(24)*. The local negative charge may attract more hydrogen ions and create more acidic microenvironment for immobilized enzyme. On the contrary, the optimum of CI has an acidic shift toward pH 2.4. The decrease in optimal pH

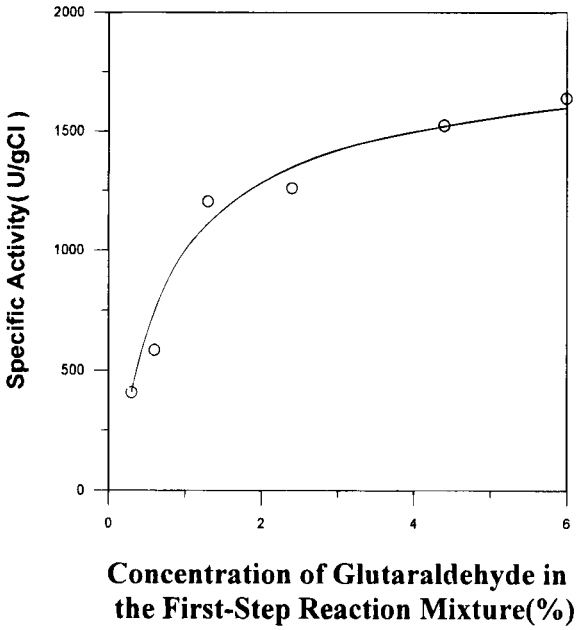

Fig. 6. The effect of glutaraldehyde concentration for chitin activation on the specific activity of CI.

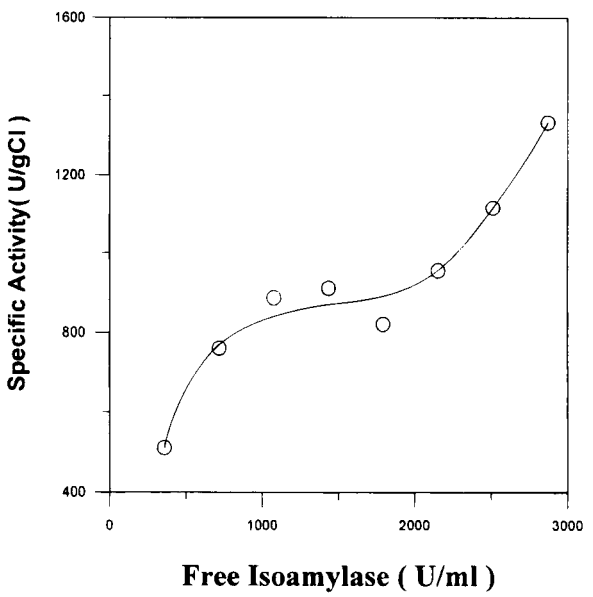

Fig. 7. The specific activity of CI as a function of free isoamylase concentration.

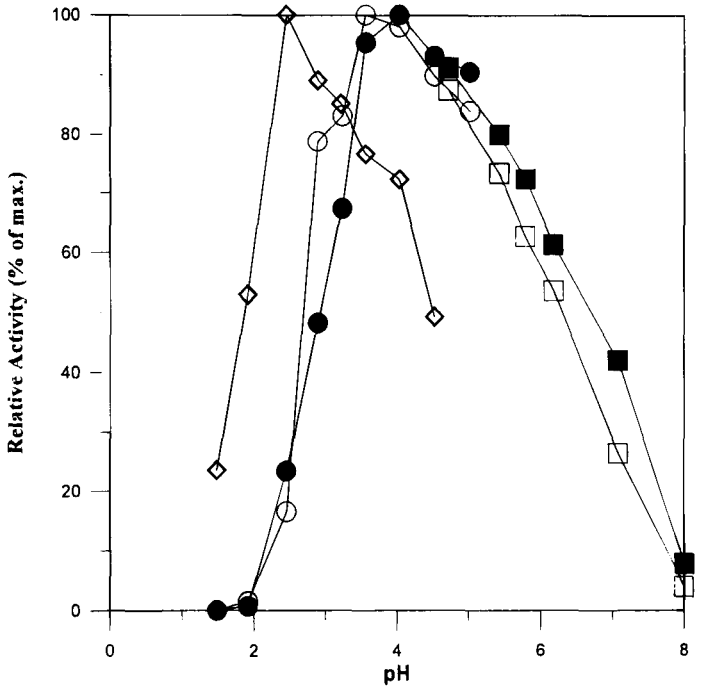

Fig. 8. The comparison of activities of free isoamylase, CMCI, and CI at various pH. Symbols: ⊖ 0.1*M* acetate-HCl buffer for free isoamylase; ⊟ 0.1*M* phosphate-citric acid buffer for free isoamylase; ● 0.1*M* acetate-HCl buffer for CMCI; ■ 0.1 *M* phosphate-citric acid buffer for CMCI; ◇ 0.1*M* acetate-HCl buffer for CI.

may be related to the existence of positive charges on the surface of chitin, probably associated with residual amino groups *(25)*. As for thermal effect, CMCI shows close tendency to free enzyme with slight improvement for the temperature other than the optimal temperature (50°C) as shown in Fig. 9. On the other hand, CI gave better result at temperature higher than the optimal temperature, but performs worse in the temperature range lower than 50°C. The straight lines of Lineweaver-Burk plot for the free isoamylase, CI, and CMCI indicate a good agreement of Michaelis-Menten kinetics (data not shown). The corresponding kinetic parameters, K_m (Michaelis-Menten constant) and K_{cat} ($=V_{max}/[E]$; maximum reaction velocity divided by enzyme activity) are listed in Table 1. Larger value of K_m of CMCI system suggests the existence of steric hindrance and significant mass transfer resistance. However, CI has a similar K_m to that of free isoamylase. Since the porous structure (pore size 100–500 nm) of chitin might make substrate molecules more accessible to the interior of CI *(26)*, the mass transfer resistance would be relaxed more or less. Besides, chitin activated with glutaraldehyde (surface-N-C-C-C-C-C=N-isoamylase) provide "longer arm" for enzymes immobilization than that of CM-cellulose azide (surface-O-C-C-N-isoamylase). This makes it easier for large molecules, such as starch, to approach the immobilized enzymes.

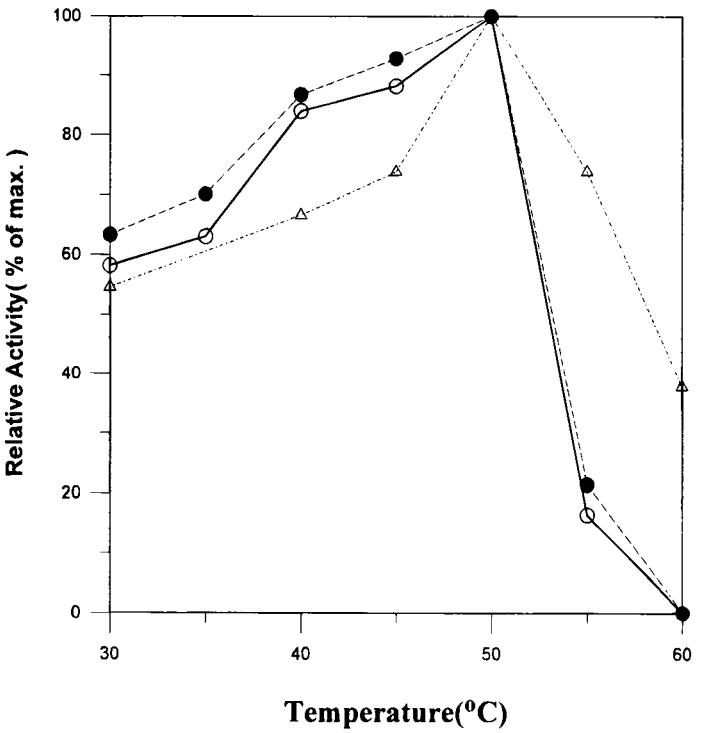

Fig. 9. The comparison of activities of isoamylase, CMCI, and CI at various temperature. Symbols: ●-CMCI; ⊖ free isoamylase; △-CI.

Table 1
Kinetic Parameters for Free Isoamylase, CMCI and CI

	K_m (g/l)	K_{cat} (min^{-1})[a]
Free Isoamylase	0.67	1.47
CMCI	3.57	2.93
CI	0.69	0.72

[a]K_{cat}: turnover number (= $V_{max}/[E]$)

CONCLUSIONS

The optimal conditions for immobilization of isoamylase on CM-cellulose azide are found to be 0°C, pH 3.5, for about 90 min. The maximum activity and activity retention obtained in this studies were 1422 U/g-CMCI and 24%, respectively. On the other hand, the optimal conditions for immobilization on chitin are 2% glutaraldehyde as activation agent for the first step and pH 3.3 (not shown) for the second step. The maximum specific activity and activity retention were U/g-CI and 46% whereas the methods of physical adsorption and one-step chemical binding only showed about 10% activity retention. The kinetic analysis indicated the mass transfer

resistance of CI was similar to that of free enzyme for the two-step method. The results of this study indicated that chitin activated by glutaraldehye seems to be a good support for isoamylase immobilization.

ACKNOWLEDGMENT

The support from Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC is deeply appreciated.

REFERENCES

1. Shuler, M. L. and Kargi, F. (1992), in *Bioprocess Engineering: Basic Concept*, Prentice-Hall, Englewood Cliffs, NJ, p. 78.
2. Whelan, W. J. (1971), *Biochem. J.* **122,** 609.
3. Abe, J. I., Hizukuri, S., Koizumi, K., Kubota, Y., and Okada, Y. (1989), *Carbohyd. Res.* **185,** 191.
4. Lynn, A. and Stark, J. R. (1992), *Carbohyd. Res.* **227,** 379.
5. Kitagawa, H., Amemura, A., and Harada, T. (1975), *Agr. Biol. Chem.* **39,** 989.
6. Harada, T. (1984), *Biotech. Gene. Eng. Rev.* **1,** 39.
7. Stanley, W. L., Waters, G. G., Kelly, S. H., and Olson, A. C. (1978), *Biotechnol. Bioeng.* **20,** 135.
8. Fujita, M., Sakai, S., Futai, M., and Amenura, A. (1990), *Agri. Biol. Chem.* **54,** 2315.
9. Wu, D. H., Wen, C. Y., Chu, W. S., Lin, L. L. and Hsu, W. H. (1993), *Biotech. Lett.* **15,** 883.
10. Mitz, M. A. and Summaria, L. (1961), *Nature* **189,** 576.
11. Stanley, W. L., Waters, G. G., Chan, B., and Mercer, J. M., (1975), *Biotechnol. Bioeng.* **17,** 315.
12. Harada, T., Yokobayashi, K., and Misaki, A. (1968), *Appl. Microbiol.* **16,** 1439.
13. Epstein, C. J. and Anfinsen, C. B. (1962), *J. Biol. Chem.* **237,** 2175.
14. Micheel, V. F. and Ewers, J. (1949), *J. Makromol. Chem.* **3,** 200.
15. Melrose, G. J. H. (1971), *Rev. Pure Appl. Chem.* **21,** 83.
16. Hornby, W. E., Lilly, M. D., and Crook, E. M. (1966), *Biochem. J.* **98,** 420.
17. Wharton, C. W., Crook, E. M., and Brocklehurst, K. (1968), *Eur. J. Biochem.* **6,** 565.
18. Fukushi, T. and Isemura, T. (1967), *J. Biochem.* **64,** 283.
19. Kim, C. H., Lee, G. M., Abidin, Z., Han, M. H., and Rhee, S. K. (1988), *Enz. Microb. Technol.* **10,** 426.
20. Stanley, W. L., Waters, G. G., Kelly, S. H., Chan, B. G., Garibaldi, J. A., and Schade, J. E. (1976), *Biotechnol. Bioeng.* **18,** 439.
21. Iyengar, L. and Rao, A. V. S. P. (1979), *Biotechnol. Bioeng.* **21,** 1333.
22. Kim, C. H. and Rhee, S. K. (1989), *Biotechnol. Lett.* **11,** 201.
23. Muzzarelli, R. A. A., Barontini, G., and Rocchati, T. (1976), *Biotechnol. Bioeng.* **18,** 1451.
24. Zaborsky, O. (1973), Immobilized Enzymes, CRC.
25. Freire, D. G. and Santanna, G. L. (1990), *Biomass* **23,** 71.
26. Muzzarelli, P. A. A. (1980), *Enz. Microb. Technol.* **2,** 177.