Immobilization of α-Amylase on Polymeric Carriers Having Different Structures

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Synopsis

α-Amylase was immobilized onto several polymeric carriers having carboxyl groups by the Woodward's reagent *K* better than by other methods. The amount of immobilized α-amylase mainly depended on the surface area of carriers, while the enzymic activity depended on the texture of carrier surface. Flat surface was favorable for making the enzyme exhibit high activity and for keeping it active in wide pH range. The durability of immobilized enzyme was excellent at 20°C and pH 5.5.

INTRODUCTION

Immobilization of enzyme facilitates the purification of reaction system and the recovery of enzyme and makes it possible to use the enzyme repetitively or continuously. Immobilization is often accompanied by some changes in the enzymic activity, optimum pH, affinity with substrate, durability of the enzyme, etc. Such changes depend on the combination of enzyme and carrier and the immobilizing conditions. In this study α-amylase was immobilized onto several kinds of polymeric particles. The appropriate condition for immobilization and some changes observed in the immobilized enzyme were investigated. In particular, the influence of the structure of carriers on the amount and activity of immobilized enzyme was discussed in detail.

EXPERIMENTAL

Materials

α-Amylase was Type-II-A, crystal from bacillus subtilis (Sigma Chemical Co.). *N*-ethyl-5-phenylisoxazolium 3'-sulfonate, commonly called the Woodward's reagent K, (WR-K), was purchased from Tokyo Kasei Co. and used without further purification. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide HCl (CI, Sigma Chemical Co.) was used as the other coupling agent. ε-Amino caproic acid (Tokyo Kasei Co.) was employed as a spacer. Phosphoric buffers were used when the pH of the system must be fixed.

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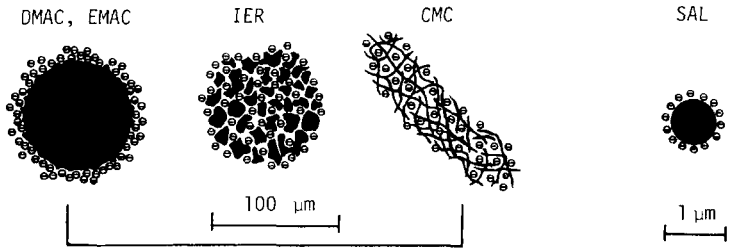


Fig. 1. Schematic presentation of carriers used: DMAC-diallyl phthalate–methacrylic acid copolymer gel particle; EMAC-ethylene glycol dimethacrylate–methacrylic acid copolymer gel particle; IER-weakly acidic cation–exchange resin Bio-Rex 70; CMC-partially carboxymethylated cellulose CM 52; SAL-partially hydrolyzed styrene–acrylamide copolymer latex particle.

Three substrates were used: soluble starch (Katayama Chemical Co., special grade), amylose (Tokyo Kasei Co., A846), and amylopectin (Tokyo Kasei Co., A456). A dye, amidoshwarz 10B (AS10, Merck Art 1166), was used for determination of α-amylase in the solution.

Carriers

The structure of four kinds of carriers employed is presented schematically in Figure 1. In the figure the black part indicates hydrophobic domain, and the minus sign represents the carboxyl groups on polymer chains.

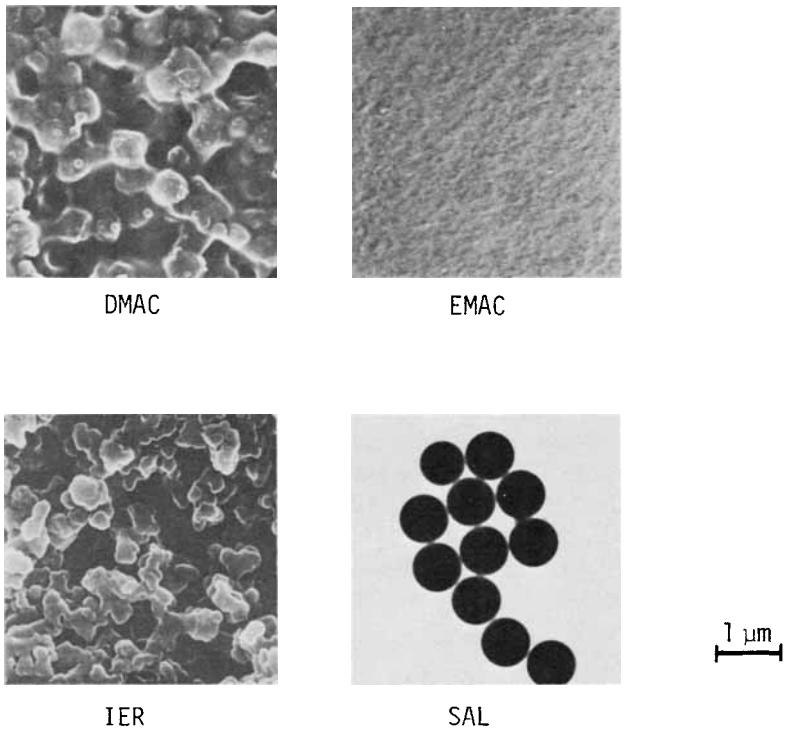


Fig. 2. Scanning electron micrographs of surfaces of DMAC, EMAC, and IER and transmission electron micrograph of SAL.

Gel Particles Having Hydrophobic Core and Carboxylated Shell. Diallyl phthalate (DAP) or ethylene glycol dimethacrylate (EDMA) was suspension-copolymerized with methacrylic acid (MAc) to form copolymer gel particles having a hydrophobic crosslinked core covered by MAc unit-rich polymer chains.[1] Gel particles composed of poly-DAP core and MAc-rich shell were abbreviated as DMAC and gel particles composed of poly-EDMA core as EMAC.

Weakly Acidic Cation-Exchange Resin. Macroreticular type cation exchange resin used was Bio-Rex 70 (Bio Rad Laboratories Co.). The particles were 100–200 mesh (average diameter ~100 μm) in size and had 10.2 meq COOH/g dry resin. A part of resin was ground into smaller size (average diameter ~1 μm) to examine the dependence of the activity of immobilized enzyme on the size of carrier. The original and ground resins were refered to as IER and IER′, respectively.

Crosslinked Carboxymethyl Cellulose (CMC). Microgranular ion exchange cellulose, CM 52 (Whatman Co.), was used as received. The ion capacity was described to be 1.0 meq/g dry resin.

Carboxylated Latex Particle (SAL). Styrene-*N*-hydroxymethyl acrylamide copolymer latex particles[2] were partially hydrolyzed by mixing the latex of 20% solid with the same volume of 20% NaOH at 30°C for 4 h. The carboxyl group formed by hydrolysis was determined by conductometric titration and found to be 4 units/nm^2, which corresponded to 0.053 meq/g particle.

Characterization of Carriers

Scanning and transmission electron micrographs were obtained by using a FESEM-JFSM 30 and a Hitachi HU-12AF, respectively. The electron micrographs of DMAC, EMAC, IER, and SAL are shown in Figure 2. Surface area of carriers was measured by a Kinoshita BET apparatus.

Immobilization

Ionic Bonding. α-Amylase and polymer carrier were mixed in a buffer solution, and the dispersion was stirred at an appropriate temperature and pH. After an appropriate duration, the carrier was washed with fresh buffer. α-Amylase in the recovered buffer was determined to calculate the amount of α-amylase bound to the carrier by ionic bonding.

Covalent Bonding with Woodward's Reagent *K*. The process to bond α-amylase onto carriers covalently is composed of two steps, that is, the activation of carrier with coupling reagents such as Woodward's reagent K (WR-K) or carbodiimide (CI) and the immobilization of α-amylase onto the activated carrier:

In covalent bonding of α-amylase on each carrier by WR-K, the method of Wagner et al.[3] was referred to. Three grams of the carrier was mixed with WR-K of the same equivalent with the carboxyl groups on the carrier. The mixture was stirred at various pHs and temperatures for various durations to find out the optimum condition for activation. After that, the carrier was washed with fresh buffer to remove free WR-K. The activated carrier was mixed with 80 mg α-amylase at various pHs and temperatures.

DMAC, EMAC, IER, and CMC covalently bonding α-amylase were cleaned by filtration and rinse. SAL immobilizing α-amylase was purified by repetitive centrifugation. When a spacer was used, the process consisted of activation by WR-K, coupling of spacer, activation by WR-K, and immobilization. The same condition with the immobilization was taken for the coupling of the spacer.

Covalent Bonding with Carbodiimide. Refering to the method of Marumoto et al.,[4] 2 g DMAC was mixed with CI in the same equivalent with the carboxyl groups on carrier. The mixture was stirred at pH 7.0 and 4–5°C for 5 h. After removing free CI, the activated carrier was mixed with buffer solution (pH 5.5) containing 50 mg α-amylase at 5°C for 24 h.

Determination of α-Amylase

α-Amylase remaining in the buffer solution after immobilization was determined by the method of Nakao et al.[5] with some modifications. This method is based on a stoichiometrical reaction between enzyme protein and AS10. In a test tube, a 0.1-mL solution containing α-amylase was mixed with 0.2 mL of 0.1% AS10 solution [solvent = propanol/acetic acid/water (3/1/6 in volume)]. The solution was shaken at 20°C for 20 min and then diluted to 2 mL. During this treatment a complex of enzyme and AS10 is denatured to precipitate. The precipitates was filtrated with a membrane filter TM2P (Toyo Roshi Co., pore size 0.45 μm) and rinsed. The colored membrane filter was put into 1.5 mL of 25 m*M* NaOH solution of ethanol/water (50/50 in volume) and the solution was shaken for 20 min so that the dye AS10 was eluted from the membrane filter. The absorption of the solution was measured at 630 nm.

Determination of Enzymic Activity

After each enzymic reaction (at pH 5.5 and 37°C unless otherwise mentioned), the amount of undecomposed substrate was measured by the Carawey method.[6] Five milliliters of 0.01*N* iodine solution was added into 5 mL solution or dispersion of enzyme and substrate to stop the reaction and to color the solution. The solution was diluted to 50 mL and the absorbance (E) of the solution was measured at 660 nm. E was proportional to the concentration of substrate (C) in the range up to 6 m*M* substrate in the original solution. Specific activity was calculated from

$$\text{specific activity (I.U./mg enzyme)} = \frac{1}{X}\frac{5(E_b - E_s)}{7.5E_b}C_0 \qquad (1)$$

where X is the amount of α-amylase (mg), E_b and E_s are the absorbances of the samples at the incubation time = 0 and 7.5 min, respectively, and C_0 is the concentration of substrate (m*M*) at the incubation time = 0 min. The amount of substrate decomposed was less than 50% after 7.5 min incubation and almost proportional to the incubation time in all of the experiments. For the determination of the Michaelis constant, however, the more precise initial velocity of decomposition must be measured from a truely linear part of the decomposition vs. time plots, and, therefore, the

incubation time was reduced to 1.5 min for this purpose. The Michaelis constant was determined from the Hofstee plots (C/v vs. C plots, where v is the rate of enzymic reaction) in the range of C from 0.2 to 5 mM.

Continuous Hydrolysis of Starch by Column Method

In a column of 2.4 mm diameter and 25 cm length was packed 0.90 g DMAC immobilizing α-amylase. Aqueous solution of soluble starch (1.0 mg/mL, pH 5.5) was passed into the column at the flow rate of 0.2 mL/min at 20°C. The operation was done for 9 h each day. Decomposition of starch was determined by measuring the absorbance of the effluent after addition of iodine solution.

RESULTS AND DISCUSSION

Optimum Conditions for Immobilization of α-Amylase onto DMAC and EMAC

α-Amylase was bound to DMAC or EMAC by the following methods: (a) ionic bonding, (b) covalent bonding by the Woodward's reagent K (WR-K), (c) covalent bonding by WR-K—spacer—WR-K, and (d) covalent bonding by carbodiimide (CI).

In the ionic bonding, release or desorption of α-amylase from the carrier must be taken into account. Actually, the result in Figure 3 indicates that a significant amount of once-adsorbed α-amylase desorbed when the dispersion medium was replaced with fresh buffer. A maximum followed by a slight decrease in the amount of adsorbed α-amylase was observed at the early stage of adsorption in Figure 3. This is not an unusual phenomenon for adsorption of proteins onto polymer carriers.[7] Progressive desorption of α-amylase occurred with repetitive replacements of dispersion medium although a definite amount of α-amylase remained on the particles finally as shown in Figure 4. The amount depended on the starting points on adsorption isotherms indicated by arrows in Figure 4.

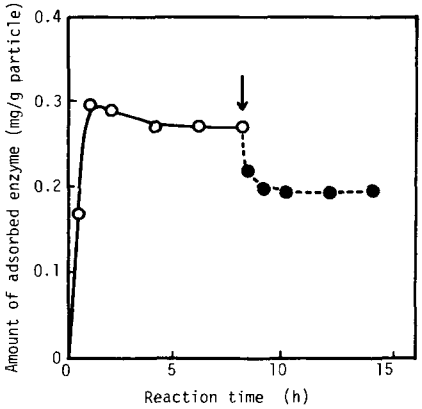


Fig. 3. Adsorption and desorption of α-amylase on and from EMAC at pH 7.0 and 5°C. The arrow indicates where the dispersion medium was replaced with fresh buffer.

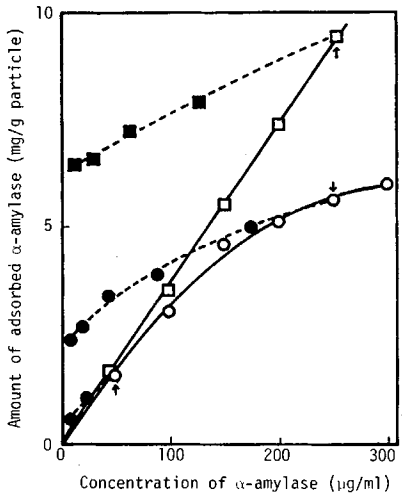


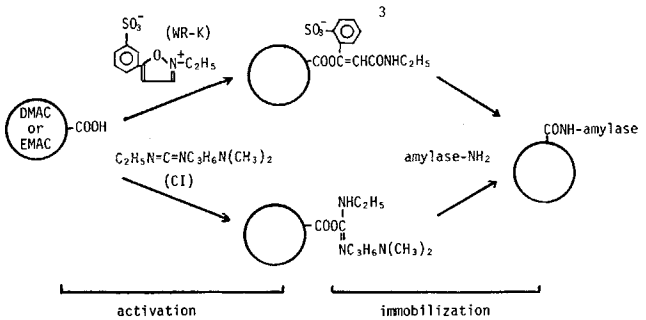
Fig. 4. Adsorption isotherms of α-amylase on gel particles at pH 7.0 and 5°C: (○,●) EMAC; (□,■) DMAC; solid mark: shifted equilibrium by replacing the dispersion medium with fresh buffer. The arrows indicate the starting points for replacement of medium.

In contrast with the case of ionic bonding, α-amylase bound covalently onto DMAC or EMAC was not released from the carrier even if the dispersion medium was replaced with fresh buffer repetitively.

Covalent bonding process of α-amylase onto carriers is composed of two steps as shown in Scheme 1.

The condition for activation of DMAC or EMAC affects the next step—immobilization step—and, consequently, the activity of immobilized α-amylase. Therefore, the optimum condition for activation was investigated at first. Activation of DMAC or EMAC was carried out at different temperatures, pHs, and durations, while immobilization was done under a fixed condition at pH 7.0 and 5°C, although this condition was not necessarily a favorable immobilizing condition for each carrier, as mentioned later. It was found from these experiments that the activation should be carried out at a temperature lower than 10°C and a pH about 8.5 for more than 2 h. Lower pH is supposed to cause deactivation of caboxyl groups on the carrier and higher temperature is said to lead to an undesirable intramolecular reaction of WR-K.[3]

The condition for immobilization is, of course, the most decisive factor determining the activity of immobilized enzyme. To investigate the opti-



Scheme 1

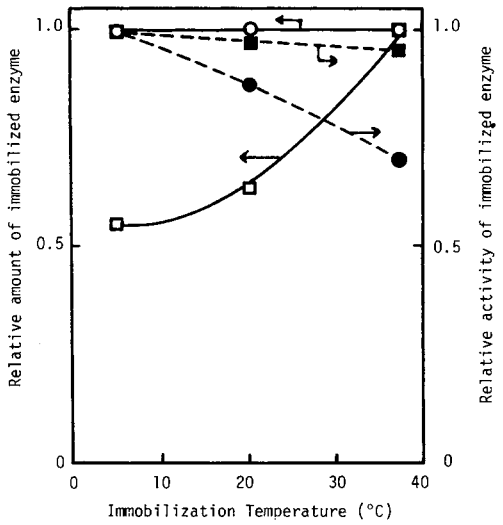


Fig. 5. Dependence of the amount and activity of immobilized α-amylase on the immobilization temperature: (□,■) ionic bonding; (○,●) covalent bonding by WR-K; carrier: EMAC; immobilization: pH 7.0 for 5 h; activity measurement: pH 5.5 and temp 37°C.

mum condition, α-amylase was immobilized under different conditions onto DMAC which was activated under the fixed condition, that is, at pH 8.5 and 5°C for 2 h. The dependence of activity of immobilized enzyme on the pH and temperature for immobilization is shown in Figures 5 and 6, in each of which the maximum value was normalized to be unity. According to Figure 5, a lower temperature was favorable for covalent bonding, although the amount of ionically bound α-amylase increased with elevating temperature in the range examined. The pH range appropriate to immo-

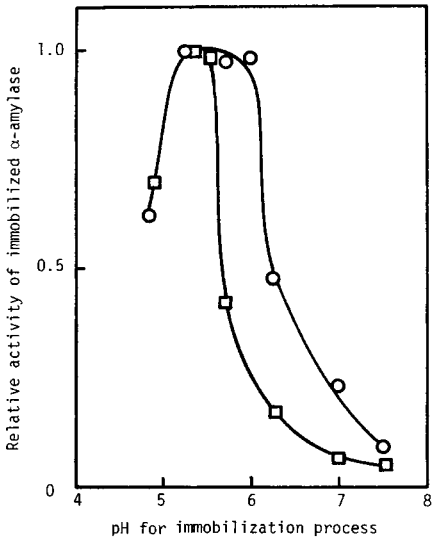


Fig. 6. Dependence of the activity of immobilized α-amylase on pH for immobilization process: (□) ionic bonding; (○) covalent bonding by WR-K; carrier: DMAC, immobilization temperature: 5°C; activity measurement: pH 5.5 and temperature 37°C.

bilize α-amylase covalently spread to higher pH range compared with that for ionic bonding (Fig. 6). The so-called optimum pH range where the enzyme exhibits the highest activity is around 5.5 for free α-amylase. Therefore, it is speculated that the covalently bound α-amylase can keep its favorable conformation without being affected by the negatively charged surface of carrier.

Covalent bonding of α-amylase by WR-K—spacer—WR-K was carried out under the following conditions which correspond to the best conditions in covalent bonding by WR-K: activation, pH 8.5, 5°C for 2 h, immobilization or spacer coupling, pH 5.5, 5°C for 5 h. On the other hand, immobilization by CI method was carried out under the condition presented by Marumoto et al.[4] without any change.

The amount and activity of α-amylase immobilized by three methods, WR-K method, WR-K—spacer—WR-K method, and CI method, were compared. The amount of immobilized enzyme by the second method was about 30% less than that by the first one. There was no significant difference between the amount of enzymes immobilized by the first and third methods, but the Michaelis constant for the third system was larger than that of the first system (0.9 and 0.3, respectively). This means a higher affinity of the substrate with the enzyme immobilized by the first method than with the other enzyme.

In the following section, therefore, only the WR-K method was employed for immobilization of α-amylase onto various carriers to investigate the dependence of the amount and activity of immobilized α-amylase on the structure of carriers.

Characterization of DMAC and EMAC

Some DMACs and EMACs having different surface densities of carboxyl groups were prepared by changing the mode of monomer charge in copo-

TABLE I
Characteristics of Carriers and α-Amylase Immobilized on Them[a]

Carrier	DMAC	EMAC	CMC	IER	SAL	Native
Diameter (μm)	109.4	85.3	—[c]	74–149	0.71	
Surface area (m^2/g)	0.049	0.061		13.8	8.05	
Charge (meq/dry g)	0.5	0.05	1.0	10.0	0.05	
(unit/A^2)	70	4.8		4.2	0.05	
Enzyme immobilized (mg/g carrier)	11.2	10.1	31.3	37.0	33.6	
Specific activity (I.U./mg enzyme)[b]	370	740	350	55	1025	1870
Michaelis const (mmol/L)[b]	0.3	2.9	2.8	8.0	3.0	0.9

[a] Immobilized with Woodward's reagent K at 5°C and pH 5.5, and then measured at 37°C and pH 5.5.

[b] For hydrolysis of soluble starch.

[c] Rodlike gel of 12ϕ × 100 μm.

lymerization of crosslinkable monomer with methacryclic acid.[1] Carboxyl groups exist mainly in the surface layer of the gel particles, but there is no clear boundary between the surface layer and interior of particles. A quick back titration[8] was carried out to obtain the apparent amount of carboxyl groups on the surface of particles. The measured amounts depends on the conditions of addition of alkali and back titration with acid. Therefore, the amounts of carboxyl groups on the surface of DMAC and EMAC in Table I are not absolute, rather relative ones.

Gel particles, either DMAC or EMAC, with larger amount of carboxyl groups had more uneven structure on their surface.[1,8] The results on DMAC and EMAC in Figure 2 were consistent with the trend. The surface areas of DMAC and EMAC were too small to be determined precisely by BET. Therefore, their surface areas shown in Table I were the ones calculated by assuming them as spheres having flat surface. The presented values might be significantly smaller than the real but unknown ones.

Preliminary experiments revealed that the difference in the kind of core components of gel particles, diallyl phthalate for DMAC and ethylene glycol dimethacrylate for EMAC, gave no effect on the amount and activity of immobilized α-amylase (data are not shown here). In this study a DMAC having a large amount of carboxyl groups and an EMAC having a small amount of carboxyl groups were mainly used as representative gel particles, as shown in Table I. Due to the reason above mentioned, the difference in the data on α-amylase immobilized onto DMAC and EMAC in Table I can be attributed to the difference in the surface structures but not in the kind of core components of two gel particles.

Amount of Immobilized α-Amylase

The amount of α-amylase immobilized onto different carriers are shown in Table I. No consistent relation can be pointed out between the amount of immobilized enzyme and the size or charge density of carriers in Table I, although, among a series of the same kind of carriers having various charge densities, immobilized enzyme trended to increase with the charge density of carriers.[9]

The dimention of α-amylase immobilized onto SAL was calculated assuming the specific gravity of enzyme would be unity. The area occupied by an α-amylase molecule and its thickness were 1800 Å^2 and 40 Å, respectively. An unstrained conformation can be supposed from these values, although no reference describing the probable dimention of α-amylase has been found out. The area occupied by α-amylase immobilized onto IER was calculated to be 2800 Å^2/molecule. This value means that α-amylase molecules were bound sparsely onto IER surface than onto SAL surface. It is likely that α-amylase (molecular weight = 45000) cannot diffuse into narrow and complicated pores so that a part of surface area of IER will remain unoccupied by enzyme. As mentioned in the previous section, DMAC and EMAC have less surface area than IER or SAL. DMAC and EMAC combined less amount of α-amylase. From these facts, it is likely that the surface area of carriers is one of the main factors to determine the amount of α-amylase immobilized onto various kinds of carriers.

Activity of Immobilized α-Amylase

The activity of the immobilized enzyme depends upon the kind of carriers in the order:

$$\text{IER} < \text{DMAC, CMA} < \text{EMAC} < \text{SAL} \quad (2)$$

as indicated in Table I. Among the carriers employed, CMC belongs to a different category from others because it is a hydrogel and has no clearly definable surface area. Therefore, the case of CMC is omitted from this discussion.

With respect to the properties and structures of carriers, the following factors are the possible ones to affect the activity of immobilized enzyme: (a) The size of carrier; which determines the movability of carrier in the dispersion. The size of each carrier is shown in Table I. (b) The texture of carrier surface (roughness, porosity, hairy structure, etc.), which influences not only the mode and degree of exposure of the active sites of immobilized enzyme, but also the easiness for the substrate to approach the immobilized enzyme; (c) the electric property of carrier surface. For example, the kind and surface density of ionic groups on the carrier would influence the pH of environment of immobilized enzyme. The surface density of carboxyl groups on each carrier is listed in Table I. (d) The hydrophilic-hydrophobic balance of carrier, which affects the affinity of substrate with carrier. SAL has the most hydrophobic core among the carriers used, but the amide groups saved from hydrolysis of SAL would cancel the hydrophobicity of the core to some extent, although the extent cannot be determined. IER and EMAC are constituted of less hydrophobic polymers than DMAC is.

The order among IER, DMAC, EMAC, and SAL with respect to each factor of (a), (c), and perhaps (d) did not coincide with the order of (2), but the order for the carriers with respect to the factor (b) did. Namely, SAL has the flattest surface, the flatness decreases in the order of EMAC, DMAC, and IER as shown in Figure 2, and it seems that the flatter the carrier surface is, the more active the enzyme immobilized on the carrier is. If the activity of immobilized enzyme is mainly affected by the flatness of carrier surface, the reduced activity of enzyme on uneven carrier is attributed to the distorted conformation of enzyme at the unfavorable environment and to the retarded diffusion of substrate and decomposed products through the uneven or narrow spaces. The latter effect was observed in some triphase catalyses.[10] If IER is assumed to be a sphere having flat surface and no pores, the area of hypothetical surface corresponds to 1/600 of the real area measured by BET (presented in Table I). The ratio implies that most α-amylase immobilized on IER would suffer these distortion and diffusion problems.

The finding that flat surface is favorable for exhibiting high enzymic activity is discussed from the viewpoint of size of substrate. Starch is composed of amylose (a linear molecule having the molecular weight of several thousand) and amylopectin (a branched molecule having the molecular weight more than 100,000). The activity of α-amylase immobilized on different carriers against amylose and amylopectin was measured separately.

TABLE II
Specific Activity of Immobilized α-Amylase for Hydrolysis of Amylose and Amylopectin[a]

Substrate	GS-1	CMC	IER	IER'	SAL	Native
Amylose (a)	450	360	60	80	1120	2020
Amylopectin (b)	270	250	20	45	970	1840
Activity ratio (b/a)	0.60	0.69	0.33	0.56	0.87	0.91

[a] IER (diameter 74-149 μm) was ground into IER' (diameter 1 μm).

The results are shown in Table II. The ratios of activity against two substrates are less than unity not only in all of the immobilizing systems but also in free enzyme system. SAL system had a comparable ratio with free enzyme system. IER had the least ratio but ground IER (IER', which had a similar appearance to IER on the scanning electron micrograph) had a higher ratio. These results show that the bulky substrate, amylopectin, has a disadvantage to diffuse into the narrow spaces of carriers and is less hydrolyzable by α-amylase immobilized on the inside of carriers. But this substrate-size effect must not be the only effect to cause the dependence of activity of immobilized enzyme on the texture of carriers, because even the activity against amylose (whose molecular weight is about 1/10 of α-amylase) is significantly different among the immobilizing systems examined.

Concerning the order (2), some other data were obtained. Figure 7 shows the dependence of the activity of immobilized α-amylase on pH for different immobilizing systems. The relative activity in Figure 7 is the ratio of the activity at each pH to the maximum activity which is shown in Table I and usually appeared at about pH 5.5. It has been supposed that the optimum pH for immobilized enzyme would shift to a higher pH than that for free enzyme because of a decrease in the local pH due to carboxyl groups of carriers which must affect the conformation of enzyme molecules and, con-

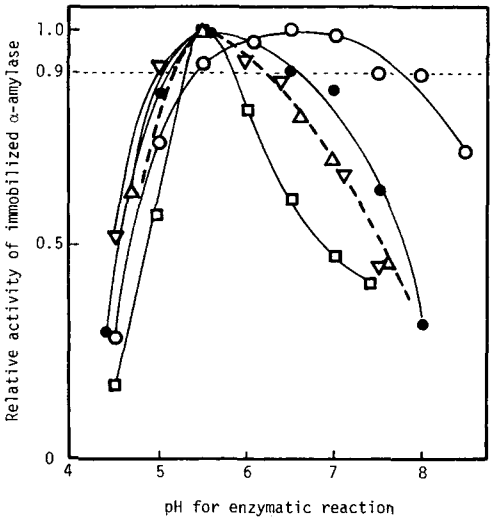


Fig. 7. Dependence of the activity of immobilized α-amylase on pH for enzymic reaction at 37°C for different carrier systems. (—) free α-amylase; (△) DMAC; (▽) EMAC; (□) IER; (○) SAL; (●) CMC.

sequently, the enzymic activity.[11] But no shift of optimum pH was observed in our experiments except for SAL system. The expected pH dependence of the activity of immobilized enzyme due to the conformational change of enzyme might be depressed by some other causes in most of the cases examined. It is likely that the increase in dissociation of carboxyl groups on the carriers with increasing pH would affect the diffusion of substrate into the narrow spaces because of conformational change in the microdomain of carriers and of electrostatic repulsion between carriers and substrate.

According to Figure 7, the width of pH range with the relative activity higher than 0.9 increased in the following order with respect to the kind of carriers: IER < DMAC < EMAC < SAL. This order is exactly the same with (2). The result means that α-amylase immobilized on the carriers having uneven surface is apt to lose its activity when pH increases, whereas the α-amylase immobilized on a flat surface seems to be able to keep its activity high in wide pH range. It is probable that the activity of enzyme molecules combined to complicated and uneven structure is more sensitive to the change in pH perhaps because of their distorted conformation.

Continuous Hydrolysis of Starch by Immobilized α-Amylase Packed in a Column

One of the purposes for immobilization of enzyme is the repetitive use of enzyme. DMAC immobilizing α-amylase was packed in a column and employed for hydrolysis of starch in the flowing solution. The reaction temperature was set to be 20°C because it was found that the activity decreased with time at a higher temperature. The operational stability of this immobilized α-amylase was excellent. The enzyme retained more than 95% of activity after 50 days operation under the condition described in the experimental section.

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