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Effects of Carbodiimide Structure on the Immobilization of Enzymes

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ABSTRACT

A series of water-soluble disubstituted carbodiimides of different structure was tested for enzyme immobilization. In the experiments, a polyacrylamide-type bead polymer possessing carboxylic functional groups was used as support. The enzymes immobilized were aminoacylase (*N*-acylamino acid amidohydrolase; EC 3.5.1.14), arginase (L-arginine amidinohydrolase; EC 3.5.3.1), cyclodextrin glycosyltransferase (α-1,4-glucan 4-glycosyltransferase, cyclizing; EC 3.2.1.19), glucoamylase (1,4-α-D-glucan glycohydrolase, EC 3.2.1.3), and carboxypeptidase B (peptidyl-L-lysine [L-arginine] hydrolase; EC 3.4.17.2). It was found that the degree of immobilization strongly depended on the structure of carbodiimide used.

Index Entries: Water-soluble carbodiimides; water-soluble carbodiimides in enzyme immobilization; aminoacylase, immobilized; arginase, immobilized; cyclodextrin glycosyltransferase immobilized; glucoamylase, immobilized; carboxypeptidase B, immobilized.

INTRODUCTION

For the production of immobilized enzyme of high catalytic activity and advantageous properties, the possible interactions existing among the enzyme, support, and the coupling agent must be considered. It has been

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found that a polyacrylamide-type bead support possessing carboxylic functional groups activated by water-soluble carbodiimide is suitable for the immobilization of various enzymes (*1–12*). It was supposed that the degree of immobilization depends on the structure of carbodiimide employed for the activation. Therefore, a series of water-soluble disubstituted carbodiimides has been synthesized (*13*), and the present report describes their application in immobilization experiments.

MATERIALS AND METHODS

Akrilex C-100, a polyacrylamide-type bead (100–320 μm) polymer possessing carboxylic functional groups (6.4 meq/g dry wt), is a commercial product of Reanal Factory of Laboratory Chemicals (Budapest, Hungary). 1-Cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-4-toluene sulfonate was purchased from Fluka Chemie AG, Buchs, Switzerland. Other carbodiimides were synthesized according to Jászay et al. (*13*). Aminoacylase was isolated from pig kidney (*14*) with an activity of 2200 U/mg. Arginase isolated from bovine liver was a Reanal product. Its activity was 30 U/mg. Cyclodextrin glycosyltransferase, isolated from *Bacillus macerans* as a practical-grade powder containing ammonium sulfate, was produced by Chinoin Pharmaceutical Workes, Ltd., Budapest, Hungary. Its specific activity after dissolution and dialysis was 27.5 U/mg protein. Glucoamylase, isolated from *Aspergillus niger*, was a salt-free, lyophilized preparation of practical grade. Its specific activity was 1.0 U/mg protein. Carboxypeptidase B was isolated from pig pancreas (*15*) with an activity of 37.5 U/mg. All other chemicals were reagent-grade commercial preparations (Reanal).

Measurement of Protein

Protein determination was performed according to the method of Lowry et al. (*16*), as modified by Schacterle and Pollack (*17*). The amount of immobilized protein was calculated as the difference between the amount of protein introduced into the reaction mixture and the amount of protein present in the filtrate and washing solutions after immobilization.

Assay of Enzyme Activities

Activity of soluble and immobilized aminoacylase was determined according to Szajáni et al. (*18*). For the measurement of soluble and immobilized arginase activities, the method of Boehringer Mannheim GmBH (*19*) was used. Activity assays of soluble and immobilized cyclodextrin glycosyltransferase were performed by the method of Kitahata et al. (*20*) and Ivony et al. (*21*), respectively. Glucoamylase activity determinations were carried out according to the method of Szajáni et al. (*22*). For the activity assays of soluble and immobilized carboxypeptidase B, the methods of Folk et al. (*23*) and Südi et al. (*12*) were used.

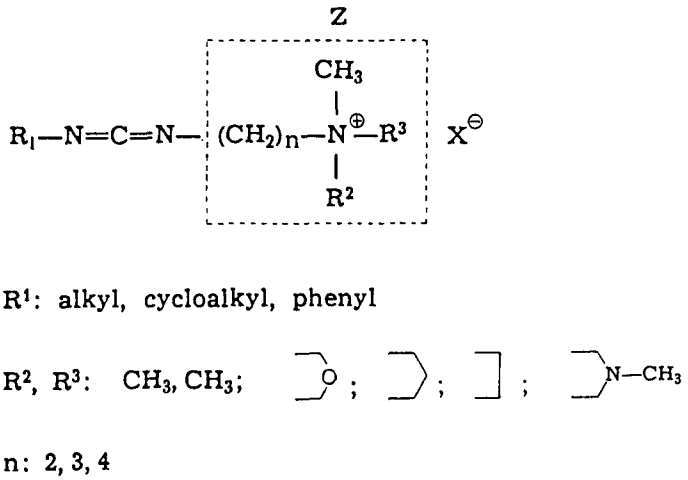

Fig. 1. General formula of water-soluble disubstituted carbodiimides.

Immobilization of Enzymes

The enzymes were covalently attached to a polyacrylamide-type bead polymer possessing carboxylic functional groups activated by water-soluble carbodiimide, as described earlier (*12,18,21,22*). The general method of immobilization was the following:

Akrilex C xerogel (1 g) was suspended and swollen in 50 mL of 0.1*M* buffer. Water-soluble carbodiimide, in a stoichiometric quantity relative to the carboxylic functional groups located on the support, dissolved in 25 mL of cold (0°C) buffer, was added, with continuous stirring and cooling in an ice bath. After 10 min, 25 mL of enzyme solution containing 0.5 g of protein was added, and the pH was adjusted to the starting pH value. The mixture was incubated at 0–4°C for 48 h, with two 6-h periods of agitation. The gel was filtered by suction and successively washed three times with 100 mL of buffer, three times with 100 mL of buffer containing 1.0*M* sodium chloride, and three more times with 100 mL of buffer to remove the unbound proteins, and, finally, with a large volume of distilled water to remove the buffer ions. The product was lyophilized.

The buffers used were 0.1*M* potassium phosphate (pH 7.0) for aminoacylase (*18*), 0.1*M* potassium phosphate (pH 6.0) for arginase, 0.1*M* sodium citrate/sodium phosphate (pH 4.5) for carboxypeptidase *B* (*12*), 0.05*M* sodium acetate (pH 6.5) for cyclodextrin glycosyltransferase (*21*), and 0.1*M* potassium phosphate (pH 7.5) for glucoamylase (*22*).

RESULTS AND DISCUSSION

Carbodiimides are excellent reagents for the activation of carboxylic groups of supports in mild conditions (*24–26*). Probably the relatively bulky substituents make it possible to avoid reacting with the side chains

Table 1
Effects of Carbodiimide Structure on the Immobilization

Enzyme	Carbodiimide structure					Activity on dry wt. basis (unit. g^{-1} solid)	Activity on protein basis (unit. g^{-1} protein)		Activity bound (%) [b]	Activity loss (%) [b]	Protein bound (mg. g^{-1} solid)
	R_1	R_2	R_3	n	X						
Aminoacylase	$CH_3-(CH_2)_2$	CH_3	CH_3	2	$H_3C-\langle O \rangle-SO_3^-$	42,000	720	32.7	12.3	0	58.3
	$CH_3-(CH_2)_2$	CH_3	CH_3	3	$H_3C-\langle O \rangle-SO_3^-$	46,800	380	17.3	9	8.7	123.3
	$CH_3-(CH_2)_2$	CH_3	CH_3	3	J^-	42,000	340	15.4	10.6	2.4	123.5
	$\langle O \rangle$	CH_3, $\overset{+}{N}$		2	$H_3C-\langle O \rangle-SO_3^-$	35,040	272	12.4	10.9	15.6	128.6
Arginase	$\langle O \rangle$	CH_3	$O_2N-\langle O \rangle-CO-CH_2$	3	Br^-	1,190	3.51	11.7	65.3	9.1	339.0
	$\langle O \rangle$	CH_3	$\langle O \rangle-CO-CH_2$	3	Br^-	710	1.65	5.5	30.1	43.5	430.1
	$\langle O \rangle$	CH_3, $\overset{+}{N}$		2	$H_3C-\langle O \rangle-SO_3^-$	340	0.89	3.3	5.5	77	327
	CH_3-CH_2	CH_3	CH_3	3	Cl^-	330	7.5	25.0	4.3	15.7	44

Carboxypeptidase B		CH_3	CH_3	3	$H_3C-\langle O \rangle-SO_3^-$	4,140	44.4	118.4	37	31	93.3
		CH_3	CH_3	3	J^-	3,240	47.9	127.7	29	40	67.8
				2	$H_3C-\langle O \rangle-SO_3^-$	3,200	32.3	86.1	29	62	99.2
		CH_3	CH_3	2	J^-	3,090	15.4	41.0	18	67	200.7
Cyclodextrin glycosyl-transferase				2	$H_3C-\langle O \rangle-SO_3^-$	407	1.26	4.6	1.7	46.2	322
				2	J^-	285	0.41	1.5	0.7	55.7	692
		CH_3	CH_3	2	J^-	317	0.9	3.3	0.9	39	352
		CH_3	CH_3	3	Cl^-	320	0.6	2.2	1	21.1	530
Glucoamylase			CH_3	3	J^-	41.6	0.1	10.0	3.8	5.7	424.8
			CH_3	3	$H_3C-\langle O \rangle-SO_3^-$	37.8	0.05	5.0	4.2	2.2	711.6
				2	$H_3C-\langle O \rangle-SO_3^-$	17.7	0.05	5.0	2.2	7.8	240
		CH_3-CH_2	CH_3	3	Cl^-	13.5	0.13	13.0	1	21.1	102.5

[a]The activity of the soluble enzyme was taken as 100%

[b]The total activity introduced into the coupling reaction mixture was taken as 100%.

localized in the active center of enzyme molecules. In current practice, 1-cyclohexyl-3-(2-morpholinoethyl)-carbodiimide metho-4-toluene sulfonate and 1-ethyl-3-(3-dimethylamino-propyl) carbodiimide hydrochloride is generally used. On a theoretical basis, it was supposed that the structure of disubstituted carbodiimides could influence the immobilization process. Therefore, over 30 disubstituted carbodiimides of different structure were synthesized (*13*) and screened for enzyme immobilization. The general formula of carbodiimides is shown in Fig. 1. The results of immobilization strongly depend on the structure of carbodiimide used for the activation of support (Table 1). It appeared from the experiments that the most favorable carbodiimide structures were those in which R^1=propyl, *tert*-butyl, or cyclohexyl; R^2=methyl; n=2–3; and X=bromide, iodide, or 4-methyl-toluene sulfonate.

A possible explanation of the results presented might arise from the steric interactions between the *O*-acyl-isourea moieties of the activated supports and the enzymes to be immobilized. For successful immobilization, the amino acid side chains essential for the catalytic activity must remain intact during the immobilization. Therefore the reactive sites of the activated support should react with the amino acid side chains on the enzyme surface, rather than with those of the active site. In the case of an activated support bearing bulky reactive groups of the *O*-acyl-isourea type, the access to the active site hole is sterically hindered and, consequently, the undesirable reactions become inhibited. On the basis of the structural characteristics of an enzyme to be immobilized, the most adequate carbodiimide can be selected for activation of the support.

We suggest that the proper selection of the carbodiimide used might contribute to the "tailor-made" immobilization of enzymes.

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