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INVESTIGATION OF THE ACTIVATION OF CROSS-LINKED AGAROSE WITH CARBONYLATING REAGENTS AND THE PREPARATION OF MATRICES FOR AFFINITY CHROMATOGRAPHY PURIFICATIONS

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SUMMARY

The activation reaction of cross-linked agarose with 1,1′-carbonyldiimidazole (CDI) has been extended to other carbonylating reagents, and has confirmed that CDI allows the facile preparation of activated matrices suitable for affinity chromatographic supports. These studies showed that 1,1′-carbonyldi-1,2,4-triazole (CDT) gave a more reactive activated matrix, while 1,1′-carbonyldi-1,2,3-benzotriazole reacted only slowly and inefficiently. Phosgene, in addition to the disadvantage of toxicity, does not give a high level of activation. The introduction of imidazolyl carbamate groups onto cross-linked agarose by generating CDI *in situ* from phosgene and imidazole gave one-third of the level of activation of that obtained with pure CDI.

All of the activated matrices had sufficient stability to aqueous conditions to allow unhurried isolation of the washed, activated product. All carbonylated matrices when subsequently coupled with monoalkylamines were found to be devoid of any additional charged groups due to the activation process.

The studies have demonstrated that CDI is the most effective and convenient of the carbonylating reagents studied for the preparation of activated matrices to be used in affinity chromatographic experiments. However, the CDT-activated matrix is much more reactive than the CDI matrix and may be useful for the coupling of unstable protein ligands where short coupling times are essential.

INTRODUCTION

In a previous communication[1] we demonstrated that 1,1′-carbonyldiimidazole

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SCHEME A

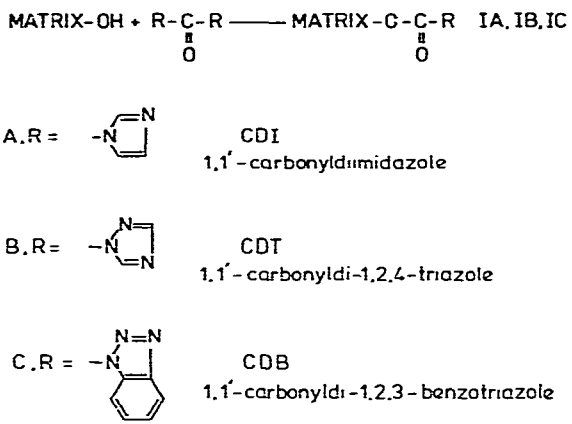

Fig. 1. The chemical reactions involved in the carbonylation of a polysaccharide (Scheme A).

(CDI) could be used to activate cross-linked agarose beads. The imidazolyl carbamate groups (see structure IA, Fig. 1) formed by using this reagent were found to be susceptible to nucleophilic attack by amines in aqueous or organic solutions. Furthermore, the carbamate derivative was found to be relatively stable to oxygen nucleophiles, which thereby facilitated the handling of the activated matrix under aqueous conditions. The attachment of ligands was extended from simple amines to proteins, and the use of the coupled agarose as an affinity chromatography support was subsequently demonstrated[2].

This report investigates the use of other carbonylating reagents *e.g.* 1,1'-carbonyldi-1,2,4-triazole (CDT) and 1,1'-carbonyldi-1,2,3-benzotriazole (CDB) for the activation process shown in Fig. 1. The report describes the activation yield, the stability of the activated matrix, the coupling of amine ligands, and the charge properties of the coupled matrix.

EXPERIMENTAL

Chemicals

CDI and Reactigel™ (6X, 20–25 μmoles of active groups per ml of gel) were purchased from Pierce (Rockford, IL, U.S.A.). CDT and CDB were prepared by standard methods[4]. Phosgene was used as a 17.8% solution in toluene. Acetone was analytical-reagent grade and all other chemicals were of reagent grade.

Solvent exchange of matrices from water

The aqueous suspension of a matrix was washed sequentially with water, water–organic solvent (70:30, v/v), water–organic solvent (30:70, v/v) and five portions of the organic solvent. The matrix was used immediately.

Typical activation procedure

The insoluble polysaccharide (0.2 g dry weight or its moist cake equivalent,

which is *ca.* 3 g in the case of Sepharose CL-6B) was presoaked overnight in, or solvent exchanged into, the appropriate organic solvent (6 ml). It is important that the solvent is anhydrous as the activation reagents used are readily hydrolysed. The activation reagent was added (1 mmol) and the suspension shaken gently for 0.5 h or left longer if desired. The product was washed with the same solvent and either stored under fresh solvent or used immediately.

Typical coupling procedure for simple amines

The activated matrix (from 0.2 g dry weight as above) was sucked dry to a moist cake and added to a solution of amine (5.4 mmol) in aqueous buffer (8 ml), generally either 1 *M* sodium carbonate (pH 10) or 1 *M* N,N,N′,N′-tetramethylethylenediamine (pH 9). For matrices of low degree of activation, a less concentrated buffer may be used. The suspension was shaken overnight at pH 10 or 72 h at pH 9 at room temperature or below. Washing was then carried out with water, 1 *M* salt solution and water again.

Analysis of all carbonylated matrices

The activated matrix was added to a solution of carbonate-free sodium hydroxide (0.15 *M*, 50 ml) in a volumetric flask. The flask was shaken periodically for 0.5 h and the contents allowed to settle. A portion (25 ml) of the supernatant liquid was titrated under nitrogen between the limits of pH 9 and 4 with 1 *M* HCl. This gives the total amount of carbon dioxide and imidazole formed from the active groups. The carbon dioxide was expelled in a stream of nitrogen at pH 3 and the sample was retitrated between the same limits to obtain the amount of imidazole present. The number of active groups present was calculated from the difference between the two figures, that is based on the amount of carbon dioxide expelled. All titrations were carried out using a Radiometer TTT2 pH titrator.

Analysis of coupled matrices

Coupled matrices could generally be analysed by titrations of their amino or carboxyl end-groups. For example, the coupled matrices were washed with HCl (pH 2, 250 ml) if they contained carboxyl end-groups, or NaOH (pH 11.7, 250 ml) if they contained amino end-groups. They were then titrated using the method described above in the presence of 1 *M* salt (10 ml) to pH 7.5 with 0.2 *M* HCl or NaOH as appropriate.

RESULTS AND DISCUSSION

Comparison of activation reagents

CDI was considered on the basis of our preliminary study[1] to be the carbonylating reagent of choice for the activation of cross-linked agarose. In the present study a range of other carbonylating reagents were examined and the results obtained compared with those derived from both the CDI and cyanogen bromide methods. It was apparent from these experiments that other carbonylating reagents could be successfully employed. Data for these experiments are listed in Table I, together with activation yields and efficiencies. None of the listed reagents offers any advantages over CDI in terms of degree of substitution on the matrix. CDT is of comparable efficiency

TABLE I

A COMPARISON OF ACTIVATION YIELDS OF CARBONYLATING REAGENTS ON CROSS-LINKED AGAROSE

Reagent	*Solvent*	*Amount of reagent used (mmol)*	*Yield of active groups (mmol/3 g)*	*Yield (%)*
CDI*	Dioxane	0.93	0.40	43
CDI*	Dioxane–Et_3N(1:1)	0.93	0.26	28
CDT*	Dioxane	0.93	0.32	35
CDB*	Dioxane	0.63	0.08	13
Phosgene*	Dioxane	0.93	0.08	9
Phosgene	Dioxane	4.45	0.31	7
CNBr**	Water	6	0.10	2
CNBr/HCl***	Water	6	0.10	2

* The activation procedure was carried out as described in the Experimental section on 3 g of moist cake.

** This was carried out by the method of March *et al.*[5], on 3 g of moist cake, with 1 *M* Na_2CO_3 as the buffer.

*** The activated agarose (3 g of moist cake) from the CNBr activation was shaken in 1 *M* HCl (10 ml) for 0.5 h. It was washed with deionised water and used immediately.

but is a much less stable reagent and is difficult to handle. CDB reacts only very slowly and inefficiently, possibly because of the steric effect of the bulky benzotriazole groups. In addition, di(*p*-nitroaniline)urea, di-(2-aminopyridine)urea and di-(2-hydroxypyridine)carbonate were tried as activating reagents, but were found to be quite unreactive with cross-linked agarose.

Phosgene, in addition to the disadvantage of toxicity, does not give a high level of activation. The active groups derived from the phosgene reaction were shown to be cyclic carbonates by the absence of chlorine in the product. This cyclisation did not occur with CDI, CDT or CDB in inert organic solvents, and in each case the carbamate derivative (structure I, Fig. 1) was the only identified product. However, if the activation with CDI was carried out in the presence of 50% triethylamine, some cyclisation did occur, and the yield of active matrix was reduced, *i.e.* the active groups present were shown to be 50% cyclic carbonates and 50% imidazolyl carbamates by titrimetric analysis. No cyclisation occurred during the activation of cross-linked agarose with CDB in the presence of triethylamine.

An attempt was made to introduce imidazolyl carbamate groups onto cross-linked agarose by generating the CDI *in situ* from phosgene and imidazole. This experiment was partially successful, but the yield of the imidazolyl carbamate was only *ca.* one-third of that obtained with pure CDI. Furthermore, it was necessary in this case to use an aqueous washing step to remove the amine salts, a procedure which resulted in the hydrolysis of some of the activated matrix. This approach, however, produced little of the cyclic carbonate product. In addition, the procedure could be used successfully to couple 6-aminohexanoic acid, with yields similar to that obtained for the normal CDI-activated matrix.

Cross-linked agaroses, activated by the CDI, CDT or CDB methods, were subjected to hydrolysis under various conditions of pH. The results obtained are

TABLE II

SUSCEPTIBILITY OF ACTIVE CROSS-LINKED AGAROSES TO HYDROLYSIS AT ROOM TEMPERATURE

The matrices, activated as described in the Experimental section (from 3 g of moist cake), were kept at pH 5 by the automatic addition of 2 *M* HCl or at pH 8.5, 10 or 11 by the addition of 2 *M* NaOH. The rate of addition of the acid or alkali was recorded on a Radiometer TTT2 automatic titrator.

Activation	*Reaction time for complete hydrolysis*			
	pH 5	*pH 8.5*	*pH 10*	*pH 11*
CDI	20	30	10	1.5
CDT	–	4	1.5	–
CDB	–	1	1	–

shown in Table II. Clearly all of the activated matrices have sufficient stability to aqueous conditions to allow unhurried isolation of the washed, activated product.

When an active matrix such as a CDI-activated polysaccharide is used to couple proteins it is clearly desirable to have no active groups remaining at the end of the reaction. This is because further nucleophiles inevitably come into contact with the matrix during an affinity chromatography separation. The results shown in Table II therefore give a measure of the time for which a coupling reaction must be left unless the excess active groups are quenched, *e.g.* by the addition of ethanolamine.

The CDT-activated matrix is much more reactive than the CDI matrix and may be used where short coupling times are essential. In particular it could be useful for a more facile coupling of biological molecules sensitive to high pH values. Typically coupling reactions to the CDI matrix should preferably be left 10–18 h at pH 10, or for 36–48 h at pH 8.5 to 9 (both at 4°C). These reactions must be adequately buffered, particularly if high substitution matrices are used.

An investigation of the coupling reaction on activated cross-linked agaroses

A series of couplings was carried out using three simple nucleophilic ligands of differing pK_a values to evaluate the susceptibility of the active groups towards aminolysis compared with hydrolysis. The couplings were carried out on cross-linked agarose activated by the six methods listed in Table III. The ligands selected for this study were 6-aminohexanoic acid, glycine and glycylglycine. In addition, 1,6-diaminohexane was tried in one case to compare couplings under aqueous and non-aqueous conditions. The ligands, all of which have titratable end-groups, were added to the reaction mixture in large (more than ten-fold) molar excess over the active groups on the matrix. The reactions were carried out under well buffered conditions, and their pH values were rechecked just prior to washing. The pH of the glycylglycine reactions, which were carried out at an initial pH of 10, increased to pH 10.5 at the end of the reaction, but in all other cases no pH shift was observed.

The data in Table III show that with the matrices activated by carbonylation, each amine couples best at a pH value within one unit of its pK_a value. For example, under the conditions studied 6-aminohexanoic acid ($pK_a = 11$) couples best at pH 11, glycine at pH 10 ($pK_a = 9.8$) and glycylglycine at pH 9 ($pK_a = 8.1$). The coupling of ligands to the CNBr-activated matrix appears to be less pH dependent, and ap-

ABLE III

OUPLING YIELDS FOR AMINOLYSIS OF ACTIVE CROSS-LINKED AGAROSES

ross-linked agarose (3 g of moist cake) was activated as described above with either CDI or CNBr[5]. For the ›upling reactions a constant amount of amine (5.4 mmol) was used in each case. Aqueous coupling reactions were ırried out at pH 9 and 10 as previously described, except 6-aminohexanoic acid which was left for 5 h at pH 11, ıd without additional buffer. The coupling of CDI activated cross-linked agarose to 1,6-diaminohexane in dioxane as carried out overnight. The matrix was solvent-exchanged back into water and washed in the usual way.

ctivation	*Amount of active groups on matrix (mmol)*	*Coupling yield (%)*				
		6-Aminohexanoic acid	*1,6-Diaminohexane*	*Glycine*	*Glycylglycine*	
		pH 9	*pH 10*	*pH 11*	*pH 9*	*pH 10*
DI	0.280	20	40	50	70	25
DT	0.315	27	49	–	49	22
DB	0.070	–	61	–	–	–
nosgene	0.080	–	35	–	–	25
NBr	0.105	61	62	–	32	29
NBr/HCl	0.105	–	–	–	29	13

TABLE IV

THE TRYPSIN CAPACITIES OF AFFINITY COLUMNS PREPARED BY THE IMMOBILISATION OF SOYABEAN TRYPSIN INHIBITOR TO SEPHAROSE CL-6B WITH DIFFERENT CARBONYLATING REAGENTS

*Method**	*Active groups***	*STI coupled at pH 9*		*STI coupled at pH 10*	
		*Trypsin bound**** *(mg)*	*Total column volume (ml) in distilled water*	*Trypsin bound**** *(mg)*	*Total column volume (ml) in distilled water*
CDI	66	0.68	0.64	1.18	0.46
	192	0.97	0.4	2.1	0.38
CDT	52	0.51	0.6	0.51	0.48
CDB	27	0.18	0.64	0.2	0.58
Phosgene	52	0.37	0.52	1.04	0.38

* The activation procedures described in the Experimental section were used. In this case 1 g of moist cake was activated with the following amounts of carbonylating reagent: CDI, 0.47 and 1.21 mmol; CDT, 0.31 mmol; CDB, 0.63 mmol; phosgene, 4.45 mmol. The activation reaction was left for 0.5 h except for the CDB reaction which was left for 5 days.

** Measured by titration analysis and expressed in μmoles for the whole sample.

*** Soyabean trypsin inhibitor (15 g) was coupled to the activated matrix at pH 9 or at pH 10 using conditions described previously[1]. Crude bovine trypsin (20 mg) was loaded into the column equilibrated in 500 m*M* NaCl–50 m*M* Tris, pH 8.0. The buffer was changed to 500 m*M* NaCl–3 m*M* HCl to elute the bound trypsin. The concentration of bound trypsin was determined spectrophotometrically at 280 nm and values expressed as milligrams per total sample.

parently a higher percentage of activated groups are coupled with this activated matrix than with the CDI-activated matrix, *e.g.* 61% compared with 20% for 6-aminohexanoic acid at pH 9. However, the concentration of ligand groups attached to the matrix is the same for both products (*ca.* 60 μmol) owing to the higher starting concentration of active groups with the CDI-activated matrix.

The coupling of trypsin inhibitor to the activated matrices

Proteins can be attached to the matrix under mildly acidic conditions via a diimide-mediated coupling to a preformed leash with either a free amino or carboxyl group. This procedure has been used in the preparation of high capacity trypsin affinity columns[1]. In addition we described the immobilisation of trypsin inhibitor via CDI-activated cross-linked agarose at pH 9, and the subsequent use of the generated affinity support for the purification of bovine trypsin. These studies have been extended to the other carbonylating reagents, and the results are summarised in Table IV. The results shown in this table confirm that CDI is a very satisfactory reagent for the preparation of an affinity column, although CDT also gives a suitable product. Two levels of CDI were used in this study and it can be seen that the matrix with the higher level of activation (192 μmole per gram of moist cake) showed a decreased swollen volume presumably caused by cross-linking reactions. The matrix still was suitable for the purification of trypsin, as can be seen by the high capacity of the column for this protein. Although soybean trypsin inhibitor is stable to the coupling conditions used in this study, many proteins would be degraded under these conditions. As was shown in the companion paper[6] the coupling reaction can, however, be carried out at pH 8.5, or alternatively a diimide-mediated coupling of the protein to a leash can be carried out at mildly acidic pH values[1].

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