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Short Communication

Efficient substitution of 1,1′-carbonyldiimidazole activated cellulose and Sepharose matrices with amino acyl spacer arms

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ABSTRACT

Previous procedures for addition of the spacer arm, 6-aminocaproic acid, to 1,1′-carbonyldiimidazole (CDI) activated matrices used dilute, largely aqueous reaction conditions. Only 20–50% of the activated groups were substituted. Treatment of CDI activated matrices in an organic solvent, with a concentrated aqueous solution of sodium 6-aminocaproate, allowed efficient (up to 95%) substitution of CDI activated groups. The substitution efficiency of aminocaprylic acid was also improved considerably using an organic solvent although the maximum efficiency was lower (66%). The new procedure allows reproducible levels of spacer arm substitution to be obtained. A benzamidine affinity resin prepared by the new method had a 50% higher capacity for trypsin than one prepared by the original method.

INTRODUCTION

1,1′-Carbonyldiimidazole (CDI) activation of hydrophilic matrices is a useful technique for affinity chromatography because it produces an uncharged, relatively stable urethane link between matrix and ligand [1–4]. Previous work demonstrated that very high levels of CDI activation (up to 10 mmol/g) could be obtained on cellulose and agarose matrices. However, substitution of activated matrices with a spacer arm or ligand was inefficient (typically in the range of 20 to 40% and occasionally up to 50% [4–6]. This phenomenon was particularly noticeable with the use of dilute buffered solutions of amino acyl spacer arms. The inefficiency of coupling resulted in poorly reproducible substitution levels.

Of particular concern has been the coupling of spacer arms, aminocaproic and aminocaprylic acid. This report investigates their coupling to activated matrices in a range of solvent environments to determine whether reduction of water content improves efficiency. Other variables tested were: reaction time, ratio of moles of spacer arm added to moles of active groups and solvent type. The results reported here present an improved method for the attachment of ligands with limited solubility in organic solvents.

EXPERIMENTAL

Perloza bead cellulose was purchased from Tessek (Prague, Czechoslovakia); Sepharose CL 6B from Pharmacia (Uppsala, Sweden); trypsin (type IX), CDI, 6-aminocaproic acid, 7-aminocaprylic acid, 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide (EDC) and dimethyl sulphoxide (DMSO)

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(99+%) from Sigma (St. Louis, MO, USA); and *p*-aminobenzamidine from Aldrich (Germany). Dioxane, acetone and N,N-dimethylformamide (DMF) were analytical reagent grade and ethanol was technical grade. DMF was degassed and then distilled under vacuum from calcium hydride before use. Lithium, sodium and potassium aminocaproate salts were prepared by dissolving 6-aminocaproic acid in an equimolar amount of 1 *M*-LiOH, NaOH and KOH respectively, and freeze-drying the solutions.

CDI activation of matrices and titration methods were similar to the method of Bethell *et al.* [1]. Carbon dioxide was not titrated. Imidazole released by hydrolysis of active groups was titrated between pH 5 and pH 8.5 after carbon dioxide removal. The carboxyl groups of spacer arm substituted matrices were titrated to pH 8 with 0.1 or 1 *M* NaOH using a Radiometer autotitrator. Titrated samples were washed, oven-dried and weighed.

Solvent exchange

The matrix was washed sequentially with deionised water, dioxane–water (25:75), dioxane–water (50:50), dioxane–water (75:25) and 100% dioxane. A minimum of 5 matrix volumes was used for each stage. The final 100% dioxane washes were analytical grade to ensure minimal water content. For some experiments dioxane was replaced by acetone to solvent exchange the matrix to the anhydrous state.

Mixing procedure

All reactions were mixed mechanically at room temperature in sealed vessels. CDI activation mixtures were agitated mildly using an Ika Vibra-mix shaker. Spacer arm–activated matrix mixtures were mixed by rotation on a Ballmill roller.

CDI activation

CDI was added to the solvent-exchanged matrix and mixed for 1.5 h. The mixture was transferred to a sintered glass funnel and washed with 10 × resin volumes of solvent (acetone or dioxane) to remove unreacted CDI and imidazole produced by the reaction. Weighed samples were taken from the activated matrix. One sample was kept for titration. The other samples were either maintained in the organic solvent used for CDI activation or solvent exchanged to another solvent, water or a solvent–water mixture. Activation levels of 2.5–3.5 mmol/g dry matrix were obtained using 0.7–1 g CDI per g dry matrix (determined from known wet weight:dry weight ratios).

Spacer arm addition

A 5-fold excess of 6-aminocaproic acid (5 mmol of spacer arm per mmol of active group on the matrix) was the standard amount used. A 3-fold excess of aminocaprylic acid was used because of its lower aqueous solubility. The surplus of spacer arm removed the need for additional buffering and ensured a reproducible, complete reaction. A 50% solution of aminocaproic acid, pH 11.3, was produced using 10 *M* NaOH and water. These pH conditions were higher than those used previously [1,2,5] and were chosen so that 80 to 90% of amino groups were in the reactive (unprotonated) form. Aminocaprylic acid is less soluble and was prepared as a 20% solution at the same pH.

The CDI-activated matrix in the appropriate organic solvent was mixed with spacer arm solution for 0.5–24 h. Afterwards mixtures containing organic solvent were solvent exchanged to water as described previously. All matrices were then washed thoroughly with deionised water. The substitution of spacer arm groups on the matrix was determined by titration (ml NaOH)/g dry weight of matrix. The dry weight value included the weight of spacer arm groups attached to the matrix. For comparison with the original CDI substitution, corrected spacer arm substitution values were calculated using corrected dry weights (dry weight − weight of spacer arm groups). The accuracy of the corrected values were validated in one experiment (Table I).

Ligand binding and capacity testing

Two samples of 6-aminocaproate bead cellulose were used to produce affinity matrices for trypsin. For each sample a slurry was prepared (5 g wet weight of resin in 2 ml of water) and the pH adjusted to 4.7 with 1 *M* HCl. The affinity ligand (*p*-aminobenzamidine hydrochloride, 0.2 g dissolved in 2 ml water) and the condensation agent (EDC, 0.2 g dissolved in 0.5 ml water) were each pH adjusted to 4.7 and added to the slurry. The slurry was maintained at pH 4.7, while mixing, at room temperature for 48 h [7]. Both samples were retitrated

TABLE I

VERIFICATION OF CORRECTED SUBSTITUTIONS AND COMPARISON OF SOLVENTS

Weighed samples of CDI-activated matrices in dioxane were solvent exchanged using mixtures of either DMSO, DMF or water with dioxane to produce samples in 100% DMSO, 100% DMF, 100% water, 50% dioxane and the original 100% dioxane form. Another sample (CDI*), solvent exchanged to 100% water, was used to determine the concentration of activated groups on the matrix. W. W. is the original wet weight of CDI-activated matrix in dioxane, D.W. is the dry weight of the sample. Titre/D.W. is the titrated substitution whereas substitution* is the corrected substitution. The accuracy of the corrected values is indicated by the correlation between corrected substitution percentages and percentages of substitution per gram wet weight.

Sample	W.W.	Titre	D.W.	Titre/W.W.		Titre/D.W.		Substitution*	
	(g)	(ml 1 M NaOH)	(g)	ml/g	%	ml/g	%	mmol/g	%
CDI*	3.035	0.895	0.3518	0.295		2.54		2.54	
Dioxane	3.034	0.753	0.4679	0.248	84	1.61	63	2.26	89
Dioxane 50%	3.010	0.575	0.4438	0.191	65	1.30	51	1.69	66
Water	3.033	0.437	0.4239	0.144	49	1.03	40	1.27	50
DMSO	3.056	0.844	0.4972	0.276	94	1.70	67	2.44	96
DMF	3.145	0.820	0.5049	0.261	89	1.62	64	2.29	90

to ensure completeness of carboxyl group substitution.

Resin capacity was tested by frontal analysis [8] on 1-ml samples packed in 2-ml polystyrene columns. Equilibration, loading and washing buffer was 25 mM Tris, 10 mM $CaCl_2$ (pH 8.2). Trypsin was eluted with 0.05 M formic acid and total protein (mg) determined by UV spectroscopy at 280 nm and bicinchoninic acid assay [9].

RESULTS AND DISCUSSION

Spacer arm solubility

The solubilities of lithium, sodium and potassium aminocaproate salts were tested in a range of solvents to see if a nonaqueous or near nonaqueous medium could be used for coupling. Solubilities were generally poor in the absence of water. Addition of a small volume of water dramatically improved solubility. Therefore it was essential to dissolve the spacer arm in a small volume of water before addition to the activated matrix. The concentrated solutions used did not appear to precipitate when added to organic media. Aminocaprylate was also dissolved in water prior to addition to organic media. Water could not be eliminated from the reaction mixture, in either case, but could be kept to a minimum.

Comparison of coupling efficiency in different environments

Initial studies used dioxane as solvent. Reducing the water content of the reaction mixture resulted in a steady improvement in the efficiency of substitution on both cellulose and agarose matrices (Table II). For bead cellulose the substitution efficiency using aminocaproic acid was 63% in water, 82% in dioxane–water (50:50) and 93% when water content was minimised. For Sepharose CL-6B the respective substitution efficiencies were 50%, 76% and 96%. Thus using a CDI-activated matrix in 100% dioxane and treating this with a concentrated aqueous solution of sodium aminocaproate produced 90–95% substitution of active groups.

Bead cellulose samples in DMSO or DMF had similar substitution efficiency to dioxane samples (96% and 90%, respectively) using minimum water content. In this experiment the values for water, water–dioxane (50:50) and minimum water were 50%, 66% and 89%, respectively (Table I). Comparison of results for bead cellulose in dioxane from separate experiments (Table I, Table II) showed that variation between samples was least when water content was minimised. The substitution efficiency obtained using acetone as organic solvent was intermediate between aqueous and dioxane samples. Bead cellulose substitution values ranged from 73 to 79% with minimum water whereas in 100% water the corresponding values where 56 to 59%. Sam-

TABLE II

COUPLING YIELDS OF CDI-ACTIVATED CELLULOSE AND SEPHAROSE WITH 6-AMINOCAPROIC ACID

CDI-activated matrices in 100% dioxane, dioxane–water (50:50) or 100% water were mixed with spacer arm solutions and substitution determined by titration. Corrected substitutions were determined using a corrected dry weight (dry weight − weight of spacer arm attached). Reaction efficiency was calculated using corrected substitution values.

Matrix	Active groups (mmol/g)	Media water (%)	Titrated substitution (mmol/g)	Corrected substitution (mmol/g)	Efficiency (%)
Bead cellulose	3.5	100	1.6	2.2	63
		50	1.88	2.85	82
		0	2.05	3.25	93
Sepharose CL-6B	2.47	100	1.01	1.23	50
		50	1.4	1.87	76
		0	1.67	2.38	96

ples in acetone developed a brown colour. This did not occur in the other solvents, suggesting a side-reaction and/or decomposition was occurring, presumably via enolisation of the acetone. The coloured products were soluble and readily washed out. The percentage substitutions using ethanol were lower than aqueous results and are not recorded here.

For bead cellulose, using aminocaprylic acid, the substitution efficiency could be improved from 27% in water to 66% when water content was minimised. The lower efficiency compared to the aminocaproic acid example was accounted for by the greater volume of water required to solubilise the 7-carbon spacer arm.

The use of a concentrated spacer arm solution improved the efficiency of active group substitution compared to previous results [5,6], even when the CDI-activated matrix was in aqueous media. The increased efficiency obtained by using an organic solvent to minimise the water content of the reaction mixture could be due to a higher molar ratio of spacer arm to water. This would favour spacer arm substitution of active groups rather than base-catalysed hydrolysis. Moreover the organic solvent is likely to affect the rates of the competing substitution and hydrolytic reactions and hence the percentage of active groups substituted.

Therefore the choice of solvent is important. The protic solvents (ethanol and water) gave the lowest results. The aprotic solvents (acetone, dioxane, DMSO and DMF) gave the highest results. The inference is that replacement of water with an aprotic solvent alters the relative rates of the hydrolytic and substitution reactions. Aprotic solvents increase the enthalpy of anionic forms and increase entropy. Overall the free energy differences between ground state and transition state for the reactions are apparently modified to favour the substitution reaction [10]. The more polar aprotic solvents (DMSO and DMF) are the best choices to solvate the CDI-activated matrix because solvent shock damage upon addition of the aqueous solution is less likely. If dioxane is used, the spacer arm solution can be diluted with solvent prior to addition to the matrix.

Other variables

The effect of reaction time and spacer arm concentration was also studied. The shortest time used for the reaction was 30 min. The reaction proceeded to effective completion within this time whether the solvent used was water or acetone. Only a 3–4% variation occurred from 0.5–20 h.

A 3- to 5-fold excess of aminocaproic acid (over active groups) was required for maximum substitution efficiency. For bead cellulose in acetone the substitution efficiences were 60% (1), 72% (2), 75% (3), 79% (4) and 79% (5 equivalents).

Trypsin-binding capacity

Two samples of CDI-activated bead cellulose (1.4 mmol/g) were substituted with aminocaproic acid; the first in dilute aqueous reaction conditions [5], the second using dioxane and minimising water content. The uncorrected spacer arm substitutions were 0.42 mmol/g and 1.09 mmol/g, respectively.

Following coupling with *p*-aminobenzamidine the samples were capacity tested by frontal analysis. The capacities for trypsin were 18 mg/ml and 28 mg/ml, respectively. Otherwise their chromatographic behaviour was the same. Thus the new technique allowed a considerable improvement in capacity without apparent side-effects.

Several advantages result from efficient substitution of a CDI-activated matrix. CDI is a moderately expensive, unstable compound requiring special storage conditions (below 0°C, sealed from atmosphere). A reduced requirement for CDI with increased efficiency of substitution is obviously an advantage. Aminocaprylic acid is expensive and efficient use will provide a considerable cost saving. If the substitution of the CDI-activated matrix is low for any reason (*e.g.* unreactive CDI, poor solvent exchange) efficient substitution of activated groups is more likely to yield a useful product. Extremely high substitution levels can be obtained using this technique. Such high levels of substitution can be undesirable for affinity chromatography [11,12]. However for some applications, highly substituted matrices allow very high capacities to be obtained [13]. The example of trypsin capacity quoted above demonstrates how capacity can be increased by efficient substitution. Alternatively lower substitutions may be obtained with more precision by minimising the hydrolysis reaction. This new method for spacer arm addition to a CDI-activated matrix does not require any additional steps compared to previous techniques. It can produce a significant cost saving when scale-up is considered.

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