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Chemical Coupling of Amino Acids, Peptides and Proteins to Sephadex

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Immunosorbents may be synthesized by coupling antigens to solid hydrophilic supports.[1-4] The supports should be devoid of fixed ionic groups and other potential non-specific adsorption sites. Cross-linked dextrans (Sephadex) possess the required properties and have been used already as matrices for specific sorbents.[5]

A few years ago we fixed human and porcine blood group substances A on Sephadex G25 (Porath and Killander, unpublished) by diazo coupling with *p*-aminobenzyl Sephadex. Although the results were promising, the work was discontinued for several reasons. We are now following up our earlier investigations with a wider scope of application in mind and are exploring other methods of coupling.

Kent and Slade have suggested condensation of proteins with isothiocyanate fixed to a polymer (polyaminostyrene).[6] This communication describes the results of some exploratory studies using the mode of fixation suggested by Kent and Slade but with *p*-amino-phenoxy-hydroxypropyl and *β*-amino-ethyl ethers of Sephadex G25. These starting materials were kindly supplied by Mr. Björn Söderquist and Dr. Bertil Gelotte of Pharmacia, Uppsala.

The amino ethers were converted to the corresponding isothiocyanate derivatives by treatment with thiophosgene.

The amino derivatives of Sephadex were allowed to swell in potassium hydrogen phosphate buffer (3.5 M KH_2PO_4/K_2HPO_4, pH 6.8). A 10 % solution of thiophosgene in carbon tetrachloride was added (3 ml/g of amino derivative) and the reaction was carried out under vibration for 1 h. The gel was then washed for 5 min with 0.5 M sodium hydrogen carbonate, followed by sequential washings with water and portions of water-acetone-mixtures of increasing acetone concentrations to remove solids and to shrink the gel.

In order to estimate the isothiocyanate content a portion of the dried product was hydrolyzed with hydrochloric acid and the hydrogen sulphide formed was iodometrically determined.

The reactions between the isothiocyanate gel and the amino acids or amino acid derivatives were performed in aqueous sodium hydrogen carbonate solution or in formamide containing triethylamine. After mixing the suspension at room temperature for 20–30 h the gel was washed with 0.5 M sodium hydrogen carbonate, water, 0.5 M hydrochloric acid, with water-ethanol mixtures of increasing ethanol concentration, and finally, with pure ethanol.

In Table 1 are compiled relevant data for a number of experiments with *p*-isothiocyanato-phenoxy-hydroxypropyl Sephadex with an isothiocyanate-content of 120–150 *μ*equiv./g dry gel substance.

The amino acid analyses were performed according to Spackman, Moore and Stein using the Spinco automatic amino acid analyzer. To determine the effect of the carbohydrate on the recoveries of amino acids, mixtures of the free amino acids and Sephadex were "hydrolyzed" and analyzed. No losses were observed with the particular amino acids used in these experiments. Since, in the coupled products the amino acids presumably have been converted to substituted thioureas a quantitative reversion to amino acids upon hydrolysis cannot be expected. Therefore, analytical data in Table 1 are minimum estimates of the extent of substitution.

Table 1. Coupling of amino acids or amino acid esters to *p*-isothiocyanato- phenoxy-hydroxypropyl Sephadex containing 120–150 μequiv. isothiocyanate per gram dry substance.

Amino acid or ester	Solvent. Gel substance/amino acid (or ester) ratio	Yield of amino acid in μmole/g after	
		alkaline hydrolysis	acid hydrolysis
Methionine methyl-ester hydrochloride	3 ml 0.1 M $NaHCO_3$. 200 mg/50 μmole	13	7
Methionine methyl-ester hydrochloride	5 ml 0.5 M $NaHCO_3$. 260 mg/50 μmole	38	20
Methionine methyl-ester hydrochloride	3 ml formamide + triethylamine. 200 mg/50 μmole	53	25.5
Methionine	3 ml formamide + triethylamine. 100 mg/60 μmole	20	11
Histidine methyl-ester dihydrochloride	2 ml 0.5 M $NaHCO_3$. 50 mg/60 μmole	96	65
Histidine methyl-ester dihydrochloride	2 ml 0.5 M $NaHCO_3$. 50 mg/10 μmole	25	—
Histidine hydro-chloride	2 ml 0.5 M $NaHCO_3$. 50 mg/60 μmole	98	—
Alanine methyl-ester hydrochloride	2 ml 0.5 M $NaHCO_3$. 50 mg/10 μmole	26	10

Table 2. Coupling of peptides to *p*-isothiocyanato-phenoxy-hydroxypropyl Sephadex containing 150 μequivalents per gram dry gel substance.

Peptide	Reaction conditions: solvent; mg gel/mg peptide; time, days	Yield of peptide after acid hydrolysis	
		μmole/g	mg/g
Oxytocin	10 ml 0.1 M $NaHCO_3$; 200/15; 3	16.5	16.6
Oxytocin	10 ml 0.5 M $NaHCO_3$; 200/15; 2	15.5	15.6
Glucagon	5 ml 0.1 M $NaHCO_3$; 120/10; 3	4.6	16.0
Glucagon	5 ml 0.5 M $NaHCO_3$; 120/10; 3	5.5	19.1
Insulin	10 ml 0.1 M $NaHCO_3$; 320/80; 3	10.3	59.5
Insulin	5 ml formamide + tri-ethylamine; 100/50; 7	2.5	14.5
γ_G-Globulin	1 ml 0.2 M $NaHCO_3$; 100/20; 3	0.21	34.0

Acid treatment causes some cyclization to thiohydantoins, which are not converted to amino acids in good yield by further acid treatment. The lack of agreement between the results of acid and alkaline hydrolysis is therefore evidence for chemical fixation of the amino acids or their derivatives to Sephadex. Simple physical adsorption should not cause such a discrepancy. Some of the figures are surprisingly large in view of the fact that competing reactions take place. For example, about half of the isothiocyanate groups in the Sephadex are hydrolyzed after exposure for 7 h to the conditions used in the coupling reaction (0.5 M sodium hydrogencarbonate at 30°C). Hydrolysis and other side reactions are under study at present.

The coupling of amino acids and amino acid esters to β-isothiocyanato-ethyl Sephadex was found to be less efficient. The yield obtained was only about half that observed with the corresponding aromatic isothiocyanato ether of Sephadex.

Control experiments were performed to determine whether the amino acids were coupling to groups other than the isothiocyanate. When the regular sequence of treatments was carried out without including thiophosgene, no traces of amino acids were detected in the analyses of the hydrolyzed Sephadex products. However, when ordinary Sephadex is treated with thiophosgene fixation does occur to a limited but significant extent. In such an experiment with methionine methylester 1.5 μmole of this substance was coupled per gram of dry Sephadex.

In order to investigate the effects of molecular size on the extent of substitution some experiments were made with oxytocin, glucagon, insulin and γ_G-globulin. The conditions for coupling were essentially the same as before. To exclude physical adsorption the reaction products were washed thoroughly with 30 ml portions of the following solutions: 0.5 M sodium hydrogen carbonate (3 times), water, 0.5 M hydrochloric acid (3 times), concentrated sodium chloride in 0.5 M hydrochloric acid, (twice), water (3 times), portions of ethanol-water with increasing concent ration of ethanol and finally with pure ethanol. The gel was separated from the solvent by centrifugation after each washing step.

The coupled products were hydrolyzed with hydrochloric acid and analyzed as before. The results are shown in Table 2. The yields have been calculated on the basis of the amino acid analysis and the known composition of the substances. Upon acid hydrolysis the N-terminal amino acids and lysine are partly destroyed. For example, the recovery of phenylalanine and glycine from insulin corresponded to 2.4 and 3.6 residues as compared to the theoretical 3 and 4 residues, respectively. Low recoveries of tyrosine and cystine were always found.

The fixation of the high molecular weight reactants is surprisingly large. The effect of the degree of cross-linking on the coupling of proteins and the comparative reactivities of different amino acids are interesting subjects for further investigations.

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