# PREPARATION OF CELLULOSE DERIVATIVES FOR AFFINITY CHROMATOGRAPHY AND IMMOBILIZATION OF ENZYMES. ACTIVATION BY EPICHLOROHYDRIN

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Cellulose derivatives were prepared by bonding aliphatic and aromatic diamines and amino acids to cellulose *via* epichlorohydrin. The immobilization of enzymes on these derivatives was tested with amylase. The use of the cellulose derivatives in affinity chromatography was essayed in experiments with the bonding of *p*-chloromercuribenzoate and with affinity chromatography of amylolytic enzymes. The results are comparable with those obtained with supports activated by cyanogen bromide.

During the past few years the activation of polysaccharide supports has still less been effected by cyanogen bromide which is being replaced by less toxic reagents such as ethyl chloroformate[1] or bis-oxiranes[2]. In this study an effort has been made to replace oxiranes, which do not belong to common compounds produced on a large scale, by epichlorohydrin.

## EXPERIMENTAL

Ethylenediamine, tetramethylenediamine, and hexamethylenediamine used in this study were from Koch-Light (England). Epichlorohydrin was a technical grade product (Spolek pro chemickou a hutní výrobu, Ústí n/L.) which was dried and redistilled before use.

The amylolytic enzymes were commercial preparations and their specific activities were the following: mold α-amylase (Lachema) 35 EU/mg, bacterial α-amylase (NOVO Industri) 10 EU/mg, and amyloglucosidase (NOVO Industri) 14·5 EU/mg.

The amylolytic activity was determined as described before[3-6] by the method using 2,5-dinitrosalicylic acid.

The concentration of amino groups attached to cellulose was determined by potentiometric titration in 0·5M-NaCl after preceding washing of the support to neutral reaction. The concentration of carboxyl groups was determined by the same method except that the carboxyl derivatives were first converted into the $H^+$-form.

The protein concentration of the effluent from affinity columns was determined in a UV-Analyzer (Instrument Development Workshops, Czechoslovak Academy of Sciences, Prague). The amines were coupled to cellulose in two different manners:

*A*) 100 g of cellulose (Whatman Chromedia CC 31) was suspended in 400 ml of 1M-NaOH and stirred with 25 g of epichlorohydrin 2 h at 60°C. The cellulose derivative was filtered off, washed with 400 ml of 1M-NaOH and 1000 ml of water. The washed product was suspended

in 400 ml of 1M-NaOH and treated with 0·1 ml of the amine coupled. The addition of sodium hydroxide can be omitted if ammonia is bonded. The suspension was stirred 2 h at 60°C and the cellulose derivative was then washed with water until the washings were neutral.

*B*) 100 g of cellulose (Whatman Chromedia CC 31) was suspended in 400 ml of 1M-NaOH and treated with 25 g of epichlorohydrin and 0·1 mol of the amine chosen. The suspension was then stirred 2 h at 60°C. The cellulose derivative was then filtered off, washed with 400 ml of 0·1M-NaOH and water until the the washings were neutral.

## RESULTS AND DISCUSSION

Ammonia was the most reactive of all the amines tested. Method *A* afforded 3-amino-2-hydroxypropylcellulose containing 245 μmol of amino groups per 1 g, whereas method *B* gave a derivative with 302 μmol of amino groups per 1 g. Cellulose activated according to method *A* looses its ability to bind amines when stored at 4°C. This is caused most likely by additional cross-linking of the cellulose. Fig. 1 shows that the substitution by amino groups is the higher the shorter the interval between activation and coupling of the amine. Method *B* is therefore more appropriate. Since, however, there is another reaction which takes place when method *B* is used, *i.e.* cross-linking of two amine molecules with epichlorohydrin, the bonding of amines was effected by method *A*, the interval between activation and coupling being 1 h. The results were compared with those obtained by method *B* under identical amine concentrations. The degree of substitution was the same in both cases and therefore method *B* only was used in the subsequent experiments.

There are differences in the final degree of substitution which reflect the activity of the individual amines. The degree of substitution was the lowest when amino acids were bonded (glycine, ε-aminocaproic acid). The concentrations obtained with the individual amines are listed in Table I. It is obvious that the degrees of substitution obtained with even the least reactive amines are essentially the same as those obtained after cyanogen bromide activation.

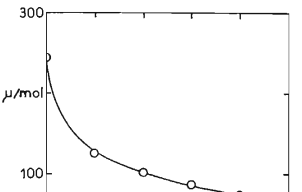


FIG. 1

Binding Capacity of Epichlorohydrin-Activated Cellulose as Function of Storage Time

Cellulose activated according to *A* was stored at 4°C and the binding capacity was determined in terms of the quantity of ammonia covalently attached and expressed in μmol of $NH_2$ per 1 g of dry support.

The properties of the cellulose derivatives prepared in this study permitted the testing of their use for immobilization of enzymes and affinity chromatography.

Direct immobilization of enzymes by method *A* or *B* is impossible because of the high alkalinity of the reaction mixture. An effort was therefore made to immobilize the amylolytic enzymes by method *B* as follows: the activation was carried out as described under Experimental and the immobilization was effected in 0·2M carbonate buffer at pH 9·0. The results are shown in Table II (experiment 1, 2, and 3). An active immobilized enzyme was obtained only when the procedure was applied to bacterial α-amylase. When the amylolytic enzymes were coupled to the carboxyl derivatives the immobilized enzymes were active in all cases. Cellulose derivatives prepared by bonding of *p*-phenylenediamine and benzidine were also employed for the immobilization of enzymes by diazotization and coupling. Active immobilized enzymes were obtained with both cellulose derivatives (Table II).

Table I

Concentration of Substituents after Bonding of Various Amines

| Amino compound | Concentration μmol/1 g dry weight | Amino compound | Concentration μmol/1 g dry weight |
|---|---|---|---|
| Ammonia | 302 | ε-Aminocaproic acid | 48 |
| Ethylenediamine | 127 | Glycine | 62 |
| Tetramethylenediamine | 120 | *p*-Phenylenediamine | 108 |
| Hexamethylenediamine | 118 | Benzidine | 112 |

TABLE II

Binding of Enzymes to Cellulose Derivatives

| No | Enzyme | Method | Method of immobilization | Retention % | Ref. |
|---|---|---|---|---|---|
| 1 | Bacterial α-amylase | *A* | 0·2M carbonate, pH 9·0 | 2·6 | — |
| 2 | Mold α-amylase | *A* | 0·2M carbonate, pH 9·0 | 0 | — |
| 3 | Amyloglucosidase | *A* | 0·2M carbonate, pH 9·0 | 0 | — |
| 4 | Bacterial α-amylase | *B* | glycine, carbodiimide | 36 | 4 |
| 5 | Amyloglucosidase | *B* | carbodiimide ε-aminocaproic acid | 36 | 4 |
| 6 | Amyloglucosidase | *B* | diazotization, coupling | 8 | 7 |
| 7 | Amyloglucosidase | *B* | benzidine, diazotization, coupling | 28 | 7 |

TABLE III

Affinity Chromatography of Amylolytic Enzymes on *p*-Chloromercuribenzoyl Derivative of Cellulose

The affinant was bonded through hexamethylenediamine. Elution by 0·05M cysteine. Adsorption and elution at pH 5·6.

| Enzyme | Specific activity, EU/mg | |
|---|---|---|
| | initial | after chromatography |
| Bacterial α-amylase | 10 | 118 |
| Mold α-amylase | 35 | 102 |
| Amyloglucosidase | 14·5 | 94 |

The amino derivatives of cellulose, obtained by bonding of aliphatic diamines, were used for the preparation of the affinity adsorbent by bonding of *p*-chloromercuribenzoate (Table III). In all cases a preparation of high specific activity was obtained after elution, yet the protein concentration of the eluate was low.

The results of this study show that the bonding of amines, aliphatic or aromatic diamines, or amino acids, to epichlorohydrin-activated cellulose yields cellulose derivatives suitable for immobilization of enzymes and attachment of low molecular weight ligands for affinity chromatography. The same procedure could probably be applied also to agarose since the cross-linkage by agarose by 2,3-dibromopropanol is carried out under practically the same reaction conditions[8].

The method described is more advantageous, from the viewpoint of industrial applications than the method using cyanogen bromide mainly because of the lower toxicity of the coupling reagent.

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