PREPARATION OF p-AMINOBENZYL CELLULOSE AND ITS UTILIZATION FOR IMMOBILIZATION OF ENZYMES

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Dedicated to Professor Ľ. Drobnica on the occasion of his 50th birthday.

The O-alkylation of non cross-linked celluloses with p-nitrobenzyl chloride according to Campbell does not afford satisfying results (alkylation degree $D_a \leqq 0{\cdot}025$). On the other hand, the use of cross-linked celluloses makes it possible to prepare p-nitrobenzyl cellulose under substantially milder reaction conditions ($D_a \leqq 0{\cdot}42$); the degree of cross linking of cellulose considerably affected the D_a of p-nitrobenzyl cellulose. The same also holds for O-alkylation of cross-linked celluloses with p-nitrobenzyl chloride according to Hakomori. Reduction of p-nitrobenzyl cellulose with dithionite is considerably associated with side reactions in which sulfur became embodied, whereas reduction with titanous chloride resulted in noticeable losses of p-nitrobenzyl groups. p-Aminobenzyl cellulose is, after activation with carboxyl groups, a suitable carrier for immobilization of enzymes as acetylcholinesterase, glucoamylase and α-amylase. The respective immobilized enzymes retained relatively high relative activities, in the last two cases also for high-molecular substrates.

Polysaccharides containing primary aromatic groups have been chosen for a longer time in many fields of enzyme engineering[1-5]. Successfully were employed derivatives of cellulose, especially p- and m-aminobenzyl ethers, p-aminobenzoylates and 3-(p-aminophenoxy)-2-hydroxypropyl ethers of cellulose[2,6], the quite frequent use being reported for p-aminobenzyl cellulose[2,3,6-9]. The procedure formerly worked up for preparation of p-aminobenzyl cellulose employing p-nitrobenzyl chloride for O-alkylation of cellulose in the first step[10] did not afford, however, satisfying results, since noticeably extreme conditions leading to a partial degradation of the polysaccharide had to be applied[11]. Data informing on the alkylation degree (D_a) of p-nitrobenzyl cellulose prepared in this way are contradictory[6-10] and lack also a note on the content of NH_2 groups in the p-aminobenzyl celluloses.

This paper is intended to show that the use of a cross-linked cellulose as a starting material for preparation of p-aminobenzyl cellulose by O-alkylation with p-nitrobenzyl chloride makes it in principal possible to carry the reaction under milder conditions and attain substantially higher D_a values. Further improvement offered following procedures based on O-alkylation of polysaccharides in the presence of a methylsulfinyl anion[12]. The obtained preparations of p-aminobenzyl cellulose serve as suitable carriers for immobilization of enzymes, as *e.g.* acetylcholinesterase, glucoamylase or α-amylase.

EXPERIMENTAL

Microcrystalline cellulose Avicel PH 101 (FMC Co., Marcus Hook, Pa., USA), cellulose powder standard grade (Whatman Ltd., Maidstone, England), standard purified cotton (Rico, Veverská Bytíška, Czechoslovakia) were commercially available, the Finnish cellulose Rauma-RR-F, the spruce sulfit were donated by Dr Pastýr of this Institute. *p*-Nitrobenzyl chloride, *p*-nitrobenzyl bromide and 1-cyclohexyl-3-(2-morpholinoethyl) carbodiimide metho-*p*-toluenesulfonate (Fluka A.G., Buchs, Switzerland), 2,4,6-trinitrobenzenesulfonic acid (Serva, Heidelberg, FRG) and (chloromethyl)oxirane (Germed, Jenapharm Laborchemie, Apolda, GDR) were commercial products, cross-linked powder celluloses were prepared according to[13].

Both acetylcholinesterase from bovine erythrocytes (acetylcholine hydrolase, EC 3.1.1.7) and glucoamylase from *Aspergillus niger* (α-1,4-glucan-glucohydrolase, EC 3.2.1.3) were Koch-Light (Colnbrook, England) products, α-amylase (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) was isolated from the production strain *Bacillus subtilis* by the affinity method[14]. [1-^{14}C] Acetylcholine chloride (4·2 mCi/mmol) was purchased from The Radiochemical Centre, Amersham, England, [U-^{14}C]-α-1,4(6)-glucan (5 μCi/mg) was prepared from *Chlorella sp.* according to[15] further improved[16].

p-Nitrobenzyl Cellulose

a) To cellulose (2 g, 12·3 mmol of anhydroglucose units, U_{AG}) activated with 25% NaOH (9·2 ml, 57·5 mmol) for 1 h at room temperature *p*-nitrobenzyl chloride (2·15 g, 12·5 mmol, or 6·4 g, 37·3 mmol), or *p*-nitrobenzyl bromide (8·0 g, 37 mmol) was added and filled up with water to a hydromodul 10. The suspension was kept stirred at 45—75°C, then poured into a greater volume of cold water and washed on a sintered glass filter with water, 50% ethanol, ethanol and acetone. The product was extracted with acetone for 4—6 days in a Soxhlet extractor and finally dried at 40°C.

b) To dry cellulose (0·5 g, 3·1 mmol U_{AG}) swollen in dimethylsulfoxide (5 ml) at room temperature for 1 h methylsulfinyl anion (7—10 ml, 1·1—1·5-fold excess per hydroxyl groups of cellulose) was added and left to activate for 16 h at room temperature. *p*-Nitrobenzyl chloride (1·6 g, *i.e.* the equimolar amount, or 4·8 g, the 3-fold amount per hydroxyl groups of cellulose) was stepwise added during 5—8 min at 25°C constant temperature maintained for 3 h. This operation was carried out under nitrogen. The product was worked up similarly as in the preceding case.

p-Aminobenzyl Cellulose

a) *p*-Nitrobenzyl cellulose was reduced with sodium dithionite in an alkaline medium according to [17], or in a 90% ethanol according to[10] using a 1·45—4-fold molar excess of dithionite per D_a of nitrobenzyl groups of the *p*-nitrobenzyl cellulose.

b) For reduction of *p*-nitrobenzyl cellulose titanous chloride (a 12-fold molar excess of the reducing agent per D_a of nitrobenzyl groups) was employed in 0·96M-HCl according to procedure[18].

Preparation of Activated Carboxy Derivative

p-Aminobenzyl cellulose (30 mg) as characterized in Table III was reacted in chloroform (5 ml) with succinic anhydride (30 mg) at 80°C for 3 h and the product was washed with acetone, water

and dried. The water insoluble part (30 mg) underwent reaction with a 2% solution of 1-cyclohexyl-3-(2-morpholinoethyl)carbodiimide metho-p-toluenesulfonate at pH 4·6 at room temperature for 2 h, and the activated preparation obtained in this way was used for binding of acetylcholinesterase, glucoamylase and α-amylase.

Immobilization of Enzymes

The enzymes were bound at 0—4°C in a suspension of the activated carrier (30 mg) in distilled water (2 ml) adjusted to pH 4·8 and containing the enzyme (5 mg). The immobilized enzymes were washed successively with 10% aqueous NaCl, 2% solution of the bovine serum albumin and 10% aqueous NaCl till the protein free filtrate was obtained.

Analytical Methods

The average polymerization degree (P) of the non cross-linked celluloses was determined according to[19], the amount of cross links and swelling volumes of the cross-linked celluloses in water were estimated according to[20]. The nitrobenzyl groups content in p-nitrobenzyl celluloses was calculated from the microanalysis of nitrogen, the amino groups content in p-aminobenzyl celluloses after thiophosgenation[17] from the microanalysis of sulfur, or alternatively after diazotization and copulation with p-bromophenol[21] and microdetermination of bromine, or colorimetrically after reaction with 2,4,6-trinitrobenzenesulfonic acid[22]. The presence of isothiocyanate grouping in samples after thiophosgenation was evidenced by spectral (IR, KBr technique[4]) means.

The activity of acetylcholinesterase was measured using [1-^{14}C]acetylcholine chloride[23] as a substrate, the activity of glucoamylase and α-amylase using [U-^{14}C]-α-1,4(6)-glucan[16,24]. A water soluble (20 μl) or a immobilized enzyme (20 mg) added to a suspension containing phosphate pH 5·1 buffer (200 μl of 50 mM, glucoamylase), or pH 6·0 buffer (α-amylase) and insoluble glucan (1 mg) was incubated at 30°C for 10 min. Products of the hydrolytic reaction were analyzed in aliquot portions (10 μl) of the reaction mixture after chromatographic separation on a paper Whatman No 1 in ethyl acetate–pyridine–water 8 : 2 : 1 as a solvent system radiochromatographically for 16 h.

The protein content of the carrier after immobilization was determined according to[25] from the difference of proteins concentration in the solution before and after immobilization.

Degree of Alkylation

The degree of alkylation (D_a) of the p-nitrobenzyl cellulose derivative was defined as the average number of p-nitrobenzyl groups introduced into each anhydroglucose unit. The molecular weight of the anhydroglucose unit was 162, the end groups and trace substitutent in the cellulose being assumed as negligible. When $C_6H_7O_2(OH)_3$ is the formula of the anhydroglucose unit, then, in terms of the degree of alkylation, the formula of a p-nitrobenzyl cellulose derivative was $C_6H_7O_2(OH)_{3-D_a}(OCH_2C_6H_4NO_2)_{D_a}$. The alkylation degree of p-nitrobenzyl derivatives of cross-linked cellulose was calculated according to equation[26] $D_a = 162\,Y_W/(100W - Y_W W_I)$, where Y_W is the percentage of the substituent and W_I the net increase in formula of cellulose resulting from the introduction of one substituent group into one anhydroglucose unit (162 parts of cellulose); values Y_W were the per cents of nitrogen determined by microanalysis, W_I the difference between the molecular weight of $-CH_2C_6H_4NO_2$ grouping and that of hydrogen, W the molecular weight of nitrogen.

RESULTS AND DISCUSSION

The reaction conditions of O-alkylation of cellulose with *p*-nitrobenzyl chloride, employed in the preceding papers[6–10] in a molar ratio NaOH : U_{AG} 12·2, proved to be extremely unfavourable. Modification of these conditions associated with lowering of both temperature and concentration of NaOH does not lead to the required result (Table I). A considerable increase of the D_a in *p*-nitrobenzyl cellulose is unreal even when using various not cross-linked celluloses of the average polymerization degree $225 \leqq P \leqq 1350$ at optimum conditions (Table I). During O-alkyla-

TABLE I

Effect of the Reaction Conditions on O-Alkylation of Cellulose

Q mol/mol	q	v, g/ml	NaOH/U_{AG}	Chloride/U_{AG}	T, °C	Alkyl cellulose % N	Alkyl cellulose D_a
Cellulose powder, Whatman, $P = 643$							
—	—	6·6	4·7	3	60	traces	—
—	—	6·6	4·7	1	60	traces	—
1/0·25	16	5·4	4·7	1	60	traces	—
1/0·5	10	3·6	4·7	1	60	0·68	0·084
1/1	9	3·4	4·7	1	60	0·795	0·100
1·5/1·5	10	3·6	4·7	1	60	0·69	0·085
—	—	6·6	4·7	3	75	0·15	0·018
—	—	6·6	10·1	3	75	0·15	0·018
—	—	6·6	16·2[a]	3	95	0	—
Microcrystalline cellulose, Avicel, $P = 225$							
—	—	4·2	4·7	3	75	0·23	0·025
Woodpulp, Rauma-RR-F, $P = 791$							
—	—	—	4·7	3	75	0·15	0·018
Cotton, Rico, $P = 1342$							
—	—	—	4·7	3	75	traces	—

[a] Activation with 40% NaOH. Alkylation (3 h) with *p*-nitrobenzyl chloride after 1 h activation with 25% NaOH, in some cases after preceding cross-linking. U_{AG} anhydroglucose units, P the average polymerization degree, Q the mol ratio NaOH: (chloromethyl)oxirane, q the number of U_{AG}/one cross link, v the swelling bed volume in water, D_a the degree of alkylation.

tion mostly a 15 – 25% decrease in weight occurred with all of the above-mentioned types of celluloses due to the solubility of the degraded portions in alkaline solutions under the respective conditions.

The use of cross-linked cellulose substantially alters the course of O-alkylation and brings a considerable enhancement of the D_a of *p*-nitrobenzyl cellulose already under mild reaction conditions (Table I). Even the preceding results[27] showed unambiguously that cross linkage of cellulose and especially cross linkage with (chloromethyl)oxirane increased the number of accessible OH groups of cellulose responsible for improvement of cellulose reactivity with regard to some type of etherification reactions. In addition to enhancement of chemical reactivity of the cross--linked cellulose its stability towards alkaline degradation also increased.

During further optimalization of reaction conditions of cross-linked cellulose with 9 anhydroglucose units per one cross link it has been shown that even under mild conditions it was possible to prepare relatively highly substituted *p*-nitrobenzyl celluloses (Table II). In this reaction it was found to be advantageous to use *p*-nitrobenzyl bromide. Thus *e.g.* at 60°C and conditions given in Table II, *p*-nitrobenzyl cellulose of the D_a 0·28 was prepared. Under conditions reported in[6–10] a preparation of a high degree of substitution with a considerable degree of degradation, *i.e.* with a high content of soluble constituents (Table II) was obtained.

TABLE II

Effect of Temperature on the Degree of Alkylation of the Cross-linked Powder Cellulose in Aqueous-Alkaline, or Dimethyl Sulfoxide-Methylsulfinyl Anion Media

Temperature °C	Medium			
	H_2O—NaOH		$(CH_3)_2SO$—$CH_3SOCH_2^{(-)}$	
	% N	D_a	% N	D_a
25	—	—	0·31	0·037
45	—	—	0·15	0·018
60	1·70	0·235	0·10	0·012
75	2·41	0·365	—	—
95	4·81[a]	1·040	—	—

[a] Activation with 40% NaOH, $[NaOH]/[U_{AG}] = 16·2$.

The $[NaOH] : [U_{AG}]$ and $[chloride] : [U_{AG}]$ ratios in aqueous–alkaline medium were 4·7 and 3, respectively. q Of the starting cellulose was 9. For symbols and abbreviations see Table I.

The cross-linked cellulose proved to have almost the same bed volume in water and in dimethyl sulfoxide (3·4 ml/g at room temperature); also the thermodynamic characterization displayed many similarities[28]. In aqueous alkaline medium second-order transitions[11,29] took place under conditions of „activation" of cellulose, *e.g.* a transition of cellulose I to cellulose II, differing substantially in the accessibility of hydroxyl groups occurred.

Hakomori[12] restricted the validity of his reaction to oligo- and polysaccharides soluble in dimethyl sulfoxide. On the other hand, satisfactory degrees of alkylation of agarose (Sepharose) were achieved[30] (*e.g.* also with benzyl chloride) under condition of Hakomori reaction. Besides of the known differences in the accessibility of hydroxyl groups of agarose and cellulose, the unknown remains, in this case, the effect of methylsulfinyl anion in she process of "activation" of the cross-linked cellulose. As shown in Table II, the methylsulfinyl anion probably does not reach the effect of the smaller hydroxyl anion; the increase in temperature acted here unfavourably.

The dithionite reduction condition of *p*-nitrobenzyl cellulose prepared from the cross-linked cellulose had to be altered in respect to the original one[6,10]. Directive were results obtained with reduction of aromatic nitrobenzene derivatives of cross-linked dextrans[17]. The *p*-aminobenzyl celluloses contained in both cases the unwanted sulfur (up to 1·9%) ascribed to sulfonamide and aminosulfone acids. In accordance with our results (Table III) these reactions[31] amounted approximately 50%. Whereas a portion of such amino groups evidently underwent thiophosgenation, diazotization and copulation proceeded with primary aromatic groups only. Titanous chloride as reducing agent[18] led to a loss of nitrogen.

TABLE III

Characterization of *p*-Aminobenzyl Cellulose after Reduction of *p*-Nitrobenzyl Cellulose with Sodium Dithionite in Alkaline Medium

The binding capacity expressed by means of the NH_2 group content determined *via* thiophosgenation.

Dithionite	*p*-Aminobenzyl cellulose		
[p-$O_2NC_6H_4CH_2$—]	% N	% S	NH_2, mmol/g
1·45	1·59	0·58	0·35
3·00	1·84	1·12	0·75[a]
4·00	1·71	1·90	0·56

[a] 0·44 as determined by diazotization and copulation with *p*-bromophenol; 0·77 by colorimetric estimation after reaction with 2,4,6-trinitrobenzenesulfonic acid.

Procedures employed for quantitative determination of NH_2 groups of *p*-aminobenzyl cellulose (thiophosgenation, diazotization and copulation) were ones of the most frequently used methods of covalent immobilization of enzymes and further proteins at the time being[32,33]. In this paper another method of binding of enzymes was employed proceeding in such a pH region (4·6) which is very convenient for work with the appropriate enzymes. Moreover, a spacer was introduced between the carrier and the enzyme in the course of immobilization.

The employed method of immobilization exploited the free amino groups of enzymes. Consequently, no chemical modification of the active sites of hydrolases took place, this being indicated by relatively high activities of immobilized enzymes A good effect on the preservation of activity of the immobilized enzymes exerted, however, also the spacer through which the enzymes were bound to the carrier. Differences in the relative activities of immobilized enzymes (Table IV) could be associated with the differences in molecular weights of substrates. In contrast to glucan, the spatial mobility of acetylcholine chloride in the network of cross-linked cellulose can be hold for good. Binding of all three hydrolases should be ascribed, with regard to both the relatively high degree of cross linkage and the preparation conditions of the carboxy derivative, to the surface of the cross-linked network of the cellulose. The values of protein loading of immobilized enzymes are in favour of this effect (Table IV).

Good results have also been achieved with immobilization of glucose oxidase[34] by this procedure. Hydrolases and glucose oxidase are structurally diametral proteins. It is, therefore, possible to anticipate that this method of binding is of general use for macromolecules of protein type.

TABLE IV

Characterization of the Immobilized Enzymes

Enzyme	Activity, U/mg: before immobilization	Activity, U/mg: after immobilization	Protein content mg/g	Coupling yield %
Acetylcholinesterase	28·2	17·6	27·0	16·2
Glucoamylase	1·2	0·7	21·7	13·0
α-Amylase	3·4	1·7	24·3	14·6

Enzymes were immobilized with *p*-aminobenzyl cellulose of 1·84% N and 0·77 mmol of NH_2/g (*cf.* Table III).

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