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PROTEIN IMMOBILIZATION BY AMINOLYSIS OF CELLULOSE XANTHATE ESTERS

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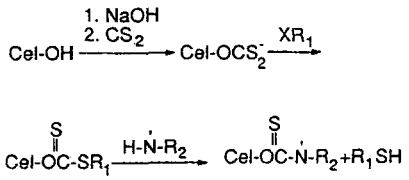
P-Nitrobenzyl cellulose xanthate (CelXNB) was obtained with a degree of substitution (*DS*) in the range 0·5–3. The first-order rate constant for hydrolysis of CelXNB at 25 °C, extrapolated to zero buffer concentration, is hydroxide ion catalysed, whereas the water-catalysed path is about 2000 times faster than that observed for *p*-nitrobenzyl ethylxanthate (EXNB), probably owing to the highly ordered cybotactic region of cellulose. Aminolysis of CelXNB produces the corresponding thioncarbamate ester; for simple alkylamines, the second-order rate constants are similar to those obtained for EXNB. The second-order rate constants at pH 11 for immobilization of bacterial α-amylase and bovine serum albumin were 13·4 and 112 l mol^{-1} s^{-1}, respectively, unexpectedly high values when compared with simple alkylamines, even considering the concentration of external reactive groups of the proteins. CelXNB with low *DS* should release 1 mol of *p*-nitro-α-toluenethiol for every mole of protein that becomes immobilized. The net weight increase of the cellulose matrix allows the calculation of the absolute molecular weight of the protein. Preliminary results support this assumption.

INTRODUCTION

Enzyme immobilization has become an important tool for the use of enzymes in industrial processes and analytical methods. The main advantages are reusability, stability and economy.[1,2] In addition to economic reasons, immobilized enzymes can be used to study changes in selectivity and as models for natural enzymes bound to cellular membranes.[3]

During the last few years, several methods have been employed for the preparation of water-insoluble derivatives of enzymes, one of them being the covalent binding to water-insoluble matrices.[2] However, to our knowledge, the rate of immobilization has never been measured, and therefore the mechanism of binding is still unknown.

In this paper we describe a method for binding an enzyme covalently to functionalized cellulose fibres, based on a sequence of simple reactions shown in Scheme 1. The first step is the xanthation of cellulose under mild conditions (1 M NaOH), which would produce mainly the monoxanthate from the β-1,4-anhydroglucopyranose unit. Cellulose xanthate was esterified by *p*-nitrobenzyl bromide to form the corresponding xanthate ester at the 2-, 3- or 6-position. Aminolysis of this ester produced the thioncarbamate ester, releasing *p*-nitro-α-toluenethiol that can be observed spectrophotometrically.



CelXNB

R_1 = *p*-nitrobenzyl

Scheme 1

The functionalized cellulose was characterized in terms of the degree of substitution, the pH profile of the hydrolysis reaction and aminolysis with simple amines and with some proteins, including enzymes. The reactivity of the immobilized enzymes was also measured. Despite the complexity of proteins, only a few groups might be reactive for immobilization. This preliminary work shows that the immobilization reaction of an enzyme on the cellulose matrix can be studied with important consequences, such as the possibility of developing an absolute method to measure the molecular weights of proteins.

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The results illustrate several interesting features of the reactions involved in the immobilization. Both the spontaneous hydrolysis of the cellulose ester and the aminolysis by proteins show unexpectedly high rate constants. This change in reactivity of groups bound to the hydrated cellulose matrix calls for more detailed studies.

EXPERIMENTAL

In general, all reagents were of analytical grade and were used without further purification, except for ethyl-, hexyl- and *n*-butylamine, which were distilled before use.

Proteins were obtained from Sigma. Bovine serum albumin was a crystallized and lyophilized sample; alkaline phosphatase from chicken intestine was partially purified and lyophilized; α-amylase, from *Bacillus subtilis*, was recrystallized four times. Alkaline phosphatase from *Escherichia coli* was obtained from Worthington Biochemical as a 65% ammonium sulphate suspension.

UV spectrophotometry and kinetic measurements were effected with a Cary Model 219 spectrophotometer; pH was measured with a Metrohm E-603 pH meter and melting points with a Uniscience Model 498 apparatus.

p-*Nitro-α-toluenethiol (NTT).* The synthesis was carried out according to the method of Price and Twiss,[4] forming the corresponding thiosulphate from *p*-nitrobenzyl bromide, followed by decomposition with sulphuric acid and final purification by molecular distillation under a nitrogen atmosphere (white crystals, m.p. 53·5 °C; lit.[4] m.p., 52·5 °C). The molar absorptivity at 280 nm [pH 11·0 (0·05 M carbonate] in 10% aqueous ethanol is $8{\cdot}15 \times 10^3$ l mol^{-1}.

p-*Nitrobenzyl cellulose xanthate (CelXNB).* About 3 g of cotton were treated for 4 h in a 500-ml stoppered Erlenmeyer flask with 250 ml of 1 M sodium hydroxide solution in a mechanical shaker. A solution of 20 g of carbon disulphide in 100 ml of acetone was then added and the suspension was allowed to react for 2·5 h with shaking. The product was filtered in a Buchner funnel and washed several times with water buffered with 0·1 M sodium monohydrogenphosphate at pH 8. The modified cellulose was then treated with a solution of 3 g of *p*-nitrobenzyl bromide in 100 ml of acetone in a 200-ml stoppered Erlenmeyer flask for 12 h with shaking. The CelXNB thus obtained was washed with distilled water, ethanol and diethyl ether in a Buchner funnel and dried under vacuum over phosphorus pentoxide.

*Determination of degree of substitution (*DS*).* The degree of substitution was defined as the average number of xanthate ester groups per 100 glucoanhydropyranose units. Weighed samples of CelXNB were allowed to react in 10-ml stoppered centrifuge tubes by adding 8 ml of 0·05 M sodium carbonate buffer solution (pH 11) and 1 ml of 0·55 M aqueous ethylamine. The tubes were mechanically shaken at room temperature, periodically centrifuged and the absorbance was read at 280 nm until a constant value had been attained. The number of moles of NTT formed per gram of CelXNB, *n*, was calculated from a plot of the final absorbance versus the weight of CelXNB. The degree of substitution (*DS*) was obtained from the equation

$$DS = 1{\cdot}62 \times 10^4 n \qquad (1)$$

where $1{\cdot}62 \times 10^4$ is the molecular weight per 100 anhydropyranose units.

Protein immobilization. The procedure was similar to that used to determine the *DS*, replacing ethylamine with the protein. Since the concentration of the latter was much lower, the time required for aminolysis of the xanthate ester was much longer.

Hydrolysis and aminolysis of CelXNB. The hydrolysis and aminolysis of CelXNB were studied at 25·0 °C in aqueous solutions by following the appearance of NBT at 280 nm. For the hydrolysis, the first-order rate constants were obtained at two buffer concentrations and extrapolated to zero buffer concentration. The second-order rate constants for the aminolyses with simple amines (ethyl-, butyl- and hexylamine) were calculated with respect to the free base. The second-order rate constants for the aminolysis by proteins (or enzymes) at pH 11 in 0·09 M sodium carbonate buffer were calculated considering the total concentration of

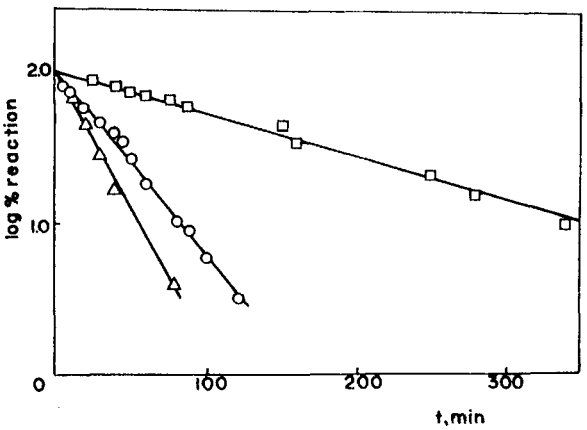


Figure 1. Kinetics of the aminolysis of CelXNB at 25 °C, pH 11 (0·048 M carbonate). □, Bovine serum albumin, $3{\cdot}33 \times 10^{-6}$ M; ○, ribonuclease A, $1{\cdot}06 \times 10^{-5}$ M; △, ethylamine, $1{\cdot}83 \times 10^{-2}$ M

the protein. In general, plots of $\ln(A_\infty - A_t)$ vs time were linear and the reaction was first order with respect to CelXNB (Figure 1). Experiments using a constant concentration of α-amylase produced the same first-order rate constant when the weight of CelXNB was changed by a factor of four at pH 10·0, 11·0, and 12·0. Experiments in which the frequency of shaking was changed by a factor of ten showed that the rate constants were not controlled by diffusion.

Activity of the immobilized alkaline phosphatase. Cellulose fibres (10–20 mg) containing the immobilized enzyme were suspended in 3 ml of 1 mM *p*-nitrophenyl phosphate and 2 ml of 1 M Tris buffer (pH 8) and mechanically shaken. The absorbance at 410 nm was read periodically. The number of milimoles per litre of *p*-nitrophenol liberated per second per milligram of enzyme was calculated according to the relationship

$$\text{activity units} = \frac{\Delta A \times 1000}{\Delta t \varepsilon_{410} p}$$

The molar absorptivity of *p*-nitrophenol at 410 nm at pH 8 is $1{\cdot}62 \times 10^4$ l mol^{-1} cm^{-1},[5] and the weight p (mg) of immobilized enzyme was calculated from the *DS* obtained using the enzyme instead of ethylamine.

Activity of immobilized α-amylase. The reagent was prepared by dissolving 1 g of 3,5-dinitrosalicylic acid in 20 ml of 2 M sodium hydroxide solution, adding 30 g of sodium potassium tartrate and diluting to 100 ml with water.

The activity was calculated according to Bernfeld,[6] adding 10 mg of cellulose with the immobilized enzyme to a test-tube containing 0·2 ml of a stock starch solution (1%, w/v) and 2 ml of water. The suspension was shaken for 5 min at room temperature and 0·4 ml of the reagent was added. The mixture was heated at 100 °C for 5 min, 2·0 ml of water was added and the absorbance was read at 400 nm. The amount of maltose produced was calculated from a calibration graph.

RESULTS AND DISCUSSION

p-Nitrobenzyl cellulose xanthate

CelXNB is a mixture of isomeric esters. Each anhydroglucose unit of cellulose has three reactive hydroxyl groups: two secondary OH at positions 2 and 3 and one primary OH at position 6. Xanthation of ethanol is faster than that of isopropanol by a factor of 1·37 at 25 °C[7] and the xanthation of cellulose should produce a mixture of about 30% of 2- and 3-xanthates and 40% of 6-xanthate. Xanthation of methyl α-D-glucopyranoside shows significant migration of the thiolthiocarbonyl group from position 2 to 3 and from position 3 to 6, giving 77–85% of the latter.[8] These results are similar to those obtained for the xanthation of starch.[9] However, the distribution of the 2-, 3- and 6-isomers in ripened viscose was found to be in the ratio 2·06 : 1 : 1·8, suggesting that the reactivity of the secondary hydroxyl groups depends on other factors, such as the preferential diffusion of the reagent to position 2 compared with position 3 and steric hindrance to operation of the migration mechanism close to polymer chains.[10]

Hydrolysis of CelXNB

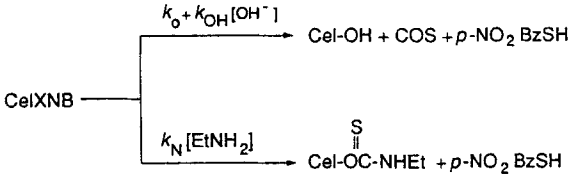
The hydrolysis was studied at 25 °C in the pH range 10–13 and was followed by the appearance of NTT (Scheme 2). The first-order rate constants were extrapolated to zero buffer concentration. The pH profile is shown in Figure 2, k_{obs} being expressed by the equation

$$k_{obs} = k_0 + k_{OH}[OH^-] \qquad (2)$$

where k_0 is the spontaneous water-catalysed rate constant and k_{OH} is the second-order rate constant for the specific base catalysis.

In Table 1, these values are compared with those for other simple xanthate esters. The rate constant ratio k_{OH}/k_0 for CelXNB is about four orders of magnitude smaller than those for the *S*-*p*-nitrobenzyl esters of ethyl xanthate (NBEX) and methyl-D-glucopyranoside 6-xanthate (NBMGX). Accordingly, the rate constant for the spontaneous hydrolysis of CelXNB is about three orders of magnitude faster than that of NBEX and NBMGX.

The rate constant for the spontaneous hydrolysis of NBEX and NBMGX were measured in 20% aqueous methanol. Addition of methanol should preferentially stabilize the initial state, producing a small decrease in the rate constants.[11] The main reason for the stabilization of the initial state can be ascribed to the fact that the organic cosolvent produces a strong perturbation on the water structure in the cybotactic region, decreasing the hydrogen bond density, and consequently reducing the free energy necessary for cavity formation.[12] The transition state of the water-catalysed hydrolysis undoubtedly prefers a more organized water structure, with the participation of at least two water molecules, resulting in a negative entropy of activation.[13,14] The net effect is thus a high free energy of activation for the spontaneous reaction.



Scheme 2

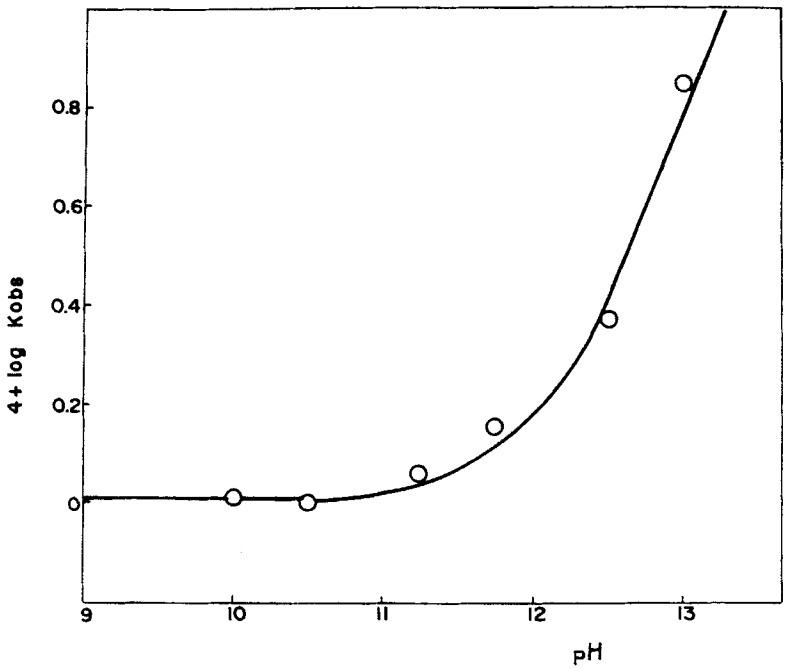


Figure 2. Hydrolysis of CelXNB at 25 °C. The curve was drawn according to equation (2); $k_0 = 1{\cdot}01 \times 10^{-4}$ s^{-1}, $k_{OH} = 5{\cdot}13 \times 10^{-3}$ l mol^{-1} s^{-1}

Table 1. Rate constants for the hydrolysis and *n*-butylaminolysis of some *p*-nitrobenzyl alkylxanthates [ROC(S)SCH$_2$C$_6$H$_4$NO$_2$] at 25 °C

ROH	k_0(s^{-1})	k_{OH}(l mol^{-1} s^{-1})	$10^2 k_N$(l mol^{-1} s^{-1})
Ethanol[a]	$3{\cdot}49 \times 10^{-8}$[b]	$4{\cdot}61 \times 10^{-2}$[b]	4·02[b]
Methyl α-D-glucopyranoside(-6-)[a]	$1{\cdot}08 \times 10^{-7}$[b]	2·05[b]	56·6[b]
Cellulose[c]	$1{\cdot}01 \times 10^{-4}$	$5{\cdot}13 \times 10^{-3}$	6·75

[a] Ref. 21.
[b] In 20% aqueous methanol, corrected from values at 35 °C.
[c] This work.

On the other hand, cellulose can form intra- and intermolecular hydrogen bonds between anhydropyranose units,[15] as well as with water. Ordinary cellulose fixes the first water molecule between two anhydropyranose units, while mercerized cellulose fixes one molecule per unit.[16] Other molecules of water are strongly adsorbed until they begin to fill up the capillary spaces between the fibres[17,18] and orient along the cellulose fibre axis.[19] Therefore, at low degrees of substitution, the *p*-nitrobenzyl xanthyl moiety should produce only a small perturbation, and the cybotactic region around it should retain the characteristics of a three-dimensional hydrogen-bonded ice structure. Therefore, very little change in the coordinates of the water molecules would be required in order to reach the transition-state structure. The entropy of activation should be very small and the free energy of activation lower than that of simple alkylxanthate esters. In line with this analysis, the observation that the spontaneous hydrolysis of NBMGX is faster than that of NBEX can be attributed to the fact that the carbohydrate moiety is less hydrophobic than an alkyl group.

Aminolysis of CelXNB

Aminolysis of CelXNB by simple alkylamines occurs with rate constants similar to those of NBEX (Tables 1 and 2). The reaction does not directly involve water molecules and the transition state is not specially stabilized as occurs for the spontaneous hydrolysis. However, the second-order rate constants at pH 11 (0·05 M carbonate) for the aminolysis by bacterial α-amylase

and bovine serum albumin are about 100 and about 2000 times faster, respectively. These values were corrected with respect to the hydrolysis of CelXNB.

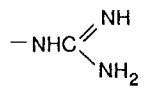
There are three amino acid residues that can react with xanthate esters (Table 3). They occur in proteins with a total average content of 13%.[20] For α-amylase and bovine serum albumin this percentage corresponds to 48 and 65 mol of residues per mole of protein, respectively. At pH 11, the guanidino group of arginine will be partially protonated and the reactivity of the groups depends on the pK_a values, which decrease in the order Arg > Lys > His.[21] Since the free energy of transfer (water to ethanol) of amino groups is positive, these will prefer to remain at the surface of the protein. However, in view of the hydrophilic–hydrophobic balance of protein interactions, only a fraction of these groups will reside at the surface.[22] Therefore, the faster aminolysis of CelXNB by these proteins cannot be explained as being due to the number of reactive residues on the surface of the protein, especially for bovine serum albumin. Indeed, we had initially expected to find a decrease in the rate constant because of the steric hindrance between the cellulose matrix and the protein, which would presumably reduce the access to the xanthate group. However, aminolysis of CelXNB can be general base catalysed,[21] and basic groups in the protein might be properly oriented, adjacent to the reactive residue so as to produce the observed rate enhancement.[23]

Table 2. Second-order rate constant for aminolysis of CelXNB at 25 °C[a]

Amine	k_N(l mol^{-1} s^{-1})[b]
Ethylamine	$5{\cdot}75 \times 10^{-2}$
n-Butylamine	$6{\cdot}75 \times 10^{-2}$
n-Hexylamine	13×10^{-2}
Bacterial α-amylase	13·4[c]
Bovine serum albumin	112[c]

[a] Carbonate buffer (0·05 M).
[b] Calculated with respect to the free base, except where indicated.
[c] At pH 11.

Table 3. Reactive amino acid residues

Amino acid	Residue	pK_a	Content(%)[a]
Arginine		12·48	3·8
Lysine	$-NH_2$	8·95	7·0
Histidine		6·0	2·2

[a] Average percentage composition of proteins.

Enzyme immobilization

Chicken intestinal and *E. coli* alkaline phosphatase and α-amylase were immobilized following the procedure described above. The reaction was monitored periodically by the appearance of NTT. When the reaction was judged to be complete, the dispersion was titrated with ethylamine to determine the number of unreacted xanthate moeities.

The activities of the chicken intestinal and *E. coli* alkaline phosphatase were $1{\cdot}12 \times 10^3$ and $4{\cdot}15 \times 10^3$ μkat mol^{-1} immobilized enzyme. The bacterial α-amylase showed an activity of 23·0 μkat mol^{-1}. The extent of immobilization on the cellulose matrix could be estimated from the weight increase of the cellulose matrix and from the number of moles of NTT produced, corrected for the hydrolysis reaction. If the xanthate ester groups were far enough away from each other that they react in a 1 : 1 ratio with the protein, it would be possible to calculate the absolute molecular weight of the protein. Preliminary results for *E. coli* alkaline phosphatase gave a molecular weight of $7{\cdot}4 \times 10^4$ as compared with the value of $8{\cdot}0 \times 10^4$ in the literature.[5]

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