Carbohydrate Research, 41 (1975) 227–233

THE USE OF CELLULOSE XANTHATE FOR THE IMMOBILISATION OF BIOLOGICAL MOLECULES

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(Received June 10th, 1974, accepted for publication, October 24th, 1974)

ABSTRACT

The mercapto groups of cellulose xanthate can reversibly form disulphide bridges with L-cysteine This property has been utilised for the immobilisation of a protein and an enzyme These macromolecules, as polythiol derivatives, formed disulphide linkages with the matrix without serious disturbance of their active sites, became firmly bound to the xanthate, and were not eluted by normal washing conditions Cellulose xanthate is a cheap, easily prepared matrix which permits a simple coupling reaction The immobilisation process is selectively reversible

INTRODUCTION

Water-insoluble polysaccharides are popular and useful as matrices for immobilisation[1], and we have used cellulose *trans*-2,3-carbonate[2,3] for the immobilisation of enzymes[4,5], antigens and antibodies[6–8], antibiotics[9], and other molecules[10], and also in a macroporous form[11,12] which protects the bound material and permits high biological activities of the product to be retained However, its production is costly and laborious because of the need for dry solvents Cellulose xanthate, which is readily available at low cost, was therefore investigated as a matrix for the immobilisation of amino acids, proteins, and enzymes

EXPERIMENTAL AND RESULTS

Cellulose xanthate — Cellulose was converted into the xanthate by a modification[13] of the methods of Chen *et al* [14] and Sanyal *et al.*[15]. Dry cellulose (5 g, Sigmacell, particle size 38μm, Sigma Chemical Co) was shaken with carbon disulphide (30 ml) for 1 h at 4°. 18% Aqueous potassium hydroxide (60 ml) was then added and the mixture was stirred at 4° for 48 h The resulting, highly viscous, orange mass was homogenised with water (90 ml) and added to methanol (900 ml) The precipitate was collected by centrifugation and washed with 5% acetic acid in anhydrous methanol (3 × 100 ml) and finally with dry ether (200 ml) The product was ground, and washed again with acidified methanol and ether, and the resulting white material

was stored *in vacuo* over phosphorus pentaoxide [Found C, 27 1, H, 4 6, S, 12 8 $C_7H_9NaO_5S_2$ (d s 1) calc C, 32 3, H, 3 5, S, 24 6%]

The thiol content of the cellulose xanthate was estimated by a modification of a method[16] based on use of 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) Cellulose xanthate (0–1 mg) was suspended in 0 1M Tris buffer (pH 8 0, 5 ml) After 20 min at 20°, 50μl of DTNB reagent (39 6 mg of DTNB in 10 ml of 0 1M sodium phosphate buffer, pH 7 0) was added and stirring was continued for 10 min The suspension was then centrifuged and the absorbance of the supernatant at 410 nm was determined The thiol content of the xanthate sample was calculated by reference to a calibration curve obtained with L-cysteine hydrochloride The assay was reproducible and showed that 1 mg of cellulose xanthate was equivalent to 150μg of cysteine hydrochloride

Reaction of cellulose xanthate with L-*cysteine* — Cellulose xanthate (100 mg) was stirred in 0 1M sodium phosphate buffer (pH 8 0, 5 ml) for 3 h at 20° A solution of L-cysteine hydrochloride (75 mg) in the same buffer (5 ml) was added to the suspension followed by a solution (11 8 ml) of potassium ferricyanide (1 82 g), ammonium chloride (4 86 g), and conc ammonia (7 1 ml) in water (100 ml) The mixture was stirred for 2 h at 20°

After centrifugation, t l c of the supernatant was performed on MN-Kieselgel G (Macherey, Nagel & Co) with 1-butanol–acetic acid–water (4 1 5), and L-cysteine (R_F 0 32) and L-cystine (R_F 0 16) as standards Detection was effected with a mixture of redistilled glacial acetic acid (15 ml), 2,4,6-collidine (2 ml), and 1% of ninhydrin in ethanol (50 ml) at 60° for 10 min Compounds having R_F values of 0 32 and 0 16 were found in the supernatant solution

The L-cysteine-treated cellulose xanthate obtained on centrifugation was stirred with portions (5 ml) of 0 1M sodium phosphate buffer (pH 8 0) at 20° for 5 min until, after centrifugation, t l c of the supernatant showed the absence of L-cysteine and L-cystine The solid was packed into a glass column (0 4 × 3 cm) and eluted with the phosphate buffer until de-aerated T l.c showed amino acid to be absent from the eluate 2-Mercaptoethanol (2 ml) was diluted under nitrogen to 10 ml with phosphate buffer previously deoxygenated by nitrogen, the solution was applied to the top of the column, and fractions (3 ml) were collected under nitrogen The column was subsequently eluted with phosphate buffer (10 ml) under the same conditions T l c showed that only fractions 1–4 contained L-cysteine Aliquots (2 5 ml) of fractions 1–4 were combined and extracted with ether (acidified with 2M hydrochloric acid) until the solution no longer possessed an odour of 2-mercaptoethanol (total extract, 200 ml) The aqueous layer was diluted with phosphate buffer to 6 ml, and an aliquot (1 ml) was diluted to 20 ml with the same buffer and assayed for L-cysteine by a modified ninhydrin method[17], as follows

A solution of ninhydrin (80 g) and hydrindantin (6 g) in 2-methoxyethanol (2 l) was flushed with nitrogen for 15 min, 4M sodium acetate (1 4 l) was added, and flushing was continued for 30 min The solution was added to a flushed, cooled mixture of 2-methoxyethanol (600 ml) and water (600 ml), flushing was continued

for 30 min, and the mixture was stored under nitrogen Using Technicon modular equipment, and a peristaltic pump to automate the process, samples of solutions containing unknown or standard (0 1 μg/ml) amounts of L-cysteine hydrochloride (pumping rate, 0 42 ml/min) were segmented with nitrogen (0 8 ml/min) and ninhydrin reagent (1 06 ml/min) After passage through a mixing coil, the solution was maintained at 95° for 12 min and then cooled After passage of the solution through a debubbler, the absorbance was determined at 570 nm in a continuous-flow colorimeter Sample solutions were introduced for 7 min to give a response on the chart recorder, and water was introduced for 15 min between consecutive samples With the aid of a calibration curve constructed for the standards, the cellulose xanthate on treatment with L-cysteine was found to have taken up 14 4 μg of L-cysteine per mg of solid

Reaction of cellulose xanthate with polythiolated albumin — Albumin was converted into a polythiol derivative[18] as follows A solution (20 mg/ml, 0 5 ml) of *N*-acetylhomocysteine thiolactone in 0 1M sodium hydrogen carbonate (pH 6 0) was mixed with a solution (10 mg/ml, 10 ml) of bovine serum albumin (Koch–Light Labs Ltd) in the same buffer After 20 min at 4°, the mixture was fractionated on a column (2 × 50 cm) of Sephadex G-25 equilibrated with 0 1M sodium hydrogen carbonate The same buffer was used as eluant, fractions (1 ml) were collected, and the elution position of the albumin was determined from the absorbance of the fractions at 280 nm Fractions containing the polythiolated albumin were combined, diluted to 100 ml with 0 1M sodium hydrogen carbonate, flushed with nitrogen, and stored at 4° under nitrogen

The protein concentration in the polythiolated albumin solution was determined by the Folin[19 20] method, with the original bovine serum albumin as a standard The polythiolated albumin solution was found to contain 90 mg of protein per 100 ml of solution

Cellulose xanthate (100 mg) was suspended in a portion (25 ml, containing the equivalent of 22 5 mg of albumin) of the solution of polythiolated albumin and, after stirring at 4° for 2 h, L-cysteine reagent (5 ml, prepared as described above) was added and stirring was continued for 48 h at 4° After centrifugation, the solid material was washed with 0 1M sodium hydrogen carbonate buffer (pH 10 6, 15 ml), and the mixture was recentrifuged The combined supernatant and washings were diluted to 50 ml with the pH 10 6 buffer This solution was found to contain 16 mg of protein A control experiment involved cellulose xanthate (100 mg) and polythiolated albumin (25 0 ml), and replacement of the ferricyanide solution with the pH 10 6 buffer. The final solution contained 21 5 mg of protein

The solid products from the treatments of cellulose xanthate with polythiolated albumin were washed initially with 0 1M L-cysteine hydrochloride in 0 5M sodium phosphate buffer (pH 7 6, 6 ml) and then with the buffer alone (4 ml) The combined washings were concentrated to 1 ml at 30° *in vacuo* and fractionated on a column (1 × 45 cm) of Sephadex G25 equilibrated with the pH 7 6 buffer The column was eluted with the same buffer and the absorbance at 280 nm of each fraction (1 ml) was determined Fractions containing protein were combined and diluted to 10 ml

with buffer, determination of protein concentration showed that 5 mg of protein were liberated from the L-cysteine-treated cellulose xanthate–polythiolated albumin mixture, but only 0 55 mg when the L-cysteine treatment was omitted

Reaction of cellulose xanthate with polythiolated trypsin — Trypsin (*ex* bovine pancreas, Koch–Light, E C 3 4 21 4) was converted into a polythiol derivative as described above, the final solution of the modified enzyme (100 ml) contained 83 mg of protein, using trypsin as standard The activity of the modified enzyme was determined by a modification of the method of Kunitz[21], using casein as substrate The solution (1 ml) of polythiolated trypsin was added to a solution (10 mg/ml) of casein in 0 1M sodium phosphate buffer (pH 7 6, 1 ml) and, after incubation for 20 min, 5% aqueous trichloroacetic acid (3 ml) was added The resulting suspension was centrifuged and the absorbance of the supernatant was determined at 280 nm One unit of trypsin activity was defined as that amount of the enzyme which liberates sufficient trichloroacetic acid-soluble products to cause an increase in the absorbance of the supernatant of one in 1 min under the conditions used The specific activity of the polythiolated trypsin was 69% of that of trypsin

Cellulose xanthate (100 mg) was reacted with a portion (25 ml, containing the equivalent of 20 7 mg of protein) of the solution of polythiolated trypsin as described above for polythiolated albumin Enzyme assay of the combined supernatant solution and washings (diluted to 50 ml) demonstrated that 64% of the original enzyme activity (1 46 units) remained uncoupled

The solids were also assayed for enzyme activity by suspension in 0 1M sodium phosphate buffer (pH 7 6, 1 ml), 16 3 and 9 3%, respectively, of the original enzyme activity was bound to the xanthate in the presence and absence of the cysteine reagent

DISCUSSION

In the modified procedure for the preparation of cellulose xanthate, the material was washed with acid, and thus it is possible that some of the material was in the form cellulose–O CS SH, although xanthic acids are unstable

The sulphur content of 12 8% does not correspond to a degree of substitution (d s) of 12 8/26 9, on account of the variation of the average molecular weight of the D-glucose residues of the chain with different d s values, and the precise degree of substitution (y) of the xanthate was calculated by using the formula

$$S = \frac{2M_s\, y\, 100}{yM_x + (1-y)M_g} \equiv y = \frac{M_g}{(200M_s)/S - (M_x - M_g)},$$

where S = sulphur content (%), M_s = atomic weight of sulphur, M_x = molecular weight of a D-glucose residue bearing one xanthate group, M_g = molecular weight of a D-glucose residue A d s (y) value of 0 40 was obtained For a similar reason, in the assessment of the –SH (≡to –SNa) content of the xanthate by the DTNB method, the first results obtained by assuming a d s of 1.0 were subjected to a

cycling improvement procedure using the formula

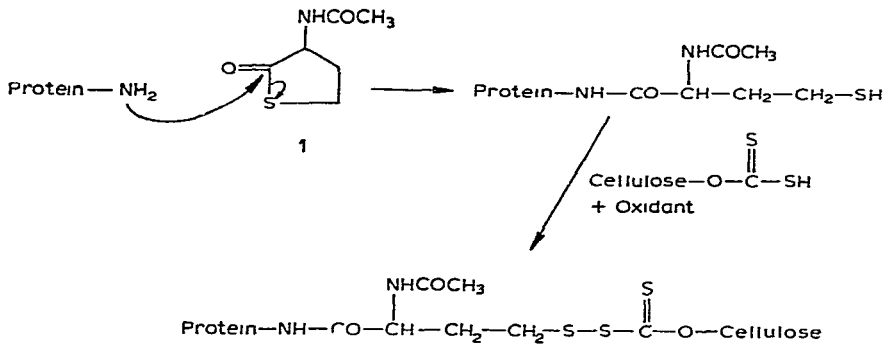
$$\frac{C}{W/[y_n M_x + (1-y_n) M_g]} = y_{n+1},$$

where C = cysteine equivalence (μmoles), W = weight of cellulose xanthate sodium salt (μg), n = number of calculation cycle, $x_1 = 1\,0$ A d s value of 0 15 was obtained Similar calculations for the cysteine coupling data gave d s 0 017 The differences between these values may be explained as follows The value based on the sulphur content is most likely to be accurate The lower value obtained by the DTNB method is not surprising since sodium cellulose xanthate itself is unstable[13] at nearly neutral pH, problems in determining free mercapto groups with the reagent have been experienced previously and would appear to be due to poor interaction of the dissolved reagent with the solid Nevertheless, the result shows that the xanthate group of cellulose xanthate can react in similar fashion to a free mercapto group This was confirmed by the demonstration that L-cysteine could be coupled to cellulose xanthate *via* a disulphide linkage, the low extent of the reaction was possibly due to instability of the cellulose xanthate at nearly neutral pH, inefficient reaction, formation of cystine, or incomplete reversal (release) The possibility of simple adsorption of the L-cysteine by the xanthate is discounted on account of the effective washing technique employed

The feasibility of reaction with a macromolecular thiol was next investigated Since proteinaceous, enzyme combination was the ultimate goal, a protein was used for the test However, L-cysteine present in proteins is usually involved in disulphide bridges Such bridges may be reduced to mercapto groups, but this is inadvisable since biological activity might be lost on disturbance of the tertiary structure It was thus considered more expedient to introduce the mercapto groups by reaction of the amino groups of the protein with homocysteine thiolactone (**1**)

After treatment of an albumin with **1**, fractionation of the reaction mixture by gel filtration separated the polythiolated material from the other species present and a good yield of polythiolated protein was obtained, as expressed in terms of the original albumin, thus avoiding the problems arising from any water content of the undried, original material Although the reaction of trypsin with homocysteine thiolactone caused some loss in enzymic activity, appreciable activity remained, demonstrating that such a derivatisation does not automatically disrupt the active site

From the results obtained for the coupling of polythiolated albumin to give **2**, as assessed by measurement of reliberated protein, it was concluded that an excess of polythiolated material was necessary and that immobilisation should be effected at nearly neutral pH In the absence of cross-linking agent, the polythiolated material was not adsorbed by the xanthate to any great extent The loading of 5 mg of protein per 100 mg of xanthate is acceptable from the point of view of enzyme immobilisation since high loading tends to impair activity by crowding effects Although cross-linking of the protein to give a homopolymer was a possibility and could therefore have decreased the efficiency of the "copolymerization", it was considered that



extensive cross-linking would be necessary to cause the protein to become insoluble without being attached to carrier

When typsin was immobilised on cellulose xanthate, 16 3% of the original activity became bound This figure represents the minimum of enzyme loaded since some decrease in specific activity always accompanies covalent immobilisation On the other hand, the figure for the control (9 3%) will not have been so affected since it is unlikely that the adsorption process extensively disturbs the active site of the enzyme In addition, adsorbed enzyme is likely to become released into solution, particularly in the presence of substrate, and thus it may be concluded that the control figure represents a maximum A figure of 16 3% retention of activity on immobilisation of an enzyme compares very favourably with figures obtained for other immobilisation systems

Thus, cellulose xanthate may be used as an effective matrix for enzyme immobilisation and for analogous applications such as production of immunosorbents Advantages of the xanthate are its easy, non-costly production and the ease with which macromolecular species may be attached The requirement for conversion of the macromolecules into the polythiol derivatives might be considered a disadvantage, but provided such a derivatisation does not adversely affect the biological activity of the macromolecules, it could prove advantageous since it introduces a spacer molecule between the macromolecule and matrix, and thereby may enhance the activity of the final product In order to conserve the derivatised macromolecule, the supernatant from the coupling could be reused Finally, the xanthate would appear to have an advantage in that the material attached may be selectively removed by a process which is not ion-dependent

ACKNOWLEDGMENTS

The authors thank Professors M Stacey, C B E , F R S , and S A Barker for their interest in this work, and Ranks Hovis McDougall Research Ltd for a research scholarship to A Z

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