Journal of Chromatography, 215 (1981) 45–53
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 14,019

COUPLING OF PROTEINS AND OTHER AMINES TO SEPHAROSE BY BROMINE OXIDATION AND REDUCTIVE AMINATION

MONICA EINARSSON* and BIRGITTA FORSBERG
Research Department, Biochemistry KabiVitrum AB, S-112 87 Stockholm (Sweden)
and
OLLE LARM, MARIA ELENA RIQUELME and ELISABETH SCHOLANDER
Department of Chemistry and Molecular Biology, Swedish University of Agricultural Sciences, S-750 07 Uppsala (Sweden)

SUMMARY

Reactive carbonyl groups have been introduced into Sephadex and Sepharose gels by oxidation with aqueous bromine at pH 7. Proteins and other amines have been coupled in high yields to oxidized Sepharose gels by reductive amination. The reactions are carried out in aqueous solutions at pH 7 with sodium cyanoborohydride as a reducing agent. The biological activity of a tested immobilized enzyme (urokinase) is not altered. The effect of the oxidation–reduction procedure on the chromatographic properties of Sepharose gels is insignificant. The Sephadexes are much altered at high degrees of oxidation.

INTRODUCTION

Carbohydrate polymers such as Sephadex, Sepharose and cellulose are widely used as solid supports in affinity chromatography[1]. The attachment of a biologically active substance to a polysaccharide often involves two stages: (1) activation of the polysaccharide and (2) coupling of the intermediate to the biologically active compound[1,2]. The conditions for activation and coupling must be mild so that the chromatographic properties of the gel are not affected and that the biologically active compounds are not inactivated. Good coupling yields and relatively short reaction times are also desirable.

In an earlier publication[3] we described a method for the coupling of proteins and other amines to carbohydrate polymers by bromine oxidation–reductive amination. Other workers have prepared synthetic glycoproteins by treating a protein with different reducing disaccharides in the presence of sodium cyanoborohydride[4] and demonstrated that the protein is linked to the carbohydrate by the ε-amino groups of the lysine residues[5].

In the present work, optimum conditions for activation (oxidation) and coupling (reductive amination) have been sought. The chromatographic properties of the oxidized–reduced gels and the biological activity of an immobilized enzyme (urokinase) have been examined.

0021-9673/81/0000–0000/$02.50

EXPERIMENTAL

Materials

All chemical reagents used were of reagent grade or better. Bromine and 1,6-hexanediamine were obtained from E. Merck (Darmstadt, G.F.R.) and sodium cyanoborohydride ($NaBH_3CN$) from Fluka (Buchs, Switzerland), Blue dextran, Sephadex and Sepharose products were purchased from Pharmacia (Uppsala, Sweden). Plasminogen (25 caseinolytic units per ampoule) and human serum albumin (HSA) of clinical grade were obtained from KabiVitrum (Stockholm, Sweden) and urokinase (10,000 Ploug units per ampoule) from Leo Pharmaceutical Products (Ballerup, Denmark). Cytochrome *c*, aldolase, catalase, chymotrypsinogen and ovalbumin (Combithek Protein Calibration Kit, Size II) were purchased from Boehringer (Mannheim, G.F.R.). Ribonuclease (25 units/mg) was purchased from E. Merck.

Oxidation of polysaccharide gels

Excess of water was removed from Sepharose CL-4B and CL-6B by suction on glass filters for 5 min. In separate experiments, 100-g samples (corresponding to 4.9 and 7.3 g dry weight respectively) were added to 0.1 *M* aqueous bromine (100, 200, 400 or 600 ml). The pH was adjusted to, and automatically (Methrom E 300 B pH-meter) maintained at, 7.0 by addition of 1.0 *M* sodium hydroxide. When the oxidant was consumed (24–48 h), as shown by a negative potassium iodide–starch reaction, the products were washed with water (1.0 l). Dry Sephadex G-50 (5.0 g) and G-200 (5.0 g) were allowed to swell in water for 24 h. After removing excess of water by suction on glass filters for 5 min, the gels were oxidized with 0.1 *M* aqueous bromine (50, 75, 100, 200 or 400 ml).

The oxidized gels turned yellow on treatment with aqueous NaOH, showing that carbonyl groups had been introduced into the gel[6]. The bromine consumption (mole percent per hexose residue) was used to characterize the degree of oxidation and is indicated in brackets in the discussion of the oxidized gels. For some gels the acid content was determined by titration[7] and the content of neutral carbonyl groups by elemental analysis of methoximated samples[8] (Table I). These parameters are also expressed in mole percent per hexose residue, regarding each dry gel as $C_6H_{10}O_5$, *i.e.*, neglecting the cross-links.

The oxidized gels were examined by electron microscopy using a Philips 400 instrument.

Reduction of oxidized gels

The oxidized gel (2.5 g dry weight, corresponding to 50–100 g suction-dried weight) was suspended in water (50 ml), and sodium borohydride ($NaBH_4$, 300 mg) was added. After 2.5 h, the sample was transferred to a glass filter funnel and washed with 1.7 *M* acetic acid (2.0 l) and water (2.0 l).

Determination of water regain

Samples of the oxidized and oxidized–reduced gels were centrifuged at 700 *g* for 30 min and the centrifuged samples (each about 1 g) were lyophilized and the water regain noted as W_r = (wet weight − dry weight)/dry weight[9].

Chromatography on oxidized–reduced gels

The oxidized–reduced gels were washed on glass filters with 0.01 *M* phosphate buffer (pH 7.0) also containing 0.15 *M* sodium chloride and 0.02% sodium azide, and were packed into columns (89.5 × 1.6 cm). In each chromatographic experiment, the flow-rate was kept constant at 5 ml/cm^2 · h with a LKB 10200 Perpex pump. Fractions of about 1.5 ml were collected and the presence of proteins was determined from the absorbance at 280 nm. Chromatography of 1.5 ml (10 mg/ml) of cytochrome *c*, ribonuclease, HSA, ovalbumin and of 0.7 ml (10 mg/ml) chymotrypsinogen was carried out in a phosphate buffer on Sepharose CL-6B and on oxidized (22, 44 and 89)–reduced Sepharose CL-6B. Cytochrome *c* and ribonuclease were also chromatographed on oxidized (89)–reduced Sepharose CL-6B, using phosphate buffers with various NaCl concentrations (between 0.16 *M* and 1.0 *M*).

Effect of the amount of $NaBH_3CN$ on the coupling yield of HSA.

HSA (250 mg) was added to each of nine suspensions of oxidized (132) Sepharose CL-4B (10 g, suction-dried, 0.37 g dry weight) in 0.2 *M* phosphate buffer, pH 7.0 (10 ml). $NaBH_3CN$ was added in amounts ranging from 0 to 1000 mg. The mixtures were gently stirred at room temperature. The reactions were terminated after 24 h by the addition of 0.15 *M* acetic acid (100 ml). Each reaction mixture was transferred to a column (K 16/20, Pharmacia), which was washed sequentially, according to a routine procedure for proteins, with 200 ml of (1) sodium bicarbonate, (2) water, (3) 1 *M* NaCl, (4) water, (5) 0.1 *M* sodium acetate buffer (pH 6) and, lastly, (6) water. The degree of substitution was determined on lyophilized samples by a Kjeldahl analysis.

Effect of the amount of $NaBH_3CN$ on the coupling yield of 1,6-hexanediamine

A solution of 1,6-hexanediamine (40 g) in water (50 ml) was adjusted to pH 7.0 by the addition of concentrated acetic acid (50 ml) at 0°C. The solution was divided into eight aliquots. To each aliquot were added oxidized (132) Sepharose CL-4B (0.37 g dry weight) and $NaBH_3CN$ in amounts ranging from 0 to 500 mg. After 24 h the reaction mixtures were transferred to columns (K 16/20, Pharmacia) which were washed sequentially with 200 ml of (1) 1% acetic acid, (2) water, (3) 35% aqueous ethanol, (4) 50% aqueous ethanol, a routine procedure for aliphatic amines, and (5) 75% aqueous ethanol, 400 ml of (6) 95% ethanol and (7) water. The products were dried at 60°C, partially hydrolysed in concentrated deuterium chloride (60°C, 10 min) and analysed by NMR spectroscopy. The degree of substitution was calculated by comparing the integrated signals of the carbohydrate and methylene protons in 1,6-hexanediamine[10].

Effect of the amount of HSA on the coupling yield

To eight identical suspensions of oxidized (132) Sepharose CL-4B (10 g, suction-dried, 0.37 g dry weight) in 0.2 *M* phosphate buffer (pH 7.0, 10 ml) were added different amounts of HSA (ranging from 0 mg to 1000 mg) together with $NaBH_3CN$ (25 mg). The reactions were terminated after 24 h and the mixtures processed as described for proteins and analysed by the method of Kjeldahl.

Effect of reduction time on the coupling yield of 1,6-hexanediamine

A solution of 1,6-hexanediamine (40 g) in aqueous acetic acid (pH 7.0, 100 ml) was prepared as described. Oxidized (132) Sepharose CL-4B (100 g suction-dried, 3.7 g dry weight) and $NaBH_3CN$ (1.2 g) were added. Samples were withdrawn from the swirled reaction mixture at various times (between 15 min and 48 h) and processed as described.

Coupling of urokinase

Oxidized (132) Sepharose CL-4B (3.6 g suction-dried, 0.13 g dry weight) and $NaBH_3CN$ (60 mg) were added to a solution of urokinase (60,000 Ploug units) in 0.2 *M* phosphate buffer (pH 7.0, 6 ml). The reaction mixture was gently stirred for 24 h, washed and analysed as described for HSA. The resulting gel contained 4.7% HSA (w/w), as determined by a Kjeldahl analysis of a lyophilized sample.

Activation of plasminogen on urokinase–Sepharose CL-4B

A column (K 16/20, Pharmacia) thermostatted at 23°C was filled with urokinase–Sepharose CL-4B (1.4 ml, see preceding paragraph) and equilibrated with 0.1 *M* phosphate buffer (pH 7.0), which also contained 0.01 *M* L-lysine monohydrochloride and 2.3 μM ε-aminocaproic acid. Human plasminogen was dissolved to give a protein concentration of about 1 mg/ml in the buffer used for equilibration. The plasminogen solution (16 ml), thermostatted at 23°C, was applied to the column and the flow-rate was maintained at 6 ml/h. Fractions of about 4 ml were collected at 4°C and analysed for plasminogen and plasmin by the caseinolytic assay method described by Sgouris *et al.*[11]. The protein concentration was determined using the absorptivity ε (1%, 280 nm) = 16.1[12].

RESULTS AND DISCUSSION

When polysaccharides are oxidized with aqueous bromine, polymers having ketoglycosyl units and units containing carboxyl functions are formed[13] (Table I). Most probably, the latter are formed through overoxidation followed by cleavage of the pyranoid ring, since no traces of glycuronic acid residues have been found in reaction mixtures of bromine-oxidized glycosides[8] or disaccharides[14,15]. Polysaccharides containing ketoglycosyl units decompose into coloured products at pH values above 7 and they are also sensitive to strong acids[6].

TABLE I

BROMINE CONSUMPTION (B) IN THE PREPARATION OF SOME OXIDIZED GELS, THEIR CARBONYL CONTENTS (C) AND CARBOXYL CONTENTS (A) IN MOLE PERCENT PER HEXOSE RESIDUE

Gel	*B*	*C*	*A*
Sepharose CL-4B	66	8.5	5.6
	132	9.4	7.4
	199	8.6	11.2
Sephadex G-50	32	18	3.0
	65	29	11.4
	130	30	11.6

Despite the same bromine consumption, oxidized Sephadexes contain more carbonyl and carboxyl functions than Sepharoses and, in proportion to the carbonyl groups, the carboxyl content rises at high degrees of oxidation (Table I).

When a degree of oxidation higher than about 30 was used for Sephadex or about 150 for Sepharose, the gels became voluminous and lost their rigidity. The W_r values (Fig. 1) of the gels revealed that they were able to hold more water after oxidation and oxidation–reduction. There was no significant difference between the W_r values (Fig. 1) of an oxidized gel before and after reduction. Examination by electron microscopy revealed that the spherical structure of the beads remained intact even at high degrees of oxidation.

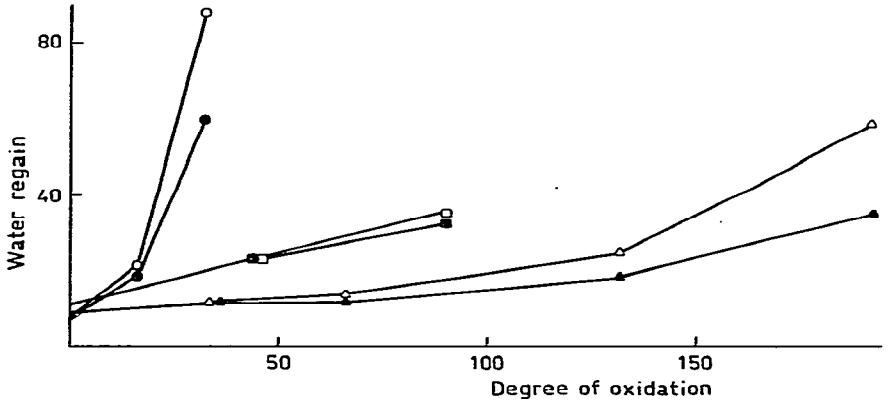


Fig. 1. Water regain (W_r) of oxidized and oxidized–reduced gels as a function of the degree of oxidation. ○, Oxidized Sephadex G-50; ●, oxidized–reduced Sephadex G-50; △, oxidized Sepharose CL-4B; ▲, oxidized–reduced Sepharose CL-4B; □, oxidized Sepharose CL-4B; ■, oxidized–reduced Sepharose CL-6B.

Since the Sephadex gels were shown to lose their rigidity at a degree of oxidation above 30, and since the bead volume and flow-rate of Sepharose did not change particularly on oxidation (< 150) and oxidation–reduction, only Sepharose gels have been investigated and discussed in detail.

The effects of the carboxyl groups on the chromatography of different proteins were studied. Since carbonyl groups of oxidized gels interact with free amino groups in proteins, these were reduced with $NaBH_4$ before the chromatographic studies. This reagent does not reduce carboxyl groups under the prevailing conditions. Upon chromatography in physiological buffers on oxidized–reduced Sepharose, cytochrome *c*, ribonuclease and chymotrypsinogen were retarded. Increasing degrees of oxidation of the gels first induced an increase in retardation, but at oxidation values above 44 there was no further increase in retardation (Figs. 2, 3). Proteins with isoelectric points below the pH of the buffer used for chromatography were not adsorbed on oxidized (44)–reduced Sepharose (Fig. 4). As expected, low-molecular-weight proteins were adsorbed to a greater extent than larger ones with comparable isoelectric points (Fig. 4). By increasing the ionic strengths of the buffers used for chromatography, the retardation that depended on the charged groups on the gels could be abolished.

The carbonyl functions of oxidized polysaccharides react with primary amines, and an equilibrium mixture of the imines, free carbonyls and amines is formed. The

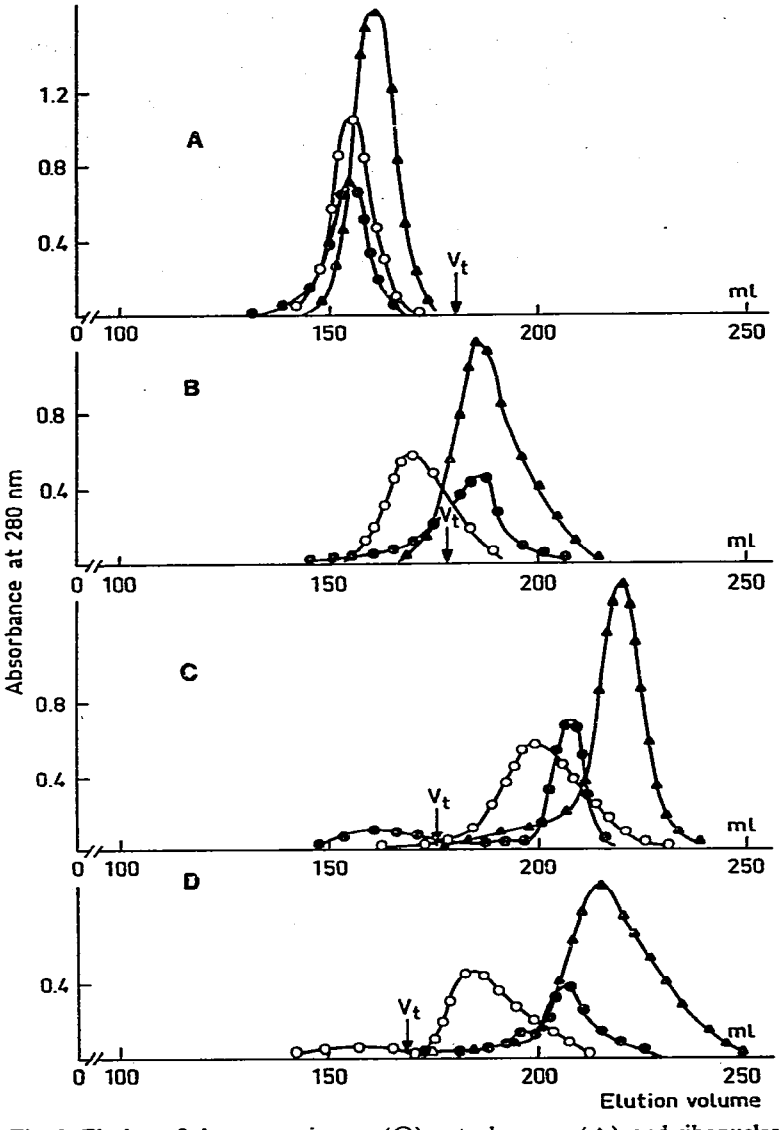


Fig. 2. Elution of chymotrypsinogen (○), cytochrome *c* (▲), and ribonuclease (●) on oxidized–reduced Sepharose CL-6B. Degree of oxidation: 0 (A); 22 (B); 44 (C); 89 (D). Eluent: 1 m*M* phosphate + 0.15 *M* NaCl + 0.02% NaN_3 (pH 7.0).

optimum pH interval for imine formation is 6–7 and reduction of carbonyl groups to alcohols with sodium cyanoborohydride is slow compared to that of imines to amines in this pH range[16]. Therefore, despite the unfavourable equilibrium for imines in aqueous solutions, a good coupling yield of secondary amines can be obtained if the reactions are performed in water solutions at pH 7. As the carbonyl groups are either reductively aminated or reduced back to alcohol functions, the original stabilities of the gels are restored on coupling.

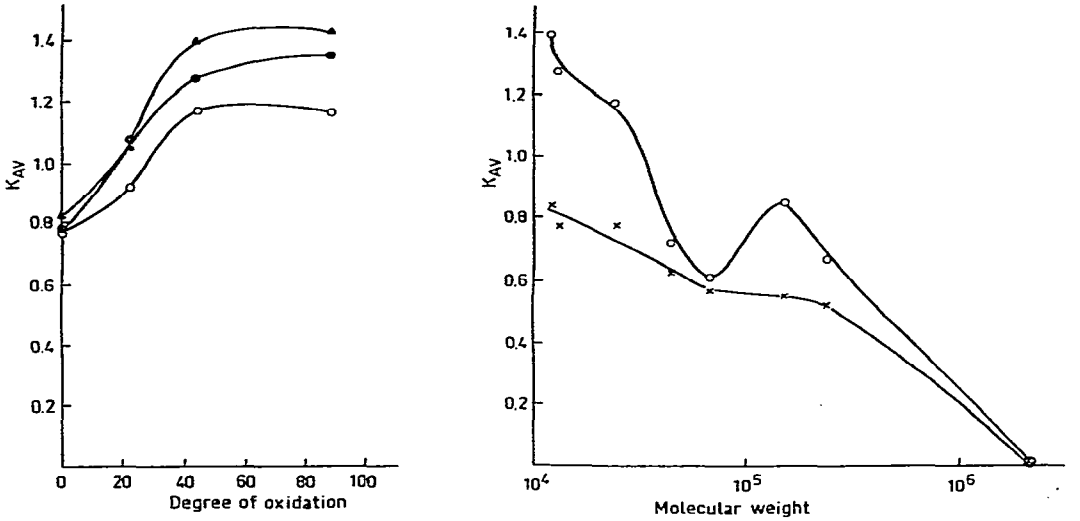


Fig. 3. Partition coefficient, K_{AV}, *versus* the degree of oxidation. Gel filtration of ribonuclease (●), cytochrome *c* (▲) and chymotrypsinogen (○) on oxidized (44)–reduced Sepharose CL-6B.

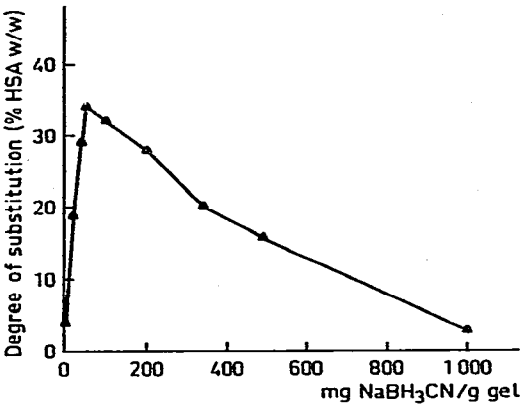
Fig. 4. Partition coefficient, K_{AV}, *versus* the molecular weight. Gel filtration of cytochrome *c* (M_w 12,500; p*I* 9.4), ribonuclease (M_w 13,500; p*I* 8.7–8.8), chymotrypsinogen (M_w 25,000; p*I* 9.6), ovalbumin (M_w 45,000; p*I* 4.7), HSA (M_w 68,500; p*I* 5.85), aldolase (M_w 158,000; p*I* 9.5) and catalse (M_w 240,000; p*I* 8.2) on Sepharose CL-6B (×) and oxidized (44) Sepharose CL-6B (○). Eluent: 1 m*M* phosphate + 0.15 *M* NaCl + 0.02% NaN_3 (pH 7.0). The void volumes were determined by chromatography of blue dextran (M_w $2 \cdot 10^6$).

When oxidized (132) Sepharose CL-4B was mixed with HSA (250 mg) and various amounts of $NaBH_3CN$, a maximum substitution was obtained using 25 mg $NaBH_3CN$ per 10 g suction-dried (0.37 g dry weight) oxidized gel (Fig. 5). When the amount of $NaBH_3CN$ was increased, the degree of substitution decreased rapidly. The results were not dependent on whether a freshly purified sample of $NaBH_3CN$ was used or a sample that had been stored for some time. In a similar experiment, 150 mg $NaBH_3CN$ gave a maximum substitution of 1,6-hexanediamine (used in excess) on the oxidized gel (Fig. 6). Increasing the amount of $NaBH_3CN$ resulted in slightly decreased substitution. Also in this case, the results were independent of the sample of $NaBH_3CN$ used.

After 2 h, 85% of the final coupling yield of 1,6-hexanediamine to oxidized (132) Sepharose CL-4B was obtained (Fig. 7). The degree of substitution then increased slowly and a maximum was reached after 48 h. Similar results were obtained when HSA was coupled to Sepharose CL-4B.

The larger the amount of protein used in the coupling reaction, the higher was the degree of substitution obtained (Fig. 8). Almost quantitative immobilization of added protein was achieved at low protein/gel ratios (Fig. 8) and for example when 250 mg HSA per 10 g suction-dried, oxidized (132) gel and 25 mg of sodium cyanoborohydride was used, 84% of the added protein was attached, corresponding to a degree of substitution of 36% (w/w).

Urokinase was used as a model for covalent coupling of an active protein to Sepharose, providing an excellent tool for activation of plasminogen. The immobi-



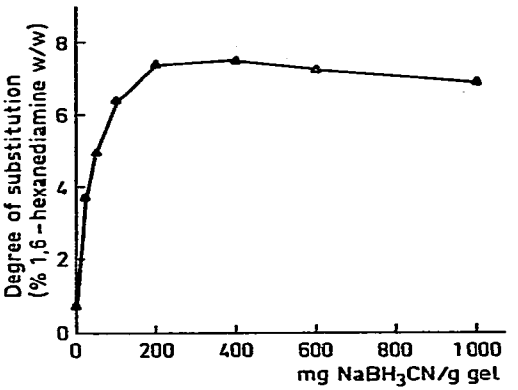
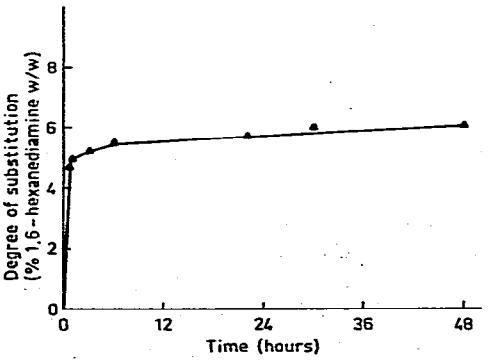


Fig. 5. Effect of the amount of sodium cyanoborohydride on the coupling of HSA to oxidized (132) Sepharose CL-4B.

Fig. 6. Effect of the amount of sodium cyanoborohydride on the coupling of 1,6-hexanediamine to oxidized (132) Sepharose CL-4B.

lized urokinase was used either for continuous or repeated activation of plasminogen to plasmin. By using a short time for contact between the effluent and the activator bed, autolysis of the generated plasmin was minimized. The activation was very efficient, as only traces of plasminogen could be detected in the generated plasmin solution. The results are in agreement with those obtained with urokinase gels prepared by the cyanogen bromide method[15,16].

The method can also be used for the immobilization of proteins and other amines on cellulose. It is evident from Table I that Sephadexes are more easily oxidized with aqueous bromine than Sepharoses. Preliminary results show that good coupling yields are obtained with Sephadexes with a degree of oxidation of about 15. The chromatographic properties of these gels seem to be unaltered under these conditions.



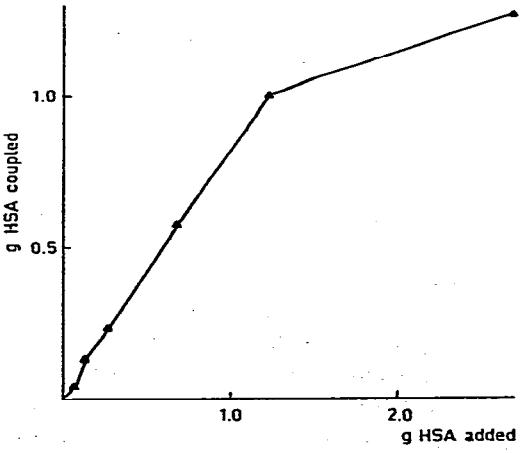


Fig. 7. Effect of reduction time on the coupling of 1,6-hexanediamine to oxidized (132) Sepharose CL-4B.

Fig. 8. Amount of HSA coupled *versus* HSA added to 1 g oxidized Sepharose (132) CL-4B and 25 mg $NaBH_3CN$.

ACKNOWLEDGEMENTS

We thank Professor Olof Theander for his interest and Docent Nigel Marsden for valuable discussions. This work was supported by the Swedish Board for Technical Developments (STU), KabiVitrum AB, Stockholm, Sweden, and Pharmacia AB, Uppsala, Sweden.

REFERENCES

1 C. R. Lowe and P. D. G. Dean, *Affinity Chromatography*, Wiley, New York, 1st ed., 1974, and references therein.
2 W. B. Jakoby-and M. Wilchek, *Methods Enzymol.*, 34 (1974).
3 O. Larm and E. Scholander, *Carbohydr. Res.*, 58 (1977) 249–251.
4 C. P. Stowell and Y. C. Lee, *Advan. Carbohydr. Chem. Biochem.*, 37 (1980) 225–281.
5 B. A. Schwartz and G. R. Gray, *Arch. Biochem. Biophys.*, 181 (1977) 542–549.
6 O. Theander, *Acta Chem. Scand.*, 12 (1958) 1887–1897.
7 R. L. Whistler and M. S. Feather, *Methods Carbohydr. Chem.*, 1 (1962) 464–468.
8 O. Larm, E. Scholander and O. Theander, *Carbohydr. Res.*, 49 (1976) 69–77.
9 K. W. Peppel, D. Reichenberg and D. K. Hale, *J. Chem. Soc., London*, (1952) 3129–3136.
10 J. Rosengren, S. Påhlman, M. Glad and S. Hjertén, *Biochim. Biophys. Acta*, 412 (1975) 51–61.
11 J. T. Sgouris, J. K. Inman, K. B. McCall, L. A. Hyndman and H. D. Andersson, *Vox Sang*, 5 (1960) 357–376.
12 P. Wallén and B. Wiman, *Biochim. Biophys. Acta*, 221 (1970) 20.
13 O. Larm, K. Larsson, E. Scholander, B. Meyer and J. Thiem, *Carbohydr. Res.*, 91 (1981) 13–20.
14 R. F. Borch, M. D. Bernstein and H. D. Durst, *J. Amer. Chem. Soc.*, 93 (1971) 2897–2904.
15 B. Wiman and P. Wallén, *Eur. J. Biochem.*, 36 (1973) 25–31.
16 M. Einarsson, unpublished results.