Journal of Chromatography, 90 (1974) 87–98

CHROM. 7171

PREPARATION OF ADSORBENTS FOR BIOSPECIFIC AFFINITY CHROMATOGRAPHY

I. ATTACHMENT OF GROUP-CONTAINING LIGANDS TO INSOLUBLE POLYMERS BY MEANS OF BIFUNCTIONAL OXIRANES

LARS SUNDBERG and JERKER PORATH

Institute of Biochemistry, University of Uppsala, Uppsala (Sweden)

(Received November 9th, 1973)

SUMMARY

A new method for preparing adsorbents for biospecific affinity chromatography is described. Bisoxiranes (*e.g.* 1,4-butanediol diglycidyl ether) have been used for the introduction of reactive oxirane groups into agarose, and for simultaneous stabilization of the gel by cross-linking. Optimal conditions for the activation and subsequent coupling of proteins, peptides and aliphatic and aromatic amines have been evaluated.

Fractionation of different forms of trypsin on soya bean trypsin inhibitor agarose is described in order to illustrate the use of oxirane–agarose in biospecific affinity chromatography.

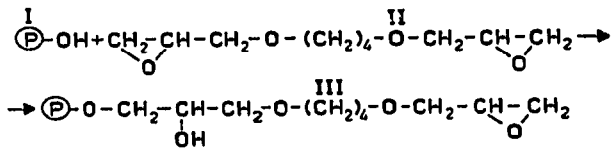
INTRODUCTION

The synthesis of adsorbents for biospecific affinity chromatography requires suitable matrices and effective coupling methods for attachment of ligands. These aspects have recently been discussed by Porath and Kristiansen[1]. Agarose fulfils most demands on a carrier for adsorptive groups or ligands, and its properties can be further improved by cross-linking with agents such as diglycidyl ethers, epihalohydrins[2,3] and divinyl sulphone[4]. A variety of coupling methods are also available but only a few of them satisfy demands such as the formation of stable bonds between ligand and matrix, high yield and no introduction of non-specific centres such as charged groups, etc.[5–7].

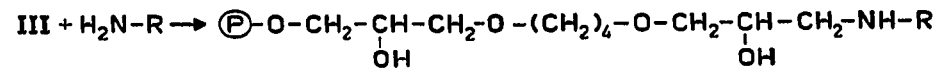
This paper describes the use of bisoxiranes for the introduction of reactive oxirane groups into agarose and for stabilization of the gel by simultaneous cross-linking. The use of bisoxiranes such as 1,4- or 1,3-butanediol diglycidyl ether consequently fulfils the dual function of bridging the gel strands as well as allowing attachment of adsorptive groups or molecules to the matrix via long hydrophilic or amphiphilic spacers[8–10]. The method has been used successfully for the immobilization of proteins, peptides and amino acids, and some of these applications have been described earlier[11,12]. A comprehensive description of the optimal conditions

for activation and coupling is included. To illustrate the use in biospecific affinity chromatography, we have chosen the isolation and fractionation of different forms of trypsins on an inhibitor agarose, because this system lends itself particularly well as a model for comparison with other methods of preparing biospecific adsorbents and immobilized enzymes. The following parameters were studied during activation: pH, reaction time, temperature, solvent and concentration of bisoxirane. Coupling was studied as a function of pH, reaction time and temperature.

For 1,4-butanediol diglycidyl ether, the activation of the polymer matrix Ⓟ–OH takes place in the following manner:



and the amino group containing compounds $R–NH_2$ are coupled to III according to the reaction:



Agarose (Sepharose 2B, 4B and 6B) was obtained from Pharmacia (Uppsala, Sweden) and 1,3- and 1,4-butanediol diglycidyl ether from EGA Chemie (Steinheim, G.F.R.). Glycyl-L-leucine, L-arginine, L-lysine, L-glycine, *p*-aminobenzamidine hydrochloride, soya bean trypsin inhibitor (STI), twice-crystallized trypsin, *p*-tosyl-L-arginine methyl ester (TAME), benzoyl-DL-arginine-*p*-nitroanilide (BAPNA) and sulphanilamide were all purchased from Sigma (St. Louis, Mo., U.S.A.).

MATERIALS AND METHODS

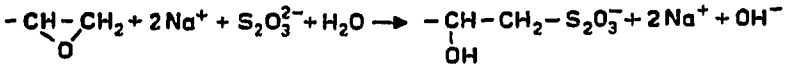
Determination of the amount of bound substance

The coupled products were analyzed with respect to amino acid[13], nitrogen (Kjeldahl method) and sulphur[14]. Prior to the analyses, the gels were washed thoroughly with water and acetone on a glass filter-funnel and freeze-dried. The water content in the dried gels was determined using a moisture analyzer. Reported values for bound substances were corrected for the residual water in the gel. The amounts of proteins coupled to the gels were determined by amino acid analysis in all experiments. Gels containing fixed amino acids and other amines were analyzed with respect to the nitrogen content. Gels with coupled sulphanilamide were analyzed for both nitrogen and sulphur.

Determination of oxirane groups

We have used the method suggested by Axén[15] involving a reaction between

the oxirane ring and sodium thiosulphate in order to determine the amount of oxirane in the solution and in the gel. The reaction is as follows:



The release of OH^- was followed by titration with 0.1 *M* hydrochloric acid in a pH-stat (pH meter 51, Autoburett ABU 12, Radiometer, titrator 11).

Oxirane in solution was determined as follows. The oxirane-containing solution (50 μl) was added to 1.5 ml of 1.3 *M* sodium thiosulphate solution and pH was kept constant by additions of hydrochloric acid until the reaction was complete. The amount of oxirane present in the solution was then calculated from the amount of hydrochloric acid needed in order to maintain neutrality.

Oxirane groups in the gel were determined in the following way. Wet agarose gel (0.5 g) was added to 1.5 ml of 1.3 *M* sodium thiosulphate solution of pH 7.0 and the oxirane content of the gel was determined by titration with hydrochloric acid. The agarose gel was suction-dried under vacuum on a glass filter-funnel for 5 min and weighed. In our experiments, the samples had a dry weight of approximately 27 mg.

Activation and cross-linking

One gram of suction-dried agarose (Sepharose 6B) was washed on a glass filter-funnel with water and then mixed with 1 ml of diglycidyl ether and 1 ml of 0.6 *M* sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre. The suspension was mixed by rotation for 8 h at 25° and the reaction stopped by washing the gel on a glass filter-funnel with large volumes of water (500 ml).

Coupling

The coupling for 1 g of suction-dried oxirane–agarose was accomplished by dissolving the desired reactant in 2 ml of a buffer adjusted to a predetermined pH. Thus proteins were coupled in the pH range 8.5–10 at a temperature of 25° and a reaction time of 15–48 h. For amino acids, amines, carbohydrates and other more stable substances, the corresponding reaction conditions were pH 9–11, temperature 25–75° and reaction time 4–15 h. An increased coupling yield was obtained at higher pH and temperature. However, the decreased yield at the low pH and temperature necessary for coupling proteins can be partially compensated for by prolonging the reaction time.

RESULTS

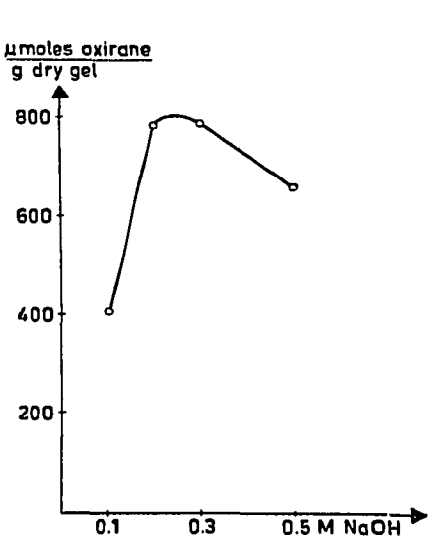
Conditions for introduction of oxirane groups in agarose gel

Optimum pH. Samples of 0.5 g of suction-dried Sepharose 6B were mixed in a 25-ml round-bottomed flask with 0.5 ml of diglycidyl ether and 0.5 ml of sodium hydroxide solution of varying molarity containing 2 mg of sodium boro-

hydride per millilitre. The flask was rotated at a slow speed at 25° for 8 h. Washing of the oxirane–agarose was performed on 25-ml plastic filter-funnels with 10-ml portions of water in order to remove excess of reagent. The last portion of the washings was found to contain no oxirane. In Fig. 1, the amount of oxirane expressed in μmoles per gram of dry gel is plotted against concentration of added hydroxide ions. The maximum activity, expressed as oxirane concentration in the gel, was found at a final concentration in the reaction mixture of 0.2 *M* sodium hydroxide, which corresponds to a concentration of 0.6 *M* of the sodium hydroxide solution added.

Optimum temperature. Samples of 0.5 g of suction-dried Sepharose 6B were mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg sodium borohydride per millilitre) at 4°. The samples were then brought to different temperatures and were slowly stirred for 8 h. Fig. 2 shows the curve for the amount of free oxirane groups in μmoles introduced per gram of dry gel as a function of temperature.

Optimum reaction time. Samples of 0.5 g of suction-dried Sepharose 6B were mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg sodium borohydride per millilitre) and rotated at 25° for different lengths of time. Fig. 3A shows the number of μmoles of oxirane groups introduced as a function of reaction time in hours while Fig. 3B displays the results from a set of experiments under the same conditions in which the amount of oxirane groups in the gel and in the solution during activation was determined. A sample of 50 μl of supernatant was withdrawn, titrated and the total amount of oxirane in the supernatant was calculated. A blank experiment was carried out in order to determine the oxirane stability in the solution in the absence of gel. In a third



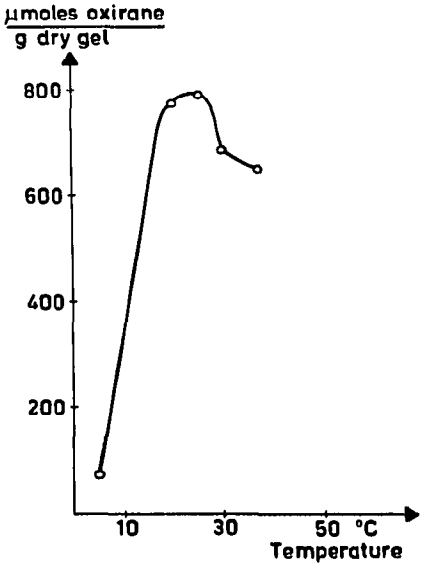


Fig. 1. Oxirane content in the gel as a function of the alkalinity of the reaction mixture.

Fig. 2. Oxirane content in the gel as a function of the temperature during the reaction.

experiment, pre-cross-linked Sepharose 6B (ref. 3) was used in order to determine the degree of cross-linking under normal activation conditions.

Optimization of diglycidyl ether concentration. Samples of 0.5 g of suction dried Sepharose 6B were mixed with 0.5 ml of 0.6 *M* sodium hydroxide solution (containing

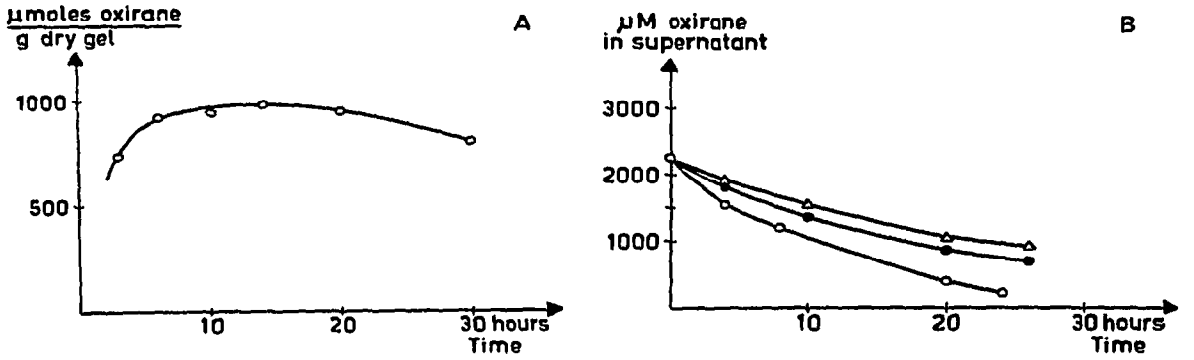


Fig. 3. (A) Oxirane content in the gel as a function of reaction time. (B) Oxirane content in the supernatant as a function of reaction time. ○—○, Oxirane in the supernatant during reaction with Sepharose 6B; ●—●, oxirane in the supernatant during reaction with epichlorohydrin cross-linked desulphated Sepharose 6B (ECD-Sepharose 6B); △—△, stability of diglycidyl ether under the conditions used for preparation of oxiran-Sepharose expressed as remaining oxirane as a function of reaction time.

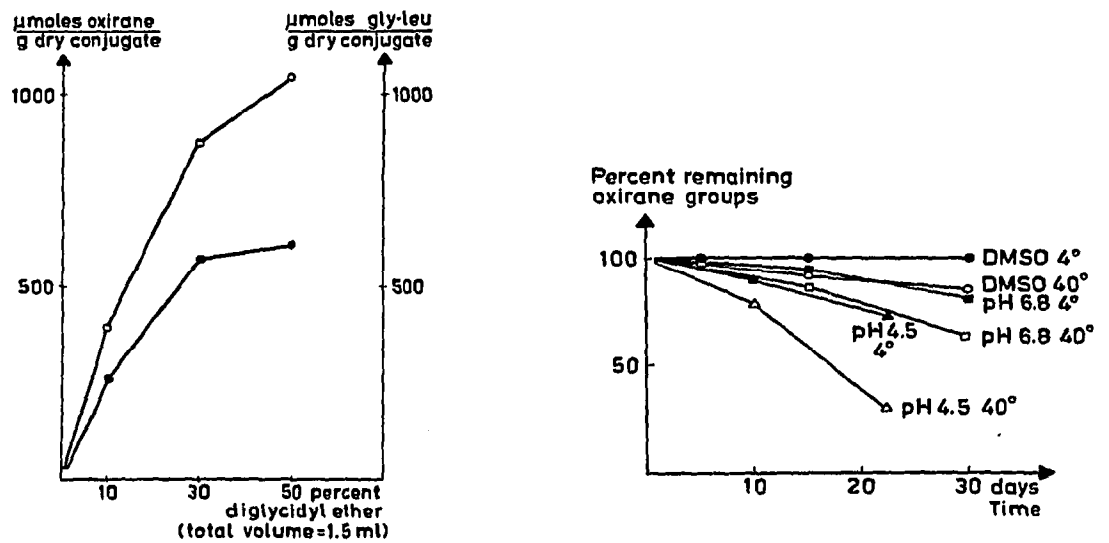


Fig. 4. Oxirane content and amount of coupled dipeptide (glycyl-L-leucine) in the gel as a function of concentration of diglycidyl ether in the reaction mixture. ○—○, Oxirane–Sepharose 6B; ●—●, amount of coupled glycyl-L-leucine in µmoles per gram of dry conjugate.

Fig. 5. Stability of oxirane–Sepharose 6B expressed as a percentage of remaining oxirane groups in the gel as a function of temperature and solvent. ○—○, Dimethyl sulphoxide, 40 °; ●—●, dimethyl sulphoxide, 4 °; △—△, 0.1 *M* sodium acetate, pH 4.5, 40 °; ▲—▲, 0.1 *M* sodium acetate, pH 4.5, 4 °; □—□, 1*M* sodium chloride, pH 6.8, 40 °; ■—■, 1 *M* sodium chloride, pH 6.8, 4 °.

2 mg sodium borohydride per millilitre) and varying concentrations of diglycidyl ether and rotated for 8 h at 25°. Fig. 4 shows the amount of oxirane coupled to the gel (μmoles per gram of dry gel) as a function of percentage of oxirane in the reaction mixture. It also shows the amount of bound glycyl-L-leucine in milligrams per gram of dry conjugate as a function of oxirane concentration in the reaction mixture. The dipeptide coupling was performed with 100 mg of glycyl-L-leucine in 1 ml of 0.5 *M* sodium carbonate buffer, pH 10, mixed with 0.5 g of suction-dried oxirane–Sepharose 6B at 50° for 15 h.

Stability of oxirane–agarose

Samples of suction-dried Sepharose 6B were incubated with a variety of solutions at 4°, 23° and 40° for different periods of time. Fig. 5 shows the stability of oxirane–agarose in distilled water (1 *M* in sodium chloride), dimethyl sulphoxide and 0.1 *M* acetate buffer, pH 4.5 (1 *M* in sodium chloride).

Conditions for coupling

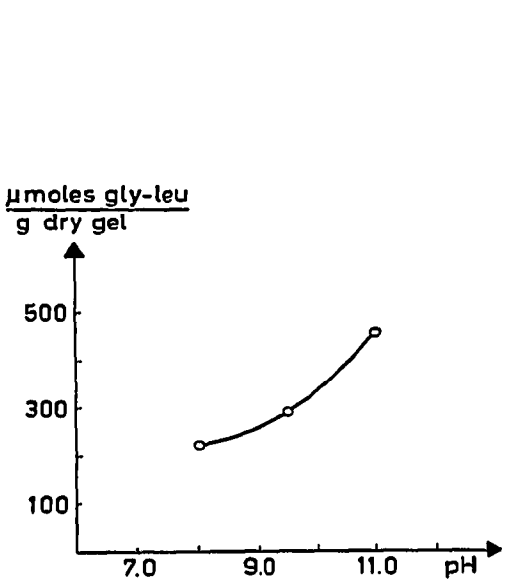
In all coupling experiments, the following activation procedure was used: 0.5 g of suction-dried Sepharose 6B was mixed with 0.5 ml of diglycidyl ether and 0.5 ml of 0.6 *M* sodium hydroxide solution (containing 2 mg of sodium borohydride per millilitre) in a round-bottomed flask and rotated for 8 h at 25°.

Coupling of dipeptide at different pH values. Glycyl-L-leucine (100 mg) was dissolved in 1 ml of buffer solutions at different pH values (0.1 *M* sodium phosphate buffer for pH 6.0 and 8.0, 0.3 *M* sodium carbonate buffer between pH 8.5 and 11) and mixed with 0.5 g of suction-dried oxirane gel. In Fig. 6 the amount of chemically bound glycyl-L-leucine (μmoles per gram of dry gel) is plotted as a function of pH. After the coupling step, the gel was washed systematically with the following solutions: 0.5 *M* sodium carbonate buffer, pH 9.5, 1 *M* in sodium chloride (100 ml); distilled water (50 ml); and 0.05 *M* glycine–hydrochloric acid buffer, pH 3.0, 1 *M* in sodium chloride (100 ml).

Coupling of dipeptide at different temperatures. Glycyl-L-leucine (100 mg) was dissolved in 1 ml of 0.5 *M* sodium carbonate buffer, pH 11, and mixed with 0.5 g of suction-dried oxirane–Sepharose 6B at 4°. The temperature was adjusted and the reaction mixture carefully stirred for 8 h. The results are shown in Fig. 7A. In another set of experiments, the contact time for the reaction between oxirane–agarose and glycyl-L-leucine was studied at 23° and 40°; the conditions were the same as above and the results are shown in Fig. 7B. The effect of concentration was studied and the conditions used were as follows: 0.5 g of suction-dried oxirane–Sepharose 6B was mixed with varying amounts of glycyl-L-leucine dissolved in 1 ml of 0.5 *M* sodium carbonate buffer, pH 11, under rotation at 50° for 15 h. Fig. 8 shows the amount of coupled dipeptide in μmoles per gram of dry gel as a function of the concentration of glycyl-L-leucine.

Coupling of proteins. Soya bean trypsin inhibitor (200 mg) was dissolved in 20 ml of 0.5 *M* sodium carbonate buffer, pH 9.5, and carefully stirred with 10 g of suction-dried oxirane–Sepharose 6B for 24 h at 25° (activation: 10 g of suction-dried agarose was mixed with 5 ml of diglycidyl ether and 5 ml of 0.6 *M* sodium hydroxide solution containing 2 mg of sodium borohydride per millilitre) for 8 h at 25°). The inhibitor–gel was then washed with the following solutions in order to remove

unbound protein: sodium carbonate buffer, pH 9.5, 1 *M* in sodium chloride (250 ml); distilled water (100 ml); 0.1 *M* glycine, pH 3.0, 1 *M* in sodium chloride (250 ml); and finally 0.05 *M* Tris–hydrochloric acid buffer, pH 7.8, 0.5 *M* in sodium chloride and 0.02 *M* in Ca^{2+} (250 ml).



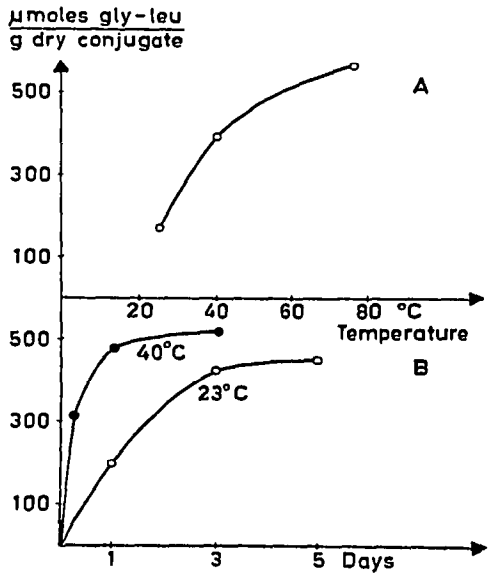
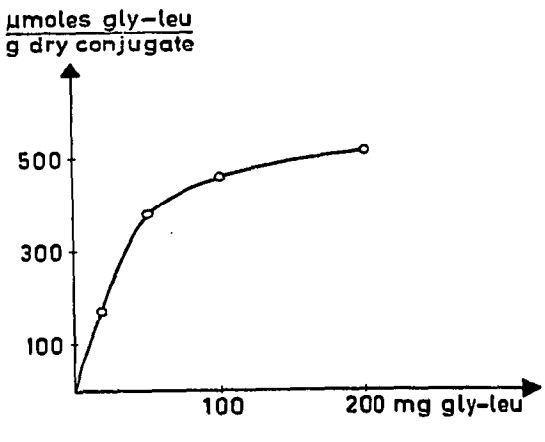


Fig. 6. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of the pH in the reaction mixture.

Fig. 7. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of reaction temperature.



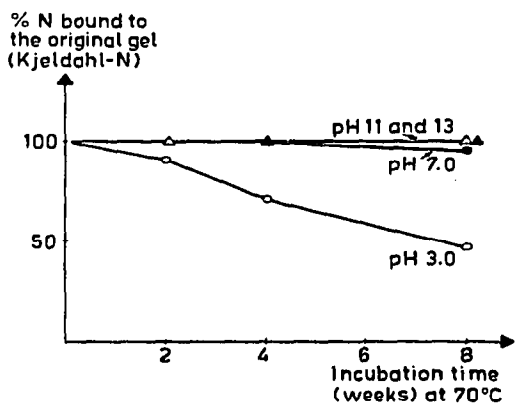


Fig. 8. Amount of coupled glycyl-L-leucine (expressed as μmoles of dipeptide per gram of dry conjugate) as a function of peptide concentration in the reaction mixture.

Fig. 9. Stability of coupled glycine expressed as a percentage of remaining nitrogen in the gel as a function of pH and incubation time at 70 °. ○—○, 1 *M* sodium acetate buffer of pH 3.0; ●—●, 0.5 *M* sodium phosphate buffer of pH 7.0; △—△, 1 *M* sodium carbonate buffer of pH 11; ▲—▲ 0.1 *M* sodium hydroxide solution (pH 13).

Deactivation of remaining oxirane groups after coupling. Immobilization of proteins results in a gel that still contains oxirane groups capable of further coupling. These groups can be blocked by treatment of the gel with a solution of, for example, 2 *M* glycine or ethanolamine, preferably at a pH above 8.5, at 23° for 24 h. Such deactivation blocks oxirane groups that are sterically available for coupling. Titration with sodium thiosulphate solution shows that some groups remain, presumably within sites that do not interfere in the chromatographic experiments.

Stability of coupled glycine

The stability of glycine coupled to Sepharose 6B (450 mg per gram of conjugate) was studied by incubation of the mixture at various pH values (1 *M* sodium acetate buffer, pH 7.0, 1 *M* sodium carbonate buffer, pH 11, and 0.1 *M* sodium hydroxide solution, pH 13) at 70° for 8 weeks. Samples were withdrawn after different incubation times, and the amounts of nitrogen in the gel and in solution were determined.

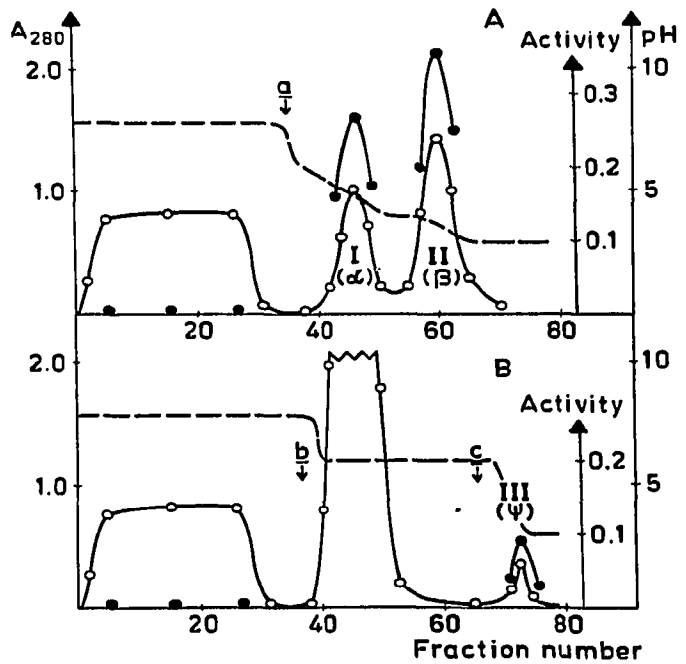


Fig. 10. (A) Chromatography of bovine trypsin on an STI–Sepharose 6B column (10 × 1.4 cm) previously equilibrated with 0.05 *M* Tris–hydrochloric acid buffer, pH 7.8 (0.5 *M* in sodium chloride, 0.02 *M* in Ca^{2+}). Amount of trypsin: 400 mg. Flow-rate: 50 ml/h. Fraction volume: 9 ml. At a, the pH gradient started (– – –). The absorption at 280 nm (○—○) and the trypsin activity (ΔE_{405}/min, ●—●) of eluted fractions were measured. The N-terminal amino acid residues of fractions I and II indicated the composition of mainly α- and β-trypsin (see Table I). (B) The same conditions as in A. At b, specific desorption was performed with citrate–phosphate buffer, pH 6.0, containing 50 mg of benzamidine per millilitre, and at c elution with 0.05 *M* glycine buffer, pH 3.0 (0.5 *M* in sodium chloride) was started. The eluted trypsin–benzamidine complex could be split and separated by chromatography on Sephadex G-25 previously equilibrated with 10^{-3} *M* hydrochloric acid. N-Terminal amino acid analyses of fraction III indicated the presence of ψ-trypsin (see Table I).

In Fig. 9, the stability is expressed as percentage of original nitrogen remaining in the gel after the incubation.

Demonstration of bioaffinity chromatography employing adsorbents produced by bisoxirane coupling

Soya bean trypsin inhibitor agarose obtained as described in the section *Coupling of proteins* was used to isolate and separate different active trypsins from commercial preparations. Adsorption was performed at pH 7.8 (0.05 *M* Tris–hydrochloric acid, 0.5 *M* in sodium chloride) and the adsorbed material displaced either by using a non-linear pH gradient (LKB 1130 Ultrograd gradient mixer operating on the following buffers: citrate–phosphate, pH 6.0 (0.05 *M* dipotassium hydrogen orthophosphate adjusted with 1 *M* citric acid), 0.5 *M* in sodium chloride; and citrate–phosphate, pH 3.0 (0.05 *M* dipotassium hydrogen orthophosphate adjusted with 1 *M* citric acid), 0.5 *M* in sodium chloride); or by consecutive displacement with citrate–phosphate buffer, pH 6.0, 0.05 *M* in phosphate and 0.5 *M* in sodium chloride containing 50 mg of benzamidine per millilitre and with 0.05 *M* glycine–hydrochloric acid, pH 3.0, 0.5 *M* in sodium chloride. Enzyme activities were determined with BAPNA as substrate[16], measuring the change in absorption at 405 nm. The amino acid terminal sequence was determined according to the method of Edman[17] and Iwanaga *et al.*[18]. As the three active trypsins, α, β and ψ, differ in their N-terminal residues, this property can be used for identification[19]. In Table I, literature values of N-terminal residues for the α-, β- and ψ-trypsins are compared with those actually found in bioaffinity chromatography of commercial trypsin (shown in Fig. 10).

TABLE I

N-TERMINAL SEQUENCE OF THE MATERIAL IN THE CHROMATOGRAPHIC FRACTIONS I, II AND III (SEE FIGS. 10A AND B)

β-Trypsin is a single polypeptide chain, whereas α-trypsin consists of two and ψ-trypsin of three polypeptide chains[19, 22].

Fraction	*N-terminal amino acid residues*
I	Ile, Ser (traces of Asn)
II	Ile
III	Ile, Ser, Asn
α-Trypsin[19, 22]	Ile, Ser
β-Trypsin[19, 22]	Ile
ψ-Trypsin[19, 22]	Ile, Ser, Asn

DISCUSSION

Treatment of hydroxyl-containing polymers with bifunctional oxiranes (*e.g.* 1,4-butanediol diglycidyl ether) in alkaline medium has proven to be an effective means of preparing adsorbents for biospecific affinity chromatography. Attempts to find the optimal conditions for activation are shown in Figs. 1–5. Fig. 1 shows

that a final concentration of 0.2 *M* sodium hydroxide in the reaction mixture is sufficient to give the maximal introduction of oxirane groups in the gel. At higher concentrations of sodium hydroxide, hydrolysis and cross-linking will produce gels with fewer free groups available for coupling. The most suitable temperature for activation is found to be about 25° (Fig. 2). Higher temperatures can be used if the reaction time is shortened. Fig. 3 shows a rather broad optimum for the reaction time; 6–10 h gives a maximum yield under the experimental conditions used.

The parameters involved in the activation and coupling are not mutually independent. The determination of exact optimum conditions of introduction of oxirane groups into the gel and for coupling would require a much larger series of experiments than those described in this paper. From a practical point of view, such an extentive study is not necessary because the approximate optimal conditions obtained simply by superimposing the results from the separate series of experiments are likely to approach the exact optimal conditions very closely.

In order to determine the degree of cross-linking and the amount of oxirane remaining in solution, experiments were performed which gave the results shown in Fig. 3A. During activation, it is necessary to work with an excess of diglycidyl ether owing to the side-reaction leading to formation of cross-links. The difference between the hydrolysis curves obtained in the absence and presence of gel is a measure of the total amount of oxirane reacted with gel, *i.e.* both involved in cross-links and available for titration. From Fig. 3, it is obvious that only a small part of the diglycidyl ether used is involved in the production of active gel-linked oxirane groups, whereas the major portion of reacting material has produced cross-links. Further evidence for this conclusion was provided by the experiment in which pre-cross-linked agarose was treated with diglycidyl ether under the same conditions. Here, less oxirane should be consumed because most of the reactive hydroxyl groups in the gel should already be involved in cross-links. The results in Fig. 3A show this to be so. The amount of available oxirane can be seen to decrease progressively when activation is allowed to continue for periods longer than 10 h, under which conditions the oxirane group is presumably hydrolyzed. The number of available oxirane groups is also a function of the initial concentration of diglycidyl ether. Fig. 4 shows that an increase in concentration gives a considerable increase in oxirane groups in the gel. For practical use, a concentration of 30% of diglycidyl ether in the reaction mixture is sufficient to give good results. One of the advantages of this method is that the oxirane gel can be stored in the wet state at 4° in 1 *M* sodium chloride solution for more than 4 weeks with less than 10% reduction in the oxirane content.

Both low- and high-molecular-weight substances have been coupled to oxirane agarose. Table II summarizes the results obtained and gives the amount of coupled substance under optimal conditions. Glycyl-L-leucine was used as a model substance in the attempts to determine optimal conditions for coupling. Figs. 6–8 show the influence of pH, temperature, time and reagent concentration on this coupling reaction. The best results were obtained at high pH (11) and a temperature of about 50°. At high temperatures, a shorter contact time can be used, but for coupling of proteins, milder conditions have to be used to retain biological activity. Table II shows the results obtained in the coupling of soya bean trypsin inhibitor, STI, to Sepharose 6B (see Methods). The amount of coupled inhibitor per gram

of dry polymer, 7 μmoles, corresponds to about 75% of that obtained with the cyanogen bromide method under similar conditions.

The STI–agarose has been used as an adsorbent for the isolation of proteolytic enzymes from crude pancreatic extracts and for separation of active and inactive trypsins in commercial preparations[11] (see refs. 20–22). From these experiments, it is clear that 1 mole of trypsin binds to 1 mole of coupled inhibitor.

Different forms of active trypsins can also be separated by bioaffinity chromatography on bisoxirane-coupled STI–agarose. Absorbed trypsin can be eluted by pH gradient elution, which completely resolved α-, β- and ψ-trypsin (Fig. 10A). The separation of ψ-trypsin from its analogues can be carried out by specific desorption with benzamidine, a strong inhibitor for α- and β-trypsin[19]. ψ-Trypsin is not eluted under these conditions, but can be subsequently desorbed by changing the pH of the eluent (Fig. 10B).

These experiments illustrate how the bisoxirane method can be used to produce effective adsorbents for bioaffinity chromatography in which non-specific adsorption is to be avoided and high selectivity is required.

In addition to active groups for coupling and cross-linking of agarose, this method provides a spacer or arm, which is of great importance in biospecific adsorption when the ligand is small and the affinity constant low[23–27]. In a study of these effects, we have used sulphanilamide coupled to agarose (Table II) to adsorb and isolate carbonic anhydrase directly from human erythrocytes (to be published). Sulphanilamide coupled to cross-linked dextran (Sephadex) with cyanogen bromide has also been used for the purification of carbonic anhydrase[28]. The corresponding agarose adsorbent possessed a very low affinity, presumably owing to more severe steric hindrance by the matrix. Adsorbents formed from oxirane agarose, however, showed a high capacity for carbonic anhydrase. The results indicate that the spacer is necessary in this case for obtaining a good adsorbent.

TABLE II

COVALENT BINDING OF SUBSTANCES CONTAINING AMINO GROUPS TO SEPHAROSE 6B

The oxirane–gel was prepared as described under *Activation and cross-linking*. Soya bean trypsin inhibitor (STI) was coupled at 25 °, pH 10.0, for 24 h, and the other substances listed at 75 °, pH 11.0, for 18 h.

Substance	*Amount of substance added to 5 g of wet polymer (mg)*	*Amount of coupled substances*	
		μmoles/g of dry polymer	*% of added substance*
p-Amino benzamidine	100	450	21
Sulphanilamide	100	430	20
Sulphanilamide	500	650	6
Glycyl-L-lysine	100	380	25
Lysine	100	428	21
Soya bean trypsin inhibitor	100	5.8	38

ACKNOWLEDGEMENTS

The technical assistance of Miss Annette Sjöström and Mr. Ingmar Olsson is gratefully acknowledged. We also thank Dr. Hendrik Arnberg for help with the N-terminal analyses. Financial support was obtained from The Swedish Board for Technical Development.

REFERENCES

1 J. Porath and T. Kristiansen, in H. Neurath (Editor), *The Proteins, 3rd ed.*, in press.
2 H. Schell and V. Ghetie, *Rev. Roum. Biochim.*, 5 (1968) 295.
3 J. Porath, J.-C. Janson and T. Lääs, *J. Chromatogr.*, 60 (1971) 167.
4 T. Låås, J. Porath and J.-C. Janson, in preparation.
5 I. H. Silman and E. Katchalski, *Annu. Rev. Biochem.*, 35 (1966) 873.
6 J. K. Inman and H. M. Dintzis, *Biochemistry*, 8 (1969) 4074.
7 R. Axén and S. Ernback, *Eur. J. Biochem.*, 18 (1971) 351.
8 P. Cuatrecasas, in G. R. Stark (Editor), *Aspects of Solid Chemistry*, Academic Press, New York, 1971, p. 79.
9 P. O'Carra and S. Barry, *FEBS Lett.*, 21 (1972) 281.
10 S. L. Marcus and E. Balbinder, *Anal. Biochem.*, 48 (1972) 448.
11 J. Porath and L. Sundberg, in H. Peeters (Editor), *Protides of the Biological Fluids*, Vol. 18, Elsevier, Amsterdam, 1970, p. 401.
12 J. Porath and L. Sundberg, in M. Hair (Editor), *The Chemistry of Biosurfaces*, Vol. 2, Marcel Dekker, New York, 1972, p. 633.
13 D. Spackman, W. Stein and S. Moore, *Anal. Chem.*, 30 (1958) 1190.
14 L. Gustavsson, *Talanta*, 4 (1960) 227.
15 R. Axén, to be published.
16 H. Fritz, G. Hartwich and E. Werle, *Hoppe Seyler's Z. Physiol. Chem.*, 345 (1966) 150.
17 P. Edman, *Ann. N. Y. Acad. Sci.*, 88 (1960) 602.
18 S. Iwanaga, P. Wallen, N. J. Gröndahl, A. Henschen and B. Blombäck, *Eur. J. Biochem.*, 8 (1969) 189.
19 R. L. Smith and E. Shaw, *J. Biol. Chem.*, 244 (1969) 4704.
20 V. Kasche, *Biochem. Biophys. Res. Commun.*, 38 (1970) 875.
21 G. Feinstein, *FEBS Lett.*, 7 (1970) 353.
22 N. C. Robinson, R. W. Tye, H. Neurath and K. A. Walsh, *Biochemistry*, 14 (1971) 2743.
23 D. Dennis, *Anal. Biochem.*, 24 (1968) 544.
24 P. Cuatrecasas, M. Wilchek and C. B. Anfinsen, *Proc. Nat. Acad. Sci. U.S.*, 61 (1968) 636.
25 T. Kristiansen, M. Einarsson, L. Sundberg and J. Porath, *FEBS Lett.*, 7 (1970) 294.
26 E. Steers, P. Cuatrecasas and H. Polland, *J. Biol. Chem.*, 246 (1970) 196.
27 J. D. Miller, P. Cuatrecasas and E. B. Thompson, *Proc. Nat. Acad. Sci. U.S.*, 68 (1971) 1014.
28 S. O. Falkbring, P. O. Göthe, P. O. Nyman, L. Sundberg and J. Porath, *FEBS Lett.*, 24 (1972) 229.