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AGAR DERIVATIVES FOR CHROMATOGRAPHY, ELECTROPHORESIS AND GEL-BOUND ENZYMES

IV. BENZYLATED DIBROMOPROPANOL CROSS-LINKED SEPHAROSE AS AN AMPHOPHILIC GEL FOR HYDROPHOBIC SALTING-OUT CHROMATOGRAPHY* OF ENZYMES WITH SPECIAL EMPHASIS ON DENATURING RISKS

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SUMMARY

The preparation of benzylated covalently cross-linked Sepharose 2B is described. Such gel was analyzed for its degree of substitution, and gels with three different degrees of substitution were used in chromatographic experiments with dextranase, α-amylase, lactate dehydrogenase, α-chymotrypsin and trypsin. Yields and chromatographic patterns for different eluting systems were determined. It was found that gradients combining an increase in ethylene glycol concentration with a decrease in salt concentration gave better results than did pure salt gradients. No denaturation was observed for dextranase or α-amylase, but the other enzymes tested were partly denatured. The most severe denaturation was observed for lactate dehydrogenase desorbed from the highest substituted gels, although the enzyme was highly active in the adsorbed state. The results and the use of amphophilic gels are discussed.

INTRODUCTION

The systematic use of the hydrophobic properties of proteins for their chromatographic fractionation has attained increased interest, and numerous papers have appeared on this subject[1–21]. The basic principles for this type of chromatography were presented by Tiselius[22] as early as 1948; he noticed that proteins and other substances (*e.g.*, dyes) that could be precipitated at high concentrations of neutral salts were adsorbed (at much lower salt concentration) to adsorbents that, without salts,

* Various names have been assigned to chromatographic methods based on essentially the same chromatographic principle: hydrophobic chromatography, hydrophobic interaction chromatography, hydrophobic salting-out chromatography, and so on. We prefer the last-named term, because it stresses that the predominent effect is a non-specific salt-dependent adsorption to hydrophobic groups in contrast to the biospecific adsorption that also often takes advantage of hydrophobically substituted gels.

showed no affinity for the substances. Tiselius called this phenomenon "adsorption by salting out", and used such adsorbents as silica and cellulose. Today, however, most studies are performed on amphophilic materials prepared by covalently coupling various hydrophobic substituents (such as alkylamines) to agarose. The most frequently used coupling technique is the cyanogen bromide method introduced by Axén *et al.*[23]; however, this technique introduces charged groups into the adsorbent, and this may give rise to difficulties in interpreting the chromatographic data[17].

A more clear-cut, purely hydrophobic effect, easier to rationalize, is to be expected if the hydrophobic substituents are bonded by totally neutral ether linkages, and this can easily be accomplished by using covalently cross-linked agarose gels as matrices [24]. Such gels can withstand alkaline media at the high temperatures necessary to bring about the following simple reaction:

$$M\text{–}OH + R\text{–}X \xrightarrow[80\text{–}100^\circ]{NaOH} M\text{–}O\text{–}R + HX$$

where M represents the agarose matrix and RX is a hydrophobic halide. The chromatographic use of benzyl ether Sepharose prepared in this way has been described[14].

Exposure of a protein to hydrophobic substances or surfaces often involves considerable risk of denaturation, but few reports dealing with chromatography on amphophilic adsorbents have taken this fact into consideration. Still fewer studies have been reported on the effects of various desorption conditions on the chromatographic elution pattern and on the yields of protein and enzyme activity. All these factors will depend on the degree of substitution, since a highly substituted gel can give multiple-point contact with proteins; this, in turn, will lead to a greater risk of denaturation and a different desorption behaviour. Another question that arises is: Will an adsorbed enzyme retain its activity? The experiments described in this paper were performed in an attempt to elucidate these problems for benzylated Sepharose.

MATERIALS

Sepharóse 2B and Dextran T150 were obtained from Pharmacia Fine Chemicals (Uppsala, Sweden), and 2,3-dibromopropan-1-ol (DBP), purity 99%, was purchased from Köln-Kalk (Köln, G.F.R.). The following chemicals were obtained from Kebo (Stockholm, Sweden): benzylchloride, 99% pure for synthesis; sulphur trioxide, minimum 98% pure for synthesis; Folin–Ciocalteu phenol reagent; starch (soluble according to Zulkowsky), analytical grade; 3,5-dinitrosalicycli acid (DNS), 97% pure for synthesis. The following enzymes and substrates were supplied by Sigma (St. Louis, Mo., U.S.A.): α-amylase, Type II-A, from *Bacillus subtilis*; α-chymotrypsin, Type II, and trypsin, Type II, both enzymes from bovine pancreas; lactate dehydrogenase (LDH), Type II, from rabbit muscle; nicotinamide–adenine dinucleotide, reduced form (NADH), 98% pure, grade III; N-acetyl-L-tyrosine, ethyl ester (ATEE); N^2-benzoyl-DL-arginine-*p*-nitroanilide hydrochloride (BAPNA); N^2-benzoylarginine, ethyl ester (BAEE). Dextranase was a partially pure preparation (Usher-400, Lot No. S-40-b) obtained from Dextran Products (Scarborough, Ont., Canada). All other chemicals were of analytical grade.

METHODS

Cross-linking of Sepharose 2B with 2,3-dibromopropan-1-ol (DBP)

The gel (800 g, wet weight) was washed with water on a glass filter and suspended in 800 ml of water in a 2-litre round-bottomed flask. DBP (80 ml) was added and allowed to dissolve partially for about 30 min, with continuous stirring at room temperature. The reaction was then started by the slow and continuous addition (from a peristaltic pump) of 5 *M* sodium hydroxide containing 1% of sodium tetrahydroborate. A total of 352 ml of the sodium hydroxide* was added during 1 h. The suspension was then allowed to react for another 90 min before the gel was washed with 5 volumes of water, 5 volumes of ethanol and, finally, with 10 volumes of water.

This treatment produced a gel in all respects equal to the previously described epichlorohydrin cross-linked and desulphated Sepharose[24], except for the alkaline desulphation, which, in this instance, was achieved simultaneously with the benzylation.

Benzylation of the DBP cross-linked Sepharose 2B

Cross-linked Sepharose 2B was treated with benzyl chloride in alkaline medium at elevated temperature according to the procedure of Porath *et al.*[14]. Variation in alkalinity and concentration of benzyl chloride gave gels with different degrees of substitution (see Fig. 1 and Table I).

Determination of degree of substitution (D.S.)

By exhaustive sulphation of benzylated and unbenzylated Sepharose 2B it was possible to calculate the D.S. by comparison of the sulphur contents. The sulphation

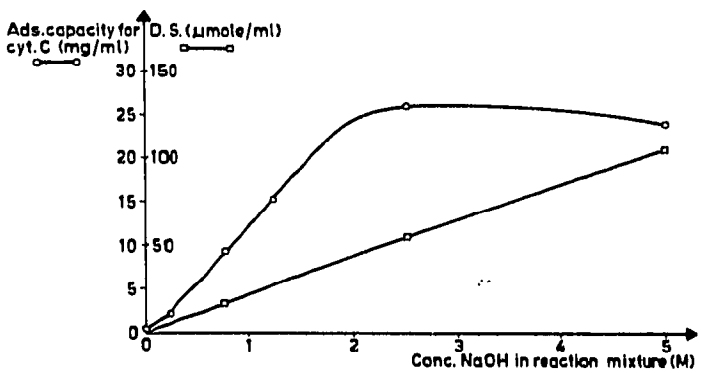


Fig. 1. Properties of benzylated Sepharose as a function of sodium hydroxide concentration in the benzylation reaction. Reaction conditions: 10 ml of wet dibromopropanol cross-linked Sepharose 2B were suspended in a total volume of 20 ml containing various concentrations of sodium hydroxide and 0.1 g of sodium tetrahydroborate (as antioxidant); 2.5 ml of benzyl chloride were added and the mixture was allowed to react for 5 h at 80°. The benzylated gels were washed with water, alcohol and water. The adsorption capacity for cytochrome *c* and the degree of substitution (D.S.) were determined as described in Experimental.

* This 352 ml of solution corresponds to the stoichiometric amount of DBP plus an excess sufficient to make the solution approx. 0.1 *M* in sodium hydroxide when the reaction is complete.

TABLE I

PROPERTIES OF BENZYLATED DIBROMOPROPANOL CROSS-LINKED SEPHAROSE 2B GELS USED FOR CHROMATOGRAPHIC EXPERIMENTS

Gel No.	*Volume compared to original gel*	*D.S.*		*Adsorption capacity for cytochrome c*	
		μmole/g	*μmole/ml*	V_e/V_t	*Benzyl/cyt. c*
I	1.0	900 ± 150	16	9.5	1.7
II	0.6	1500 ± 150	55	26	2.1
III	0.4	2500 ± 150	110	24	4.6

was performed according to a procedure devised by Porath and Fornstedt (to be published). In this 10 ml of sulphur trioxide were added dropwise 75 ml of pyridine in a round-bottomed flask fitted with a stirrer and reflux condenser and chilled in ice. About 5 g of each gel was thoroughly washed with pyridine and allowed to react with 7.5 ml of the suspension of the sulphur trioxide–pyridine complex in stoppered tubes for 24 h at 40°. The sulphated gels were then washed on a glass filter with about 200 ml of each of the following liquids: ethanol; water; 1 *M* acetic acid containing 2 *M* sodium chloride; 1 *M* sodium carbonate containing 2 *M* sodium chloride; and finally water. The gels were lyophilized and analyzed for sulphur at the Institute for Analytical Chemistry, Uppsala, Sweden (see Fig. 1).

Frontal analysis with cytochrome c

Cytochrome *c* is strongly adsorbed to benzylated gels, especially at acid pH and high ionic strength[14]. Determination of the adsorption capacity was performed as frontal analysis of a 1% solution of cytochrome *c* in 0.1 *M* ammonium formate pH 3.0 containing 3 *M* sodium chloride in small (9-mm diam.) columns containing 2–3 ml of gel. The flow-rate was 9 ml/h and the eluate was analyzed by means of a Uvicord instrument, Type 4701 A (LKB, Stockholm, Sweden). The inflexion point was determined, and, after correction for tube volumes, this value was taken as the elution volume (V_e). Normalization by division by the gel volume (V_t) gave V_e/V_t, which is a measure of the adsorption capacity under the experimental conditions (Fig. 1).

Enzyme assays

Dextranase activity was determined according to Janson and Porath[25]. The amount of reducing groups produced per minute at 40° in a 1% solution of Dextran T150 (mean molecular weight 150 000) in 0.1 *M* potassium phosphate buffer of pH 6.0 was determined by using the DNS reagent and expressed as μmoles of isomaltose; 1 enzyme unit corresponded to the production of 1 μmole of isomaltose/min under the experimental conditions.

α-Amylase activity was measured as described by Bernfield[26]. The production of reducing sugar from a 1% starch solution in 0.05 *M* sodium phosphate buffer of pH 5.9 at 25° was determined with use of the DNS reagent; 1 enzyme unit produced reducing power equivalent to 1 μmole of glucose/min.

The activity of LDH was determined by mixing NADH and sodium pyruvate

with enzyme solution in 0.1 *M* sodium phosphate buffer of pH 7.4 and recording the decrease in absorbance at 340 nm[27]; the concentrations were 0.2 m*M* NADH and 0.76 m*M* pyruvate in the reaction mixture. One activity unit catalyzed the oxidation of 1 μmole of NADH/min at 25°. The enzyme could be dissolved in buffer solution containing 50% of ethylene glycol or 1 *M* sodium chloride (but not in 3 *M* sodium chloride) without any change in activity.

The activity of LDH adsorbed to gel was determined in the following way. In a conical flask fitted with a magnetic stirrer were mixed 7 ml of water containing 0.876 mg of sodium pyruvate, 1 ml of settled gel or aqueous solution containing a known amount of LDH activity, 1 ml of 0.1 *M* sodium phosphate buffer of pH 7.4 and finally 1 ml 1% NADH solution in 0.1% sodium hydrogen carbonate solution. From this reaction mixture, 1-ml samples were withdrawn at timed intervals after addition of the carbonate solution, and the gel was rapidly filtered off. The free enzyme was treated in the same way. The absorbance of the gel-free solution was measured at 340 nm and, by comparison with the result for the free enzyme, the activity of the adsorbed enzyme could be converted into conventional units.

α-Chymotrypsin was assayed by a method based on that suggested by Schwert and Takenada[28], with ATEE (0.01 *M*) as substrate at pH 7.0 (0.067 *M* sodium phosphate) at room temperature with the aid of a Coleman double-beam spectrophotometer, Hitachi 124 equipped with a Hitachi Perkin-Elmer recorder No. 165. One activity unit caused a decrease in absorbance at 237 nm of 0.0075 per min at 25°.

The substrate used for trypsin was BAPNA (0.033%) in 0.05 *M* Tris hydrochloride buffer at pH 7.8 (Erlanger *et al.*[29]). One activity unit produced a change in absorbance at 405 nm of 3.32 per min.

The activities of α-chymotrypsin and trypsin in the adsorbed state were investigated with ATEE and BAEE, respectively, as substrates. Reactions were monitored by continuous addition of 0.1 *M* sodium hydroxide with a pH stat, Type TTTIc (Radiometer, Copenhagen, Denmark), to suspensions of the gels. In control experiments, the activities of the free enzymes were measured in the same manner.

Chromatographic experiments

The gels were packed to a volume of 1 ml in columns of I.D. 9 mm, the columns being re-packed with fresh gel for each experiment; the experiments were performed in a cold room (4°). Three columns were run simultaneously by using a three-channel peristaltic pump (Stålprodukter, Uppsala, Sweden), which gave almost exactly the same flow through all channels. The flow-rate was adjusted to 7.8 ml/h, and fractions of volume 1.00–1.30 ml were collected. With a few exceptions, the sample volume was 1 ml. Elution was always initiated with about 20 ml of starting buffer followed by a linear gradient of about 15 ml. After washing with a few column volumes of the final solvent composition, a frontal zone of buffer containing 50% of ethylene glycol or of 1 *M* sodium hydroxide was introduced in some experiments. The fractions were analyzed for protein by measuring the absorbance at 280 or 282 nm with a Hitachi Model 101 spectrophotometer or by the method of Lowry *et al.*[30]. Activity was measured on fractions and/or on pooled material; further details are given in the legends to the Figures.

RESULTS

The benzylation reaction can be easily controlled by adjusting the concentration of sodium hydroxide in the reaction mixture. The adsorption capacity for cytochrome *c* varies linearly with D.S. up to about 25 mg/ml (1500 μmole/g). This can be used as a rapid and easy method for determination of D.S. in the linear region, in which there is an almost constant value of about 2 moles of benzyl groups per mole of cytochrome *c*. The reason for the saturation behaviour of the adsorption might be purely steric, since 1 ml of gel (gel II) contains only about 20 mg of carbohydrate, but will adsorb as much as 25 mg of protein.

The determination of D.S. with sulphation was tested with chlorobenzylated cross-linked Sepharose 2B and compared with data from elementary analysis for chlorine; the agreement was within 5%. The uncertainty values in Table I are approximate estimations based on parallel determinations.

The main purpose of the chromatographic experiments was to investigate the risk of denaturation with amphophilic gels and of how this depended on D.S. and

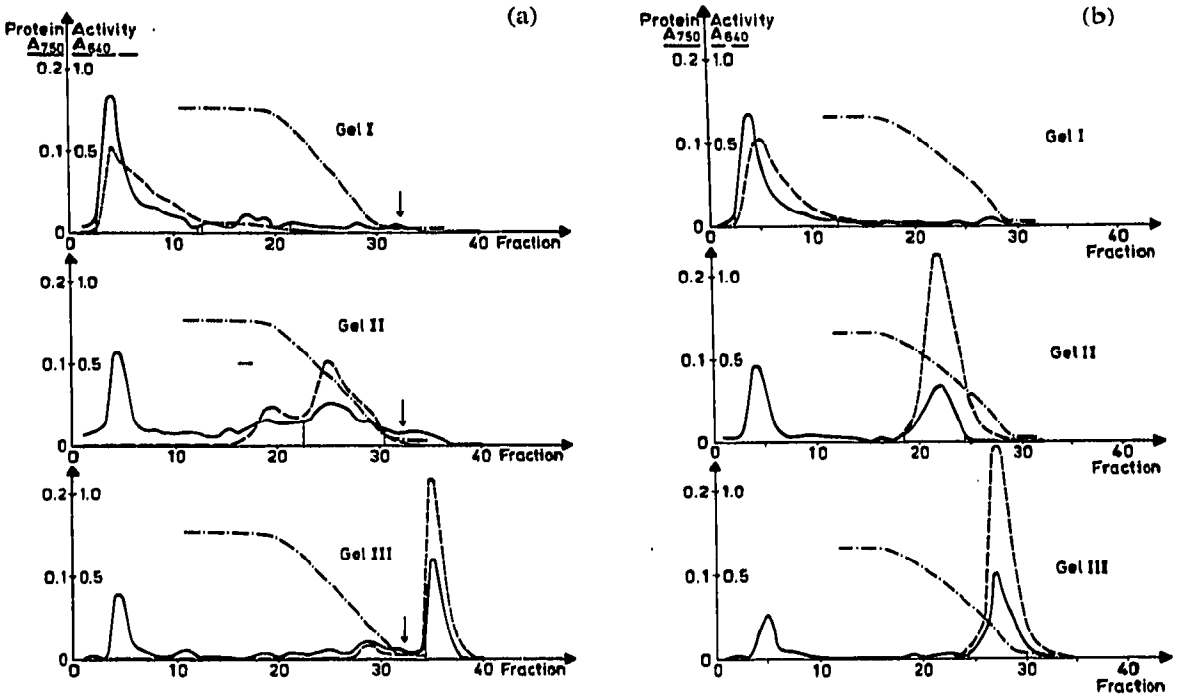


Fig. 2. Chromatography of dextranase "Usher-400". The buffers used were: 0.05 *M* Tris hydrochloride (pH 8.0) containing (A) 3.0 *M* sodium chloride, (B) no additions, and (C) 50% of ethylene glycol. Protein determined according to Lowry (A_{750}) is shown by the continuous line, and calculated protein amounts are based on bovine serum albumin. Activity: the DNS-reagent colour (A_{640}) is shown by the broken line; 3 *M* NaCl or 50% of ethylene glycol did not affect the activity. In Fig. 2a, the sample was 1.84 mg of protein in 1 ml of buffer A, and a gradient from buffer A to buffer B was used; the front of buffer C was applied as indicated by the arrow. The results are set out in Table II under a. In Fig. 2b, the sample was 1.78 mg of protein in 1 ml of buffer A, and a gradient from buffer A to buffer C was used. The results are set out in Table II under b.

elution circumstances. Consequently, gels with three different D.S. were synthesized (gels I, II and III), and an arbitrary collection of enzymes was chromatographed under standardized conditions (see Table I).

Chromatographic results

Dextranase and α-amylase. The chromatographic behaviours of these enzymes are closely similar, as is evident from Figs. 2 and 3 and Tables II and III. Both enzymes showed relatively weak interaction with the gels, and no significant denaturation occurred. Only gel III at high ionic strength effectively adsorbed the enzyme. Both enzyme preparations contained some low-molecular-weight inactive proteins in the break-through peak, resulting in an increase in the specific activity of the desorbed enzymes. It is obvious from Figs. 2 and 3 that the combined ethylene glycol and sodium chloride gradient gave the best results. Because of the presence of UV-absorbing low-molecular-weight material in the dextranase preparation, the protein content was determined by the Lowry–Folin method instead of spectrophotometrically at 280 nm.

LDH. This enzyme, with molecular weight 135 000 and composed of four

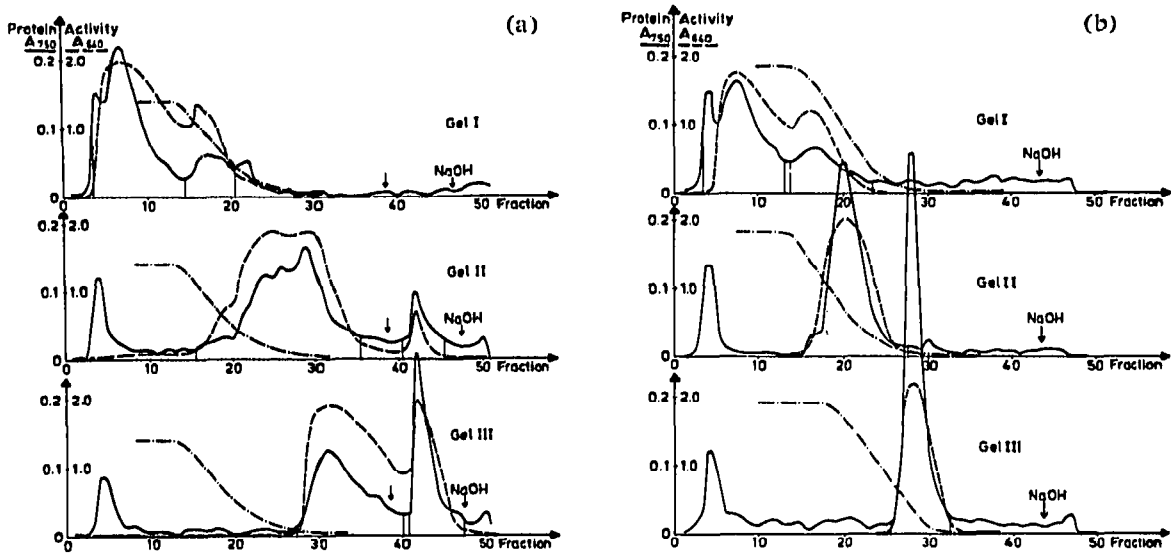


Fig. 3. Chromatography of α-amylase. The buffers used were 0.05 *M* Tris hydrochloride (pH 8.0) containing (A) 3 *M* sodium chloride, (B) no additions, and (C) 50% of ethylene glycol. Protein determined according to Lowry (A_{750}) is shown by the continuous line. Activity: the DNS-reagent colour (A_{640}) is shown by the broken line. The highest activity values plotted in the chromatogram are not proportional to the true activity, but this has no effect on the values for activity yield and specific activity (see Table III), since these are absed on values for pooled material: 3 *M* NaCl or 50% of ethylene glycol did not affect the activity. In Fig. 3a, the sample was 2.70 mg of α-amylase in 1 ml of buffer A, and a gradient from buffer A to buffer B was used; the arrow indicates the point of application of a front of buffer C. The results are set out in Table III under a. In Fig. 3b, the sample was 2.70 mg of α-amylase in 1 ml of buffer A, and a gradient from buffer A to buffer C was used. The results are set out in Table III under b.

TABLE II

CHROMATOGRAPHY OF DEXTRANASE

The yields shown for protein and activity are totals for the whole chromatogram, but the specific activities are calculated for the activity peaks. Protein values are based on the Folin method, with bovine serum albumin as standard.

	Sample	*Yield* as percentage of*		*Specific activity (units/mg)*
		protein	*initial activity*	
a	Starting material	100	100	100
	After gel I	100	110	90–100**
	After gel II	130	110	140
	After gel III	80	80	150
b	Starting material	100	100	100
	After gel I	70	100	160
	After gel II	70	120	440
	After gel III	60	110	360

* The yields of protein and activity are often approximate, as a large error was introduced when the amount of protein or activity had to be summed over large volumes in experiments where the eluted material was widely spread out.

** Referring to the two pools shown in Fig. 2a.

sub-units (presumably at least partly held together by hydrophobic forces), should be vulnerable to interaction with the benzylated gels (see Fig. 4). The decrease in specific activity with increasing D.S. is particularly obvious (Table IV). In control experiments, it was found, however, that LDH still adsorbed to gel II after elution with buffer containing 50% ethylene glycol (30 μg/ml of gel) was fully as active as the same amount of free enzyme. Enzyme adsorbed to gel III after elution with the same buffer (240 μg/ml of gel) retained 25% of its initial activity.

The desorbed enzyme was further analyzed by chromatography on Sephadex G-200. The material eluted with two distinct peaks; one (comprising about one-third

TABLE III

CHROMATOGRAPHY OF α-AMYLASE

	Sample	*Yield as percentage of*		*Specific activity [(units/mg)·10^{-3}]*
		*protein**	*initial activity*	
a	Starting material	100	100	1.2
	After gel I	110	100	1.4–1.6
	After gel II	120	90	1.6–0.5
	After gel III	110	80	1.4–1.1
b	Starting material	100	100	1.2
	After gel I	130	80	1.8–2.1
	After gel II	110	110	2.4
	After gel III	130	100	2.1

* Based on a value of 25.3 for $A_{1\,cm}^{1\%}$

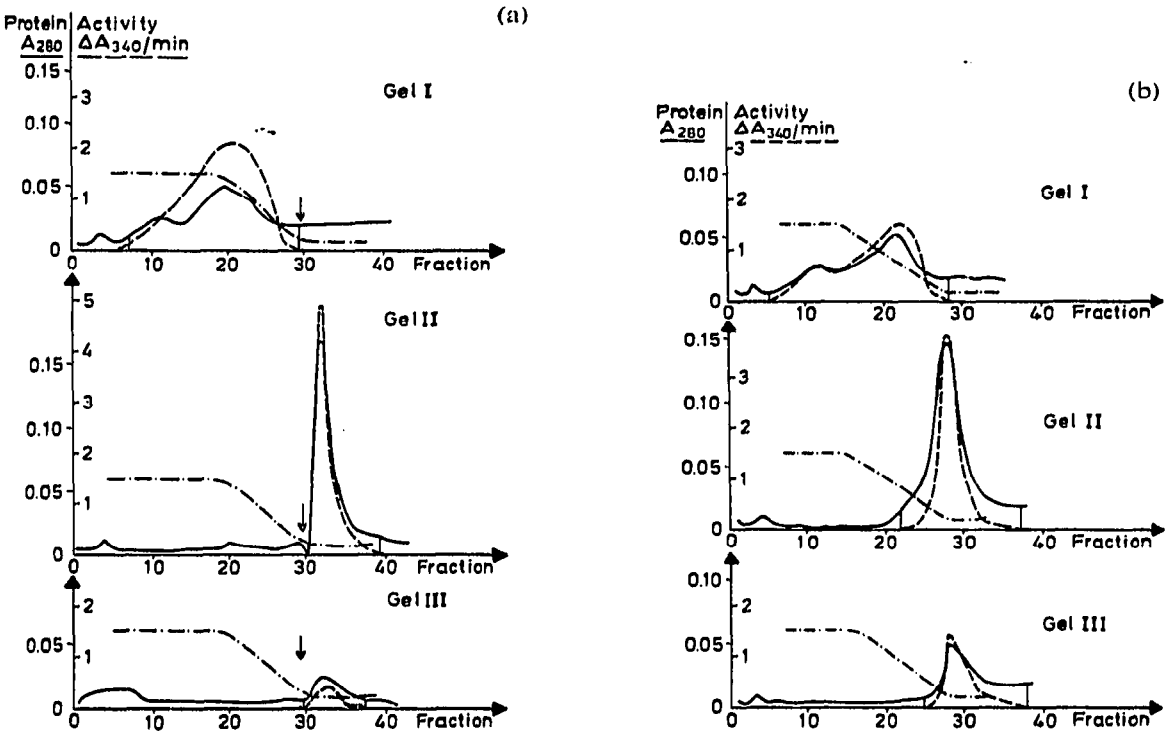


Fig. 4. Chromatography of LDH. The buffers used were 0.05 *M* sodium phosphate (pH 6.8) containing (A) 1 *M* sodium chloride, (B) no additions, and (C) 50% of ethylene glycol. High concentrations of sodium chloride affected activity; 1 *M* sodium chloride or 50% of ethylene glycol, however, had no effect. In Fig. 3a, the sample was 0.385 ml of LDH in 1 ml of buffer A, and a gradient from buffer A to buffer B was used, followed by a front of buffer C. The results are set out in Table IV under a. In Fig. 3b, the sample was 0.406 mg of LDH in 1 ml of buffer A, and a gradient from buffer A to buffer C was used. The results are set out in Table IV under b.

TABLE IV

CHROMATOGRAPHY OF LDH

	Sample	*Yield as percentage of*		*Specific activity (units/mg)*
		*protein**	*initial activity*	
a	Starting material	100	100	25
	After gel I	90	70	19
	After gel II	120	50	11
	After gel III	50	10	5
b	Starting material	100	100	24
	After gel I	100	50	20
	After gel II	100	50	13
	After gel III	60	10	8

* Based on a value of 14.9 for $A_{1cm}^{1\%}$

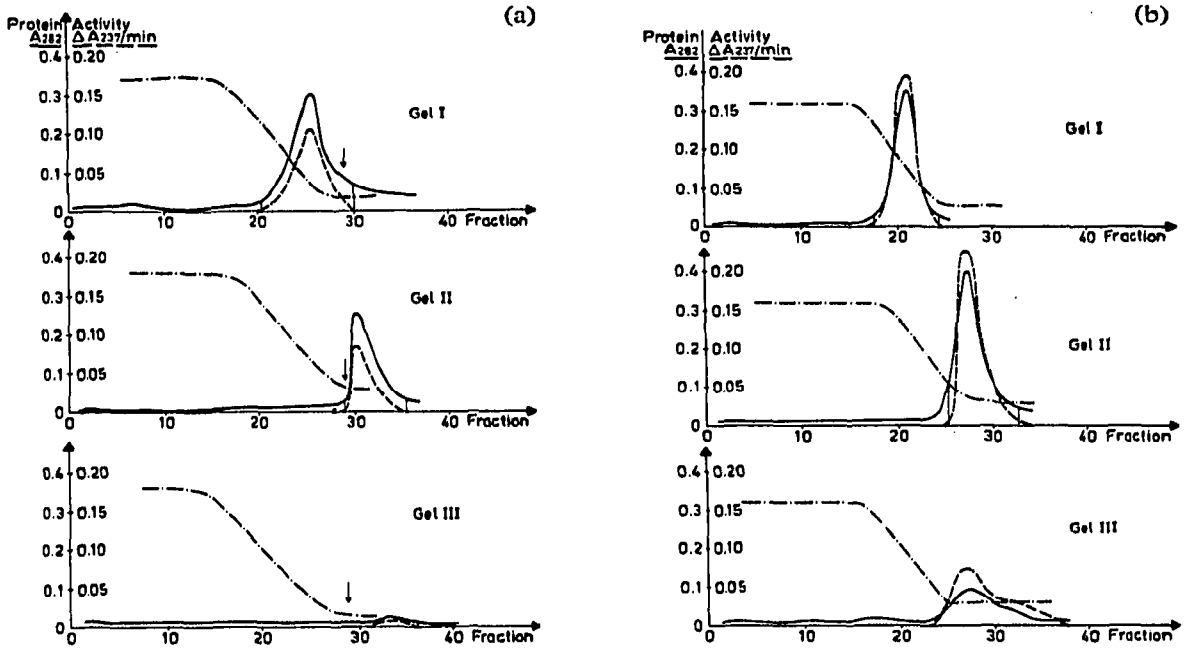


Fig. 5. Chromatography of α-chymotrypsin. The buffers used were 0.1 *M* sodium citrate (pH 3.0) containing (A) 3 *M* sodium chloride, (B) no additions, and (C) 50% of ethylene glycol. In Fig. 5a, the sample was 1 mg of enzyme in 1 ml of buffer A, and a gradient from buffer A to buffer B was used, followed by a front of buffer C. The results are set out in Table V under a. In Fig. 5b, the sample was 1.035 mg of enzyme in 1 ml of buffer A and a gradient from buffer A to buffer C was used. The results are set out in Table V under b.

of the material) was eluted with $K_{av} \approx 0.3$ and contained all the activity, whereas the rest was eluted with the void volume. The original LDH sample gave only one peak (with all the activity), which was eluted at exactly the same place as the active peak of the material chromatographed on the benzylated gels.

Trypsin and α-chymotrypsin. As with dextranase and α-amylase, very similar chromatographic results were obtained with these enzymes (see Figs. 5 and 6 and Tables V and VI). Remarkably low yields and severe losses in specific activity were obtained with the sodium chloride gradient (Tables 5a and 6a). Neither of these enzymes exhibited any activity in the adsorbed state.

DISCUSSION

Gels

The cross-linking reaction with 2,3-dibromopropan-1-ol is supposed to proceed according to the following scheme:

(1) Transformation of dibromopropanol to the more reactive epibromohydrin:

$$Br\text{-}CH_2\text{-}CHBr\text{-}CH_2OH + NaOH \rightarrow \underset{\diagdown\,O\,\diagup}{CH_2\text{-}CH\text{-}CH_2Br} + H_2O + NaBr$$

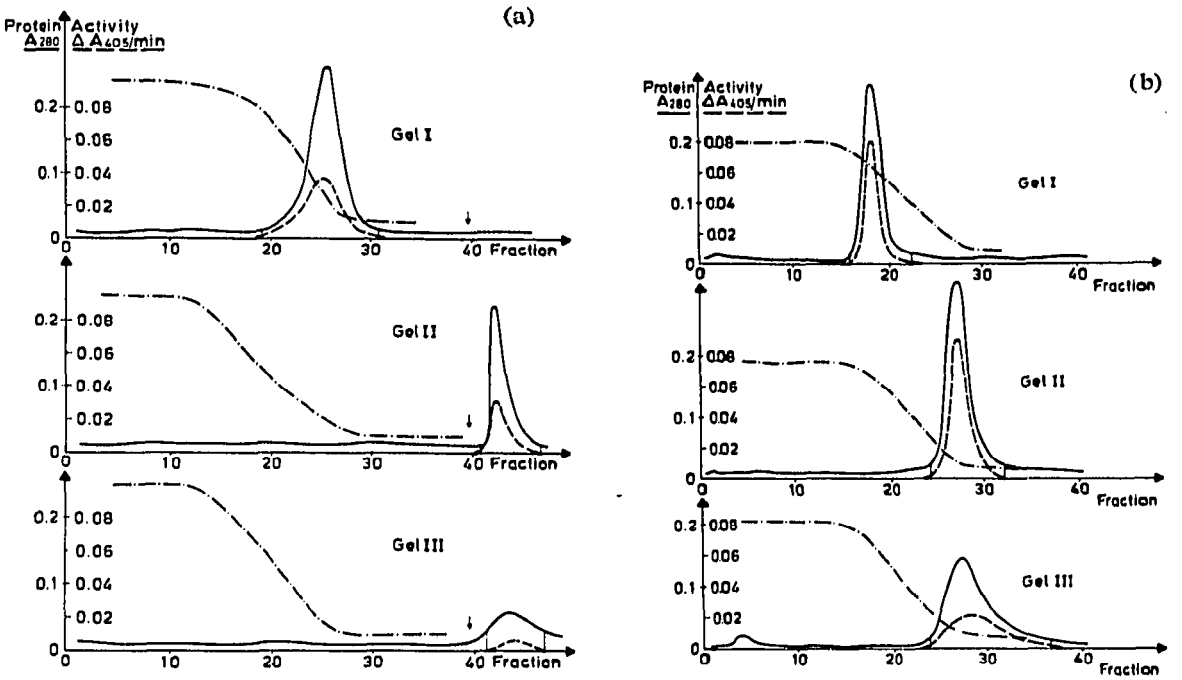


Fig. 6. Chromatography of trypsin. The buffers used were the same as in the experiment with α-chymotrypsin. In Fig. 6a, the sample was 1.13 mg of enzyme in 1 ml of buffer A, and a gradient from buffer A to buffer B was used. The results are set out in Table VI under a. In Fig. 6b, the sample was 1.07 mg of enzyme in 1 ml of buffer A, and a gradient from buffer A to buffer C was used. The results are set out in Table VI under b.

TABLE V

CHROMATOGRAPHY OF α-CHYMOTRYPSIN

	Sample	*Yield as percentage of*		*Specific activity (units/mg)*
		*protein**	*initial activity*	
a	Starting material	100	100	970
	After gel I	40	40	1100
	After gel II	28	20	790
	After gel III	8	6	790
b	Starting material	100	100	970
	After gel I	84	55	640
	After gel II	79	55	670
	After gel III	43	41	910

* Based on a value of 20.5 for $A_{1cm}^{1\%}$

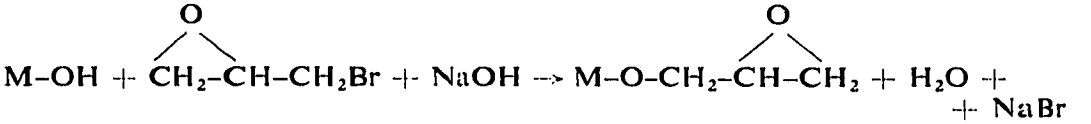
TABLE VI

CHROMATOGRAPHY OF TRYPSIN

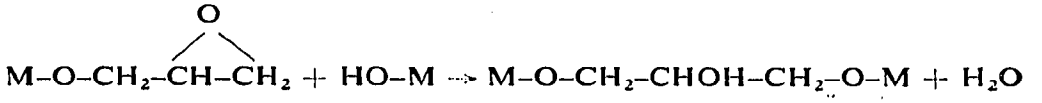
	Sample	*Yield as percentage of*		*Specific activity (units/mg)*
		protein	*initial activity*	
a	Starting material	100	100	0.90
	After gel I	34	42	1.13
	After gel II	18	6	0.30
	After gel III	15	2	0.17
b	Starting material	100	100	1.02
	After gel I	64	49	0.78
	After gel II	81	24	0.31
	After gel III	82	25	0.31

* Based on a value of 14.4 for $A^{1\%}_{1cm}$

(2) Reaction of epibromohydrin with an agarose hydroxyl group:

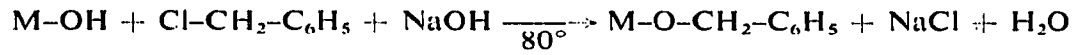


(3) Reaction with another hydroxyl group to give a cross-link:



where M denotes the agarose matrix.

The covalently cross-linked agarose gels are extremely stable[24] and can withstand drastic treatments (strong acids excluded) and high temperatures. This makes them very suitable for the facile preparation of hydrophobic derivatives from the corresponding aryl or alkyl halides. By this reaction, there is no risk of introducing charged groups into the gel. Apart from the molecular-sieving effect, the gel–solute interaction will therefore in general be of a purely hydrophobic nature. The benzylation reaction proceeds according to the following scheme:



Owing to decrease in hydrophilicity, the hydrophobically substituted gels will shrink in water when the D.S. exceeds a certain value (see Table I). Depending on the special structure of the agarose gel, the shrinkage is much less than for such synthetic gels as Sephadex or polyacrylamide. However, shrinkage must be considered when choosing the most suitable gel. In this study, the most porous agarose available was chosen in order to avoid any interference from molecular-sieving effects in the chromatographic experiments. In other instances, advantage might be taken of this effect, and then a gel with smaller pore size should be used.

Hydrophobic interaction

Generally, hydrophobic interaction is decreased by lower ionic strength and lower polarity of the solvent. Ethylene glycol has been used as polarity-reducing agent, as, in concentrations of up to about 50%, it is considered to be harmless to enzymes[31]; this has been confirmed by our experience. In addition, any change in medium that promotes a configurational change in a protein molecule will also change the hydrophobic interaction. This fact has been used in the purification of glycogen phosphorylase[3]. It is therefore not to be expected that molecules as complex as proteins will always obey these simplified rules for hydrophobic interaction; the enzymes used in this study, however, seemed to do so.

Desorption technique

In this study, desorption was performed with two types of gradients: one was a pure salt gradient (the "a"; series of Figures and Tables), and the other was a mixed gradient with high desorption power from buffer solution with a high salt concentration to buffer without salt but containing 50% of ethylene glycol (the "b" series). Although the first of these gradients was very steep in comparison with those normally used in ion-exchange chromatography, the eluted peaks were often strongly diluted. This reflects the fact that very high concentrations of salts are necessary to change the water structure in the medium and hence the hydrophobic interaction[32]. Equilibrium for the hydrophobic interaction is attained only slowly, and more favourable results would probably be obtained at a lower flow-rate.

Chromatographic results

Salting-out chromatography worked well for the hydrolytic enzymes dextran and α-amylase. Both enzymes were eluted in high yield, and, with proper desorbing procedures, high degrees of purification were achieved.

Since LDH adsorbed to the gels was highly active, it is probably during the desorption process that loss of activity occurs. Considering the data from chromatography on Sephadex G-200, it seems reasonable to presume that the desorption process causes a conformational change that exposes hydrophobic groups, which, in turn, cause aggregation of the molecules. Other enzymes also retain their activity after adsorption to amphophilic gels[10,16,33]. This seems to be a very promising field, since gels can be prepared with very high adsorption capacities. If (or when) the activity of an enzyme so bound has decreased, it can easily be regenerated simply by adsorbing more enzyme. Further, if cross-linked gels are used as the matrix, they can be efficiently cleaned by media such as strongly denaturing agents or hot alakli.

Trypsin and chymotrypsin also exhibited loss of activity during the chromatographic procedure. This can hardly depend on autolysis since the experiments were performed in the cold and at pH 3, at which the enzymes are almost completely inactive. Neither would autolysis show any differences between the different gel types. The decrease in specific activity might be due to the same mechanism as was suggested for LDH, but the poor total yield of both protein and activity is most likely to have another explanation. Even agarose of the highest quality available will contain a substantial amount of sulphate ester groups[24,34]. Some of these are resistant to alkali and are not hydrolyzed during the substitution reaction[35]; thus, 1 ml of benzylated Sepharose 2B will contain about 0.1 μmole of titratable acidic groups, which corre-

sponds to about 2.5 mg of trypsin or α-chymotrypsin, assuming equimolar interaction. Also, the higher the D.S. the more the gel has shrunk and the higher is the concentration of charged groups. Because both these enzymes have high isoelectric points and thus are positively charged, and the polarity-reducing substance increases the strength of the electrostatic forces, even small amounts of charged groups can have tremendous effects. In fact, it was noticed for other positively charged proteins (chymotrypsinogen A and lysozyme) that 50% of ethylene glycol in the buffer (0.05 *M* Tris hydrochloride of pH 8.0) caused elution of only 10–15% of the applied material, whereas 50% of ethylene glycol together with 1 *M* sodium chloride in the buffer eluted 80–90% of the material when 1 mg of protein was applied to 1 ml of benzylated gel. At low ionic strength, protein may be adsorbed by ionic interactions, whereas at high ionic strength (where these interactions are minimized), hydrophobic interactions will be strongest. There might therefore be no effective desorbing agent, since the two effects complement each other.

The lack of activity in the adsorbed state for the proteolytic enzymes indicates that the active site is occupied by the benzyl groups. With regard to α-chymotrypsin, this is easily understood on the basis of its specificity, but for trypsin this result was more unexpected.

It can be questioned whether chromatography of α-chymotrypsin on the benzylated Sepharose should be considered an example of bio-specific adsorption chromatography or salting-out chromatography. This is a question of which effect predominates, the specific interaction between the benzyl groups and hydrophobic pockets at the active sites of the enzymes or the less specific salting-out effect towards the gel. Obviously, both effects contribute in this instance.

The merit of this chromatographic method is without any doubt, as it has already been used with success for many separations[1–21]. A thorough understanding of the limitations and risks will promote a sound basis for further development and efficient use of amphophilic gels for hydrophobic salting-out chromatography and adsorption.

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