Journal of Chromatography, 209 (1981) 363–368
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,643

OPTIMAL CONDITIONS FOR THE COUPLING OF AROMATIC AMINES TO EPOXY-ACTIVATED SEPHAROSE 6B

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(Received January 12th, 1981)

SUMMARY

The influence of pH, temperature, ligand concentration and reaction time on the coupling of aniline to epoxy-activated Sepharose 6B was studied. With the optimal temperature, ligand concentration and reaction time for the coupling of aniline, the influence of pH was investigated for the coupling of two aliphatic amines, *viz.*, L-leucine and 4-aminobutyric acid, to this gel.

INTRODUCTION

Epoxy-activated agarose is a convenient starting material for the preparation of affinity gels. If a bisoxirane such as 1,4-butanediol diglycidyl ether is used in its synthesis, two desirable properties are obtained: the agarose chains are cross-linked to give a stable gel and the reactive oxirane groups are attached to the matrix by long, non-charged, hydrophilic spacers.

Epoxy-activated agarose has been shown to couple with ligands containing amine[1–4], hydroxyl[5] or thiol[6] groups. However, the reaction conditions required for maximum coupling yields with these groups have not been studied in great detail. Only one systematic study[1], involving coupling with the α-amino group of glycylleucine, has been published. It was shown that a high pH (11) and a high temperature (50°C) are necessary in order to obtain high coupling yields in this instance. Since that study was published, most workers appear to have used these or even harsher conditions, irrespective of the nature of the functional group to be reacted with the oxirane groups on the gel and in spite of the fact that the stability of oxirane groups towards hydrolysis is diminished at high pH and high temperature[1,7].

The coupling reaction with amine groups is assumed to involve the unprotonated amine, which explains the relatively high pH required for aliphatic amine groups. However, aromatic amine groups, having a much lower basicity, might couple at lower pH. This might have the advantage of giving higher coupling yields as a result of the increased stability of the oxirane groups. Moreover, conditions might be found under which a ligand carrying both an aromatic and an aliphatic amine group is specifically coupled through the aromatic amine group. Finally, coupling at

0021-9673/81/0000–0000/$02.50

lower pH is, of course, indicated in those instances where the ligand is unstable at the high pH normally used.

In this paper, a study of the optimal conditions for the coupling of the simplest aromatic amine, *viz.*, aniline, to epoxy-activated Sepharose 6B (ES) in aqueous buffers is described. The influence of four variables, *viz.*, pH, temperature, ligand concentration and reaction time, was studied. The experimental strategy of a four-factor factorial design[8], followed by the method of steepest ascent[8], was chosen. In order to investigate the possibility of selective coupling through aromatic amine groups, coupling experiments with ligands containing aliphatic amine groups, *viz.*, L-leucine and 4-aminobutyric acid, were also performed. For convenience in the measurement of coupling yields, ^{14}C-labelled ligands were used.

EXPERIMENTAL

Aliquots of 0.5 g of epoxy-activated Sepharose 6B (Pharmacia, Uppsala, Sweden) were swollen on a glass filter and washed with distilled water and coupling buffer (0.2 *M* phosphate or 0.05 *M* borate), respectively. The filter-dried material was transferred into a reaction vial and 4 ml of a solution containing the ^{14}C-labelled ligand in coupling buffer was added.

Aniline, L-leucine and 4-aminobutyric acid (Merck, Darmstadt, G.F.R.) were used as ligands; they were labelled with the corresponding ^{14}C-compounds (Radiochemical Centre, Amersham, Great Britain) to a specific activity of about 0.8 μCi/mmole.

After incubation on a test-tube rotator for a given period of time at a given temperature, the gel was filtered off and washed successively with coupling buffer, distilled water, 0.5 *M* sodium hydrogen carbonate + 1 *M* sodium chloride (pH 8), distilled water, 0.1 *M* sodium acetate + 1 *M* sodium chloride (pH 4) and distilled water; three 10-ml portions of each solution were used.

The filter-dried gel was subsequently transferred into a counting vial, 10 ml of Aquasol (NEN, Boston, MA, U.S.A.) were added and the counting rate was measured in a scintillation counter (BF 5020, Berthold, Wildbad, G.F.R.); 10^5 counts were recorded for each gel and counting rates were corrected for the background.

These corrected counting rates (cps/0.5 g ES), pertaining to a heterogeneous counting system, were transformed into coupling yields (μequiv. ligand/g ES) by multiplication with a factor $2f$, which was separately determined by the following experiments. A 1-g amount of epoxy-activated Sepharose 6B, to which an unknown amount of labelled ligand was coupled, was divided into two equal parts. One part was directly counted as described above (corrected counting rate, C_1) and the other was dissolved by heating under reflux at 100°C in a total volume of 4 ml of 0.5 *M* hydrochloric acid. A 400-μl volume of the digest and 40 μl of water were added to 10 ml of Aquasol and counted (C_2). A 40-μl volume of the stock solution of known concentration c (μequiv./ml) of labelled ligand was added to 400 μl of a digest of 0.5 g of epoxy-activated Sepharose 6B in a total volume of 4 ml of 0.5 *M* hydrochloric acid, 10 ml of Aquasol were added and the sample was counted (C_3). From these experiments f can be found:

$$f = \frac{0.4\, C_2}{C_1\, C_3} \cdot c$$

The oxirane content of the gel was determined by the method described by Sundberg and Porath[1].

RESULTS AND DISCUSSION

In the initial coupling experiments with aniline, the conditions were chosen according to a four-factor factorial design[8]. The factor levels were as follows: pH, 5.8 and 8.1; temperature, 25 and 40°C; ligand concentration, 31.25 and 62.50 m*M* (corresponding to a 2.5- and 5-fold excess with respect to oxirane groups, respectively*); and reaction time, 17 and 25 h. All experiments were performed in duplicate. The results are given in Table I.

TABLE I

COUPLING YIELDS (μequiv./g) OF ANILINE TO EPOXY-ACTIVATED SEPHAROSE 6B IN 0.2 *M* PHOSPHATE BUFFERS

32 experiments in a 2^4 factorial design, with duplication.

pH	*Reaction time (h)*	*Temperature (°C)*			
		25		*40*	
		Ligand concentration (mM)			
		31.25	*62.50*	*31.25*	*62.50*
5.8	17	26.4, 18.4	41.2, 41.9	41.6, 45.0	54.0, 65.0
	25	28.5, 24.1	39.9, 51.4	41.3, 32.7	54.3, 57.1
8.1	17	25.5, 25.9	53.1, 51.2	49.2, 42.1	59.4, 60.8
	25	23.5, 22.9	41.7, 42.1	44.2, 37.5	47.8, 44.2

The data in Table I were submitted to a four-factor analysis of variance, the results of which are given in Table II.

From the results in Tables I and II one can draw the following conclusions, which hold for coupling conditions confined to the four-dimensional factor space, limited by the pre-set factor levels:

(i) the experimental error in the coupling yield is

$$\pm\sqrt{19.11} = \pm 4.4 \ \mu\text{equiv./g ES;}$$

(ii) the pH has no significant influence on the coupling yield;

(iii) the reaction temperature and the concentration of the ligand have a strong influence on the coupling yield and their influence is interdependent; the coupling yield increases with increasing temperature and increasing concentration; and

(iv) the reaction time has a smaller but significant influence on the coupling

* We found the oxirane content of the gel to be higher than that stated by the manufacturer[7] (100 ± 2 instead of 50–70 μequiv./g ES).

TABLE II

RESULTS OF THE ANALYSIS OF VARIANCE OF THE DATA IN TABLE I

s^2 = Estimate of variance (= sum of squares/degrees of freedom); s_w^2 = estimate of variance within duplicate experiments, *i.e.*, estimate of experimental variance (= 272.48/16 = 17.03); $^*s_w^2$ = better estimate of experimental variance: as the three- and four-factor interactions are not significant [$F^{5\%}(1.16) = 4.49$], their variance estimates are pooled with that within duplicates, giving $^*s_w^2 = 401.24/21 = 19.11$. As $F^{5\%}(1.21) = 4.32$, the factors indicated with arrows in the last column are significant.

Factor	*Sum of squares*	*Degrees of freedom*	$F = \frac{s^2}{s_w^2}$	$^*F = \frac{s^2}{^*s_w^2}$
A = pH	2.15	1	0.13	0.11
B = temp.	1491.94	1	87.61	78.07←
C = conc.	2385.68	1	140.09	124.84←
D = time	142.38	1	8.36	7.45←
AB	12.38	1	0.73	0.65
AC	9.35	1	0.55	0.49
AD	109.15	1	6.41	5.71←
BC	106.22	1	6.24	5.56←
BD	73.51	1	4.32	3.85
CD	25.74	1	1.51	1.35
ABC	60.22	1	3.54	
ABD	17.85	1	1.05	
ACD	48.76	1	2.86	
BCD	0.07	1	0.00	
$ABCD$	1.86	1	0.11	
Between duplicates	4487.26	15		
Within duplicates	272.48	16		
Total	4759.74	31		

yield; increasing reaction times giving slightly decreasing coupling yields. Moreover, this effect of reaction time is significantly greater at higher pH.

Based on these conclusions, further coupling experiments with aniline were performed at pH 7.0 and at varying levels of the remaining three variables. The factor levels for the successive experiments were chosen according to the method of steepest ascent[8]. In Fig. 1 these levels are indicated on the abscissa; the ordinate gives the

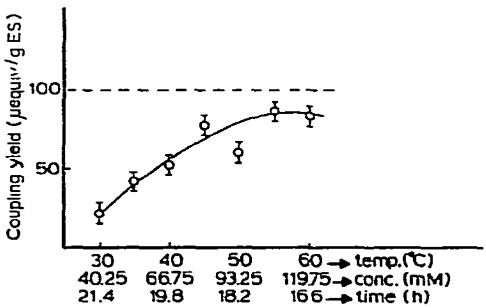

Fig. 1. Coupling yield of aniline to epoxy-activated Sepharose 6B in 0.2 M phosphate buffer (pH 7.0) under varying conditions, according to the method of steepest ascent. Broken line, oxirane content of the gel.

coupling yields found experimentally. Also indicated in Fig. 1 is the maximum coupling yield, as given by the oxirane content of the gel.

From the results in Fig. 1 it was decided to perform further experiments at the near-optimal temperature (55°C), ligand concentration (106.5 m*M*, corresponding to an 8.5-fold excess with respect to oxirane groups) and reaction time (17 h). These experiments were performed with aniline, L-leucine and 4-aminobutyric acid at varying pH with the aim of examining whether the coupling yield of aniline is indeed lower at higher pH (as a result of increasing hydrolysis of oxirane groups) and to investigate if at pH $\approx$ 7 the reaction conditions are such that aromatic amine groups are selectively coupled. The results of these experiments are shown in Fig. 2.

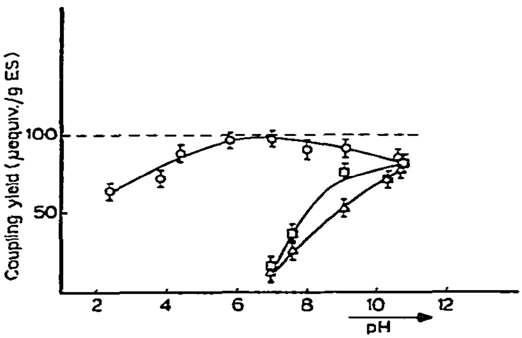

Fig. 2. Coupling yield of aniline (○), L-leucine (□) and 4-aminobutyric acid (△) to epoxy-activated Sepharose 6B in 0.2 *M* phosphate buffers (at pH $\leqslant$ 7.0) or 0.05 *M* borate buffers (at pH > 7). Reaction conditions: temperature, 55°C; ligand concentration, 106.5 m*M*; and reaction time, 17 h. Broken line, oxirane content of the gel.

It can be seen that at pH 6–7 the reaction of aniline with oxirane groups on the gel proceeds virtually to completion, whereas the aliphatic amines are not coupled at all at pH 6.5. Hence, for a ligand containing both amine functions, selective and 100% attachment through the aromatic amine group can be obtained by coupling at pH 6.5. At pH 7–10.5, the coupling yield at constant pH increases in the order 4-aminobutyric acid < L-leucine < aniline, in accordance with the decrease in the basicity of the corresponding amine groups (p*K* values 10.6, 9.7 and 4.6, respectively).

Coupling of aniline at pH 10.6 results in a reduction of about 20% in the coupling yield. It is probable that this reduction stems from a property of the gel, *i.e.*, increased hydrolysis of oxirane groups, as all three curves in Fig. 2 converge to about the same coupling yield with increasing pH.

Finally, we investigated if coupling of aniline at high pH (10.6) but at lower temperatures would result in higher coupling yields (the amount of hydrolysis of oxirane groups is known to increase with increasing pH and temperature). The results are shown in Fig. 3, and indicate that the reaction temperature has no significant influence on the coupling yield of aniline at pH 10.6. Thus, under these conditions (ligand concentration 8.5-fold excess, reaction time 17 h) the high pH (10.6) and not the high temperature (55°C) is primarily responsible for the 20% reduction in the coupling yield shown in Fig. 2.

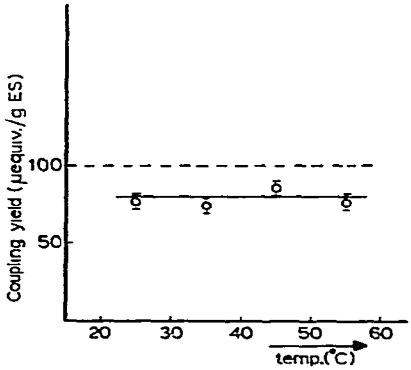

Fig. 3. Coupling yield of aniline to epoxy-activated Sepharose 6B in 0.05 *M* borate buffer (pH 10.6) at varying temperature. Reaction conditions: ligand concentration, 106.5 m*M*; and reaction time, 17 h. Broken line, oxirane content of the gel.

CONCLUSIONS

Aniline is coupled in 100% yield to epoxy-activated Sepharose 6B at pH 6–7 by performing the reaction at 55°C for 17 h using an 8.5-fold excess ligand concentration. Presumably the same holds for other ligands containing aromatic amine groups at a pH about 2 units higher than the corresponding p*K* value.

Under these optimal conditions for the coupling of aniline, aliphatic amine groups do not react. This allows the selective attachment through aromatic amine groups of a ligand containing both functions.

In coupling reactions performed at pH 10.6 with an 8.5-fold excess ligand concentration for 17 h and at temperatures between 25 and 55°C, only about 80% of the oxirane groups are substituted; there is a 20% loss due to hydrolysis of the oxirane groups.

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