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Immobilization of cholinesterase in cross-linked polyacrylamide

Trapping in the gel matrix of cross-linked polyacrylamide was first introduced by BERNFELD[1] as a method for immobilization of enzymes. The method has since been used for the preparation of insoluble derivatives of a number of enzymes[2-5] and antigens[6]. Cross-linked polyacrylamide is also being used as a medium for gel chromatography[7-9] and gel electrophoresis[10]. It has been realized that the mobility of macromolecules in this medium is markedly affected by changes in the composition (concentration and degree of cross-linking) of the polymer gel. These effects have been interpreted in terms of differences in gel porosity caused by such composition changes. Since the trapping of proteins in polyacrylamide is directly related to the non-permability of protein molecules through the polymer network, it was of interest to examine the effects of changes in the composition of the polymerization mixture on the encagement efficiency, and to compare these effects with those encountered in gel chromatography and gel electrophoresis. This report describes gel-composition effects on the trapping of butyrylcholinesterase (EC 3.1.1.8) in polyacrylamide.

The trapping was performed by copolymerization of a mixture (500 mg) of acrylamide and *N,N*-methylenebisacrylamide (Koch-Light Laboratories) of varying composition* dissolved in de-aerated veronal buffer 0.02 M of pH 7.4, in the presence of the enzyme (10 mg horse serum butyrylcholinesterase from Worthington Biochemicals; activity, 5.1 units/mg**).

The polymerization was initiated by a redox system composed of 3-dimethylaminopropionitrile (25 mg) and ammonium persulfate (15 mg). The reaction-mixture temperature was kept at 25° (water bath). After polymerization was complete (about 30 min), the stiff gel was mechanically dispersed using a homogenizer. The resulting suspension was washed with 500 ml of veronal buffer by repeated centrifugations, then washed with water and finally dried to a white powder by lyophilization. Enzymatic activity was determined by pH-static measurement of the rate of hydrolysis of acetylcholine perchlorate at pH 7.4, 25°. Trapped protein was determined by hydrolyzing the insoluble conjugate in 6 M HCl for 22 h at 110°, followed by amino acid analysis (with a Beckman automatic amino acid analyzer, Model 120 C) of the purified hydrolysate.

Table I shows the effect of cross-linking (C) on the yield of entrapped activity in the polymer. It is seen that maximum trapping of activity was achieved at $C = 5\%$. On any further increase of C the trapping efficiency greatly diminished. Table II shows the effect of total monomer concentration (T) on the yield of enzyme trapping. Immobilized activity reached a maximum at $T = 15\%$, whereas the yield of protein trapping continued to increase with increases in gel concentration.

These results can be interpreted in the light of the work of FAWCETT AND MORRIS[9] who studied the chromatography of proteins on cross-linked polyacrylamide

* For designation of the polymerization mixture composition the notation of HJERTEN[7] is used, in which T denotes the total weight of monomers (acrylamide *plus* N,N-methylenebisacrylamide) per 100 ml of solvent and C denotes the amount of cross-linking agent (N,N-methylenebisacrylamide)expressed as a percentage of the total amount of monomers.

** A unit denotes an activity unit, defined as the amount of enzyme which hydrolyzes 1 μmole of acetylcholine per min at pH 7.4 and 25°.

TABLE I

EFFECT OF DEGREE OF CROSS-LINKING ON YIELD OF ENZYME TRAPPING

Polymerizations were carried out in 3.3-ml solutions of $T = 15\%$, containing 10 mg enzyme, in 0.02 M veronal buffer, pH 7.4.

Degree of cross-linking, C (%)	*Activity of conjugate (units/ 100 mg)*	*Yield of activity trapping (%)*	*Recovery of nontrapped activity (%)*
1	1.0	8	10
5	5.4	56	3
7.5	3.7	32	9
20	0.7	6	35

and applied it to the theory of gel chromatography set forth by OGSTON[11] and LAURENT AND KILLANDER[12]. According to this theory, the swollen gel is considered to be a three-dimensional network of statistically distributed rigid fibers of thickness $2r$ and concentration L. The equations of the theory relate the chromatographic partition coefficient for a spherical solute molecule to r and L, which are, in turn, related to the mean effective pore radius p of the gel. By fitting their experimental chromatographic data from a series of polyacrylamide gel compositions to these equations, FAWCETT AND MORRIS[9] showed that at constant monomer concentration p is minimal at 5% cross-linking. At higher degrees of cross-linking there is an increase in gel permeability, accompanied by a sharp decrease in L and an increase in r. This unexpected phenomenon was attributed by the authors to an accumulation of fibers into thick strands, resulting in the widening of spaces between the strands. Our results, which show that enzyme trapping is maximal at $C = 5\%$ (Table I), suggest that matrix permeability is indeed minimal at this degree of cross-linking, in complete agreement with the chromatographic findings of FAWCETT AND MORRIS[9]. A similar effect of the parameter C on gel porosity has recently been observed in gel electrophoresis[13], where the migration velocity was found to be minimal in gels of $C = 5\%$.

At constant C, the yield of protein trapping increases with increases of monomer

TABLE II

EFFECT OF TOTAL MONOMER CONCENTRATION ON YIELD OF ENZYME TRAPPING

The degree of cross-linking (C) was 5% in all polymerizations. Reaction mixtures contained 10 mg of enzyme per 500 mg of total monomer, dissolved in the appropriate volume of 0.02 M veronal buffer, pH 7.4.

Total monomer concentration (%)	*Activity of conjugate (units/ 100 mg)*	*Yield of protein trapping (%)*	*Yield of activity trapping (%)*	*Recovery of nontrapped activity (%)*
5	0.8	7	6	53
7.7	1.0	9	7	77
10	2.3	7	18	72
15	5.4	31	56	3
20	2.3	32	19	1
25	<0.1	51	<0.1	<0.1

TABLE III

INACTIVATION OF CHOLINESTERASE IN ACRYLAMIDE SOLUTIONS

Acrylamide was dissolved in 0.02 M veronal buffer, pH 7.4. The enzyme was incubated in these solutions for 30 min at 25°. Residual activities were measured in solutions diluted 20-fold with buffer and did not change for at least 2 h after dilution.

Acrylamide concentration		*Residual activity*
%	*Molarity*	*(%)*
0	0	100
5	0.7	100
10	1.4	80
15	2.1	53
20	2.8	0.5

concentration T (Table II), as expected by the decrease in gel porosity accompanying increases in gel concentration[9]. The sharp drop in the yield of trapped enzymatic activity at monomer concentrations higher than 15%, paralleled by a similar decrease in the recovery of nontrapped activity, is probably due to an inactivation occurring in the polymerization mixture. This is supported by the results of Table III which show the gradual inactivation of cholinesterase by acrylamide solutions of increasing concentration. Acrylamide apparently acts as a denaturating agent, in much the same manner as similar amidic compounds (*e.g.* urea).

It may thus be concluded that although the effects of composition parameters on the porosity of cross-linked polyacrylamide, as observed by protein trapping, are similar to those encountered in gel chromatography and gel electrophoresis, other effects of acrylamide monomers on enzyme activity must be taken into consideration in preparing enzymes immobilized in the gel matrix.

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Department of Biophysics, Y. DEGANI
The Weizmann Institute of Science, Rehovot (Israel) T. MIRON

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