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Insoluble matrix-supported apyrase, deoxyribonuclease and cholinesterase

Matrix support of physiologically active proteins has proven useful as a method of study of environmental and conformational effects upon enzymes[1–5], and as well as a technique for possible practical application to larger-scale substrate conversion systems[6,7]. In the present investigation, polymeric matrices were used for the support of apyrase (EC 3.6.1.5), deoxyribonuclease (EC 3.1.4.5) and cholinesterase (EC 3.1.1.8).

Polymers used were CM-cellulose (Bio-Rad, Richmond, Calif.), the copolymer of L-alanine and L-glutamic acid (10:1) (Pilot Chem. Co., Watertown, Mass.), poly aspartic acid, poly galacturonic acid (Sigma Co., St. Louis, Mo.), Elvacite 2008 (methyl methacrylate; Dupont Corp., Wilmington, Del.) and ethylene–maleic acid copolymer (EMA 21; Monsanto, St. Louis, Mo.).

Acetylcholinesterase and apyrase were bonded to each of the polymers listed above (except EMA) as follows[8]: The polymer (50 mg) and *N*-ethyl-5-phenylisoxazolium 3'-sulfonate (50 mg, Aldrich Chem., Milwaukee, Wisc.) were stirred in 5 ml of deionized water for 1 h at 5°. Centrifugation of the reaction mixture provided a pellet which was the activated polymer. This was then resuspended in 5 ml of deionized water. 10 mg of the enzyme were then added, and the reaction mixture stirred for 24 h. Preparations were washed twice with water containing 0.5 M sodium citrate and resuspended in 2 ml of deionized water. (The copolymer of L-alanine and L-glutamic acid was preswollen for 24 h in acetonitrile to increase its wettability).

Deoxyribonuclease was covalently bonded to polymeric supports by reaction with azo groups and also by the technique involving *N*-ethyl-5-phenylisoxazolium 3'-sulfonate[8]. CM-cellulose, poly galacturonic acid, poly alanine–glutamic acid and ethylene–maleic acid copolymer were used as matrices for this enzyme.

CM-cellulose (Bio-Rad Labs) was converted into its azide by the method of MICHEEL AND EWERS[9] as modified by MITZ AND SUMMARIA[10]. CM-cellulose was first esterified with methanol and HCl. This was followed by treatment of the ester with hydrazine. The hydrazide obtained was filtered, washed several times with water, and then with methanol. It was then lyophilized. 1 g of the hydrazide was suspended in 150 ml of 2% HCl acid. The solution was cooled to 0–5°. Nine ml of 3% $NaNO_2$ were added and the mixture stirred for 20 min, then allowed to stand for 30 min. The insoluble azide was filtered, then washed to neutrality with water and then with diethyl ether. The preparation was then lyophilized. 100 mg of cellulose azide and 20 mg of deoxyribonuclease were stirred for 4 h at 0.5° at this time. The reaction mixture was centrifuged and pellet was washed twice with water containing 0.5 M NaCl. This final pellet was suspended in 5 ml of deionized water and deoxyribonuclease activity was determined.

100 mg of maleic anhydride–ethylene copolymer (EMA 21) was suspended in 5 ml of deionized water and 0.5 ml of 1% hexamethylene diamine cross-linking reagent was added according to the method of LEVIN *et al.*[11]. This mixture was stirred for 3 min and then 20 mg of deoxyribonuclease in 5 ml of water was added; the reaction mixture was stirred overnight (16 h) at 4°. The precipitate was centrifuged and washed with water containing 0.5 M NaCl. The washed precipitate was suspended in 10 ml of water and deoxyribonuclease activity was determined.

Catalytic activity of the matrix-supported apyrase was measured in a reaction mixture containing 0.1 ml matrix-supported enzyme, 0.8 ml substrate solution (disodium ATP in 0.1 M Tris buffer pH 7.2) and 0.1 ml H_2O. After incubation for 1 h, reaction was stopped by addition of 0.1 ml of 50% trichloroacetic acid. After centrifugation, the inorganic phosphate was measured by the method of FISKE AND SUBBAROW[12]. Protein content of the enzyme-matrix solution was determined by the phenol method[13] with correction being made for the reaction given by the matrix. pH optima and K_m values were obtained in the presence of 10^{-2} M Ca^{2+}. In Elvacite and polyaspartic acid preparations, uniform pipetting was obtained by continuous stirring.

Determination of acetylcholinesterase activity was accomplished by the method of HESTRIN[14] using nitrophenol as indicator and acetylcholine chloride as substrate.

Determination of deoxyribonuclease assay was by measurement of hyperchromicity changes[15]. 1 mg/ml DNA (Sigma) was dissolved together with 0.01 M $MgSO_4$ in 0.5 M sodium acetate buffer (pH 5.5) to serve as the substrate solution. 1.0 ml of enzyme suspension in buffer and buffer alone served as blanks. An aliquot containing 1.8 ml of buffer, 0.2 ml of substrate (200 μgm DNA) and 1.0 ml enzyme was read at 1-min intervals for 10 min (A at 260 mμ). Supported material suspensions were incubated and centrifuged. Absorbance was then measured in the supernatant after 10 min incubation.

TABLE I

CHARACTERISTICS OF POLYMER-SUPPORTED APYRASE DERIVATIVES

Derivatives	*Enzyme (wt.%)*	*Enzyme activity (%)*	*pH optimum*	*K_m (mM)*	*Ca^{2+} concn. (mM)*
Enzyme			4.0, 6.4	0.090	1
Alanine–glutamic acid copolymer	56	26	7.8	0.562	10
CM-cellulose	51	16	7.4	0.454	10
Methyl methacrylate (low mol. wt.)	35	23	7.2	0.652	10
Poly galacturonic acid	77	24	4.0, 7.8	1.143	100
Poly aspartic acid	49	22	7.2	0.670	100

Characteristics of polymer-supported apyrase derivatives are given in Table I. Dual pH optima, which we described earlier[3,4], were characteristic of some, but not all, matrix-supported preparations. Single pH optimum peaks for apyrase and apyrase derivatives have been earlier reported by WHITTAM *et al.*[16]. These authors obtained little activity for either soluble or bound apyrase in the absence of Ca^{++}. The effects of Ca^{2+} upon the present materials are described in Table II. Some of our preparations, like those of WHITTAM *et al.*, showed little activity in the absence of Ca^{2+}.

The effect of calcium varied from preparation to preparation, and was apparently affected by the local conditions imposed upon the enzyme by its supporting matrix. All supported apyrase preparations showed pH optima shifts towards higher values. Our results are similar in this regard to those of LEVIN *et al.*[11]. Higher K_m values were observed for all supported apyrase preparations as compared with that of the same enzyme in solution (Table II). Packing or stacking of apyrase molecules

TABLE II

EFFECT OF CALCIUM ON POLYMER-SUPPORTED APYRASE DERIVATIVES

Ca^{2+} concn. (mM)	Activity (nmoles/mg per min)					
	Enzyme	Alanine–glutamic acid copolymer	CM-cellulose	Methyl methacrylate (low mol. wt. grade No. 2008)	Poly galacturonic acid	Poly aspartic acid
None	90	24	16	2	22	0.6
Normalization	100	100	100	100	100	100
100	170	175	229	350	131	4167
10	170	625	275	1150	109	6500
1	170	237	191	700	109	5833
0.1	150	150	150	650	114	3000
0.01	100	133	106	150	109	500

TABLE III

VARIOUS SUPPORTS FOR ACETYLCHOLINESTERASE

Polymer	Enzyme bound to matrix (wt. %)	Activity relative to enzyme in solution (%)
Poly L-alanine–glutamic acid	33.0	42.0
CM-cellulose	35.0	35.0
Poly L-aspartic acid	30.0	48.0
Poly galacturonic acid	87.0	46.0

TABLE IV

VARIOUS SUPPORTS FOR DEOXYRIBONUCLEASE

Polymer	Enzyme bound to matrix (wt. %)	Activity relative to enzyme in solution (%)
Poly L-alanine glutamic acid	21	38
CM-cellulose	36	30
Poly galacturonic acid	61	45
Cellulose azide	28	40
Copolymer ethylene–malic acid	12	40

on the matrices in such a manner as to affect substrate interaction is a possible explanation.

K_m values for CM-cellulose apyrase in our study differ from that reported by WHITTAM *et al.*[16]. The basis for this difference is not assignable with certainty but it might be noted that the commercial source of the enzymes differed; also MICHEEL AND EWERS[9] performed the reaction at 38°, while our procedure was carried out at 25°.

The activity of acetylcholinesterase as a function of support matrix is given in Table III. Stability studies by measuring activity as a function of temperature elevation were carried out. It was found that these matrix-supported acetylcholinesterase preparations were thermolabile with a response pattern similar to that of the parent compound. This result differed from that which we reported[17] earlier for a silastic entrapped acetyl choline esterase.

Enzymatic activity of matrix-supported deoxyribonuclease preparations are given in Table IV.

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1 H. D. BROWN, A. PATEL, S. K. CHATTOPADHYAY, *Am. J. Botany*, 55 (1968) 729.
2 H. D. BROWN, A. PATEL, S. K. CHATTOPADHYAY, *Plant Physiol.*, 41, Suppl. (1966) Ixvi (abstr.).
3 H. D. BROWN, A. PATEL, S. K. CHATTOPADHYAY AND S. N. PENNINGTON, *Enzymologia*, 35 (1968) 215.
4 H. D. BROWN, A. B. PATEL, S. K. CHATTOPADHYAY AND S. N. PENNINGTON, *Enzymologia*, 35 (1968) 233.
5 H. I. SILMAN AND E. KATCHALSKI, *Ann. Rev. Biochem.*, 35 (1966) 873.
6 H. D. BROWN, A. PATEL AND S. K. CHATTOPADHYAY, *J. Chromatog.*, 35 (1968) 103.
7 T. TOSA, T. MORI, N. FUSE AND I. CHIGATA, *Enzymologia*, 31 (1966) 214.
8 T. WAGNER, C. J. HSU AND A. KELLEHER, *Biochem. J.*, 108 (1968) 892.
9 F. MICHEEL AND J. EWERS, *J. Makromal. Chem.*, 3 (1949) 200.
10 M. A. MITZ AND J. SUMMARIA, *Nature*, 189 (1961) 576.
11 Y. LEVIN, M. PRECHT, L. GOLDSTEIN, E. KATCHALSKI, *Biochemistry*, 3 (1964) 1905.
12 C. H. FISKE AND Y. SUBBAROW, *J. Biol. Chem.*, 66 (1925) 375.
13 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR, AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
14 S. HESTRIN, *J. Biol. Chem.*, 180 (1949) 249.
15 A. KALNITSKY, J. P. HUMMEL AND C. DIERKS, *J. Biol. Chem.*, 234 (1959) 1512.
16 R. WHITTAM, B. A. EDWARDS, K. P. WHEELER, *Biochem. J.*, 107 (1968) 3 P.
17 S. PENNINGTON, H. D. BROWN, A. B. PATEL AND C. O. KNOWLES, *Biochim. Biophys. Acta*, 167 (1968) 479.

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