Solubilization and purification of diphosphopyridine nucleotidase from pig brain

Enzymes which inactivate DPN+ have been known since the early studies of LENNERSTRAND[1] and VON EULER, HEIWINKEL AND SCHLENK[2]. An enzyme in animal tissues which rapidly inactivates DPN+ was found by MANN AND QUASTEL[3] to be inhibited by nicotinamide and was shown by HANDLER AND KLEIN[4] to catalyze the hydrolysis of the nicotinamide–ribose bond of the coenzyme. MCILWAIN[5] and MCILWAIN AND RODNIGHT[6] have studied in more detail the nicotinamide-sensitive DPNase of brain. The enzyme was found to be tightly bound to particles in the cell and attempts to solubilize the activity were unsuccessful. More recently ZATMAN *et al.*[7,8] have shown that the particulate DPNase of pig brain and other mammalian tissues can also function as a transglycosidase, capable of transferring the adenosine diphosphate ribose moiety of DPN from one molecule of nicotinamide to another, or to some other pyridine base. The inhibitory action of nicotinamide on the enzyme was thereby explained. The transfer reaction has since been used to prepare a large series of DPN analogs[9]. In all of the above-mentioned studies crude particulate preparations were used as a source of the enzyme. We would like to report the solubilization and 1800-fold purification of a DPNase from pig brain.

The properties of the crude pig-brain enzyme and the procedure for assay have been reviewed[10]. The starting material for the purification described here were pig brains collected at the slaughterhouse and stored at — 10°. The concentration of the enzymic activity during the purification is summarized in Table I. The brains were thawed at room temperature and allowed to autolyze for 24 h. An acetone powder was prepared by homogenizing the autolyzed tissue in 10 vol. of acetone in a large Waring Blendor. The macerated tissue was collected by filtration and washed in the Blendor successively with 2 vol. of 1-butanol, twice with 2 vol. of acetone, and finally with 2 vol. of diethyl ether. (Volume of solvents used was calculated with respect to the weight of the intact brains in each case.) All the organic solvents were chilled to —10° before use, and the blending and filtrations were done in a cold room (4°). The ether-washed powder was spread in a thin layer under a hood for 12 h to free it of solvent and stored at —4°. This powder contains virtually all of the activity of the intact brain, and at —4° the activity is stable for at least one year.

Using a Waring Blendor, 40-g portions of the acetone powder were suspended in

TABLE I

PURIFICATION OF DIPHOSPHOPYRIDINE NUCLEOTIDASE FROM PIG BRAIN

Stage	*Percent total activity*	*Specific activity (units*/mg protein**)*
1. Acetone powder (unwashed)	100	0.9
2. Supernatant after trypsin digestion	65	1.6
3. pH-3.1 supernatant (adjusted to pH 7.5)	52	2.0
4. Trichloroacetic acid precipitate	25	20
5. DEAE-cellulose supernatant	23	80
6. CM-cellulose fraction	16	1680

* 1 unit catalyzes the hydrolysis of 1 μmole of DPN/h under the conditions of assay[10].
** Protein was determined spectrophotometrically[11] or by the method of LOWRY *et al.*[12].

1 l of 0.02 *M* potassium phosphate (pH 7.5) and the suspension centrifuged at 13000 × *g* for 20 min. The supernatant was discarded and the powder resuspended in the same buffer and the washing repeated a second time. The discarded washings contained most of the soluble proteins and less than 10% of the DPNase. The extraction of the soluble proteins from the acetone powder was carried out at room temperature. All subsequent operations were carried out at 4° unless otherwise indicated.

After two buffer washings the acetone powder was suspended in 1 l of 0.1 *M* potassium phosphate (pH 7.5) and warmed to 35° in a water bath. 200 mg of trypsin (twice crystallized, salt-free, Worthington Biochemical Corp., Freehold, N.J.) in 10 ml of the phosphate buffer were added with stirring and incubation continued at 35° for 40 min. Then 250 mg of crystalline soybean trypsin inhibitor (Worthington Biochemical Corp.) in 10 ml of the phosphate buffer were added, the suspension chilled in an ice-bath, and then centrifuged at 13000 × *g* for 30 min. After the digestion with trypsin the DPNase is soluble and cannot be sedimented even at 100000 × *g* for 90 min. The supernatant, containing 65% of the activity, was decanted and adjusted to pH 3.1 by the addition of 5 *N* HCl. Insoluble protein was removed by centrifugation and discarded. The supernatant was adjusted to pH 7.5 with 5 *N* NaOH. Then 50% (w/v) trichloroacetic acid was added slowly until the pH reached 3.1. The DPNase is one of the first proteins to be precipitated by trichloroacetic acid under these conditions. The slightly-turbid preparation was centrifuged at 13000 × *g* for 30 min, the supernatant discarded, and the small pellet resuspended in 0.05 *M* potassium phosphate buffer (pH 7.5). Several 40-g portions of acetone powder were put through the foregoing procedure and the trichloroacetic acid precipitates obtained were pooled before proceeding to the final two steps of the purification.

The pooled precipitates were dialyzed for 4 h against 15 vol. of 0.005 *M* potassium phosphate (pH 7.5). The outside buffer was changed once, after 2 h of dialysis. To the dialyzed enzyme was added, with stirring, DEAE-cellulose (Brown Co., Berlin, N.H.), previously equilibrated against 0.005 *M* potassium phosphate (pH 7.5) (3.5 mg of DEAE-cellulose added/mg of protein). After 20 min of gentle stirring the cellulose suspension was filtered and the cellulose pad washed two times with small portions of buffer. The filtrate plus the washings contained 90–95% of the DPNase activity and only 20–25% of the protein of the dialyzed preparation.

Finally, the DEAE-cellulose filtrate was applied to a column of CM-cellulose (Brown Co., Berlin, N.H.), packed under a pressure of 10 lb/m^2 and equilibrated with 0.005 *M* potassium phosphate (pH 7.5). Then the column was washed with the same buffer until the effluent was free of protein. Elution of the enzyme was achieved by applying a linear gradient from 0.005–0.03 *M* potassium phosphate (pH 7.5). More than 90% of the applied protein failed to adhere to the column and was washed off before the gradient was begun. The DPNase activity emerged when the molarity of the eluting buffer had reached 0.008 *M*. 10-ml fractions were collected. The best fractions had a specific activity of 1680 units/mg protein, which represents a purification of 1870-fold over the acetone powder.

The low recovery of enzyme in the final step of the purification is partly attributable to its relative instability in solutions of low ionic strength. An alternative method which appears promising but which requires further investigation involves applying the enzyme on a CM-cellulose column in 0.1 *M* phosphate buffer (pH 5.9), followed

by elution and fractionation with buffer of the same molarity but of gradually higher pH.

No further purification of the CM-cellulose fraction has been attempted. However, methods other than cellulose chromatography have been successfully used to concentrate the activity of the trichloroacetic acid precipitates. Preparations with specific activities (units/mg protein) as high as 980 have been obtained by fractionating the precipitates first with ammonium sulfate and then with acetone at —10°. The activity is precipitated by solutions of 50–70% satd. ammonium sulfate.

The following description of the pig-brain DPNase is based on studies of soluble preparations with specific activities between 900 and 1680. In the region from 220–400 mμ the most highly purified preparation has an energy absorption spectrum typical of proteins, a single maximum occurring at 275 mμ. The enzyme in 0.1 *M* potassium phosphate (pH 7.5) can be stored in the frozen state for at least one year without loss of activity. The enzyme loses only 10% of its activity at 4° in 24 h or at 37° in 1 h. At this pH the enzyme is not destroyed at 55° for 10 min, but there is rapid loss of activity at 65°. At pH 4.8 the enzyme becomes more heat sensitive, 42% being lost at 55° for 5 min. At 4° the enzyme is stable at all pH values from 1.5–11 inclusive for at least 15 min. At this temperature the enzyme can be dialyzed against 0.05 *M* potassium phosphate (pH 7.5) for 18 h without appreciable loss of activity. The DPNase is rapidly destroyed by chymotrypsin digestion and more slowly by trypsin.

The purified DPNase demonstrates maximum activity in the pH range from pH 5–7. However, the activity varies less than 10% between pH 5 and 8.5. At pH 4 the activity is 55% of the maximum and at pH 9 it is 80% of the maximum. The soluble purified enzyme is inhibited by nicotinamide (50% inhibition at 1.5 m*M* nicotinamide). However, 3-formylaminopyridine was found to be a more potent inhibitor than nicotinamide (80% inhibition at 1 m*M* and 60% at 0.2 m*M*). The highly purified enzyme possesses transglycosidase activity as well as hydrolytic activity. This was demonstrated by the synthesis of the thionicotinamide analog of DPN^+ when the enzyme was incubated with DPN^+ and thionicotinamide in 0.1 *M* potassium phosphate buffer (pH 7.5).

TPN^+ is cleaved by the DPNase at 53% the rate of DPN^+ in the standard assay[10]. The activity of the enzyme on various DPN^+ analogs was also determined. When the nicotinamide portion of the DPN^+ molecule is modified, the following rates of hydrolysis were observed in the standard assay ($DPN^+ = 100$): 3-acetylpyridine, 58; pyridine-3-aldehyde, 55; isonicotinic acid hydrazide, 9; and nicotinic acid, 0. Modification of the adenine moiety of DPN^+ resulted in the following rates: 1-(2-hydroxyethyl)adenine[13], 60; 6-(2-hydroxyethylamino)purine[13], 53.

The soluble purified DPNase was found to be very similar to the crude particulate enzyme, described earlier[10], with respect to the reactions it catalyzes, specificity, pH profile and temperature stability.

It is interesting to note that the DPNase of acetone powder prepared from beef brain could not be recovered in a soluble state after trypsin digestion. Under the conditions described for the trypsin digestion of pig brain acetone powder, no activity was solubilized. Higher trypsin concentrations and longer digestion time resulted in the destruction of the DPNase of beef brain acetone powder.

This is publication No. 127 of the Graduate Department of Biochemistry,

Brandeis University, Waltham, Mass., U.S.A. This work was aided by Grant CY-3611 of the National Cancer Institute of the National Institutes of Health.

Graduate Department of Biochemistry, Brandeis University, Waltham, Mass. (U.S.A.)

H. G. WINDMUELLER*
N. O. KAPLAN

[1] A. LENNERSTRAND, *Biochem. Z.*, 287 (1936) 172.
[2] H. VON EULER, H. HEIWINKEL AND F. SCHLENK, *Z. physiol. Chem. Hoppe-Seyler's*, 247 (1937) iv.
[3] P. J. G. MANN AND J. H. QUASTEL, *Biochem. J.*, 35 (1941) 502.
[4] P. HANDLER AND J. R. KLEIN, *J. Biol. Chem.*, 143 (1942) 49.
[5] H. MCILWAIN, *Biochem. J.*, 46 (1950) 612.
[6] H. MCILWAIN AND R. RODNIGHT, *Biochem. J.*, 44 (1949) 470.
[7] L. J. ZATMAN, N. O. KAPLAN AND S. P. COLOWICK, *J. Biol. Chem.*, 200 (1953) 197.
[8] L. J. ZATMAN, N. O. KAPLAN, S. P. COLOWICK AND M. M. CIOTTI, *J. Biol. Chem.*, 209 (1954) 453, 467.
[9] B. M. ANDERSON, C. J. CIOTTI AND N. O. KAPLAN, *J. Biol. Chem.*, 234 (1959) 1219.
[10] N. O. KAPLAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. II, Academic Press, Inc., New York, 1955, p. 660.
[11] O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 310 (1942) 384.
[12] O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
[13] H. G. WINDMUELLER AND N. O. KAPLAN, *J. Biol. Chem.*, 236 (1961) 2716.

Received July 26th, 1961

* Research Fellow of the National Institute of Neurological Diseases and Blindness. Present address: National Institute of Arthritis and Metabolic Diseases, Bethesda, Md. (U.S.A.).