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PURIFICATION OF A LYSOSOMAL DNase FROM DROSOPHILA MELANOGASTER

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An acid DNase was purified from Drosophila melanogaster till apparent homogeneity by six consecutive chromatographic steps. The enzyme is a lysosomal DNase, because it is glycosilated and carries 1.8-2.4 mol of mannose-6-phosphate/mol of enzyme. The enzyme is fully active without any divalent cation and introduces single stranded nicks into a supercoiled DNA.

Lysosomal enzymes are acid hydrolases which are involved in the controlled intracellular digestion of macromolecules. Phosphomannosyl residues are common and unique structural features of all lysosomal enzymes which serve as recognition markers for the targeting of these enzymes to the lysosomes (1,2). The significance of the lysosomal enzymes is well documented in the numerous inherited disorders of lysosomal metabolism in humans (3). Analysis of cloned lysosomal enzymes revealed strong sequence similarities between lysosomal and nonlysosomal enzymes catalyzing closely related reactions (reviewed in 3). There is no report in the literature for the purification and characterization lysosomal DNases. Here we describe a lysosomal DNase from Drosophila melanogaster.

MATERIALS AND METHODS

Hydroxylapatite was from Bio-Rad, Aminopentyl agarose from Sigma, S-Sepharose FF, Q-Sepharose FF, Con A-Sepharose and

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Superose 12 HR 10/30 were from Pharmacia. Methyl-alpha-D-mannopyranoside, 2-(N-Morpholino)ethanesulfonic acid buffer (MES) were from Sigma, Proteinase K from Merck. Glucose-6-phosphate dehydrogenase from Leuconostoc mesenteroides, yeast phosphomannose isomerase, mannose-6-phosphate were from Sigma. Yeast phosphoglucose isomerase and glutamate dehydrogenase were from Boehringer.

Extract preparation: Drosophila melanogaster embryos (Canton S strain) were collected at 25 °C on feeding plates. The embryos were dechorionated in half strength Chlorax for 2 min. at 0 °C, then washed thoroughly with cold distilled water. Embryonic extracts were prepared by the method of Nelson et al. (4). The extract was stored frozen at -80 °C.

Nuclease assay: 1 ug of supercoiled pBR 322 plasmid DNA was incubated with an aliquot of the nuclease preparation in 40 ul of 50 mM NaCl, 50 mM MES buffer pH 5.2, 0.5% glycerol at 30 °C for 20 min. The reaction was stopped with 8 ul of 0.5 M tris.Cl pH 8.0, 5% sodium dodecysulfate and incubated at 47 °C for 1 hour with 40 ug/ml of Proteinase K. DNA fragments were separated on 0.8% agarose gel and stained with ethidium bromide. 1 unit of nuclease is defined as the amount of enzyme which converts 1 ug of supercoiled DNA to relaxed or linear DNA under conditions described above.

Detection of mannose-6-phosphate residues was done as described by Natowicz et al (5). 4 ug of purified nuclease was hydrolyzed in 1 M HCl for 4 hours at 100 °C. The sample was dried and dissolved in 50 mM Tris.Cl pH 8.1. The mannose-6-phosphate which is released from the enzyme is converted sequentially by a series of enzymatic reactions to 6-phospho-gluconate and NADPH as described by Natowich et al (5). The reaction products were amplified by the $NADP^+$-NADPH enzymatic

cycling reaction and 6-phospho-gluconate was measured fluorometrically (6,7). Mannose-6-phosphate standards were used to calibrate the assay.

Gel electrophoresis of proteins: the purification was monitored on SDS-polyacrylamide gels (8). From the last steps of enzyme purification, where the protein concentration was low an aliquot of the sample was precipitated with trichloracetic acid to obtaine detectable staining. The gels were stained with the silver stain kit of Bio Rad.

RESULTS AND DISCUSSION

Purification: In the extracts of Drosophila embryos, larvae or flies a DNase was detected which was most active at pH 5.2 and worked without any divalent cation. Purification was started from the embryonic extract as it seemed to be the optimal source of this nuclease.

All steps were done at 0-4 °C. 200 ml embryonic extract was thawed on ice, nucleic acids were precipitated with 0.2 ml of 5% streptomycine sulfate /ml extract. After 10 min. stirring on ice the precipitate was removed by centrifugation. To the supernatant 0.28 g/ml of solid ammonium sulfate was added slowly (40% saturation), the precipitated proteins were discarded. To achive 70% saturation 0.21 g/ml of ammonium sulfate was added to the previous supernatant, the precipitated proteins were collected by centrifugation, dissolved into 20 ml of 20 mM K phosphate pH 6.7, 50 mM NaCl and loaded onto an 1.5x15 cm hydroxylapatite column equilibrated with 20 mM K phosphate pH 6.7, 50mM NaCl. Proteins were eluted with a linear gradient of K phosphate pH 6.7 (20-550 mM in 50mM NaCl, 150-150 ml). The nuclease was eluted in the middle of the gradient. Active fractions were pooled (fraction I) and

dialyzed overnight against buffer A (20mM Hepes buffer pH 7.0, 0.1 M NaCl, 1mM EDTA).

S-Sepharose FF, Q-Sepharose FF, aminopentyl agarose columns were equilibrated with buffer A. The dialyzed hydroxylapatite fraction was loaded onto these columns. While a large fraction of the proteins present in fraction I bound to these columns the acid nuclease quantitatively passed through. The active flowthrough fractions were pooled, the proteins were precipitated with ammonium sulfate (0.49g/ml), the precipitated proteins were dissolved in a small volume of buffer A and dialysed against this solution for 6 hours (fraction II).

300 ul of fraction II was size fractionated by fast protein liquid chromatography on a Superose 12 HR 10/30 column. The column was equilibrated and eluted with buffer A at a flow rate of 0.5 ml/min. The active fractions were pooled (fraction III). The elution profile on the Superose 12 column is shown in Fig.1.

Con A-Sepharose column was equilibrated with 20 mM Hepes pH 7.6, 0.1 M NaCl, 1 mM $CaCl_2$, 1 mM $MnCl_2$ (buffer B). Fraction III was loaded and washed thoroughly with buffer B. The nuclease was eluted with buffer B containing 0.6 M methyl-alpha-D-mannopyranoside. To stabilize the nuclease 300 ug/ml of bovine serum albumin was added to the pooled active fractions and the enzyme was stored in small aliquots at -80 °C.

Properties: the preparation was homogeneous by the criteria of SDS gel electrophoresis and had a molecular mass of 32 kDa (Fig. 2). The purification steps of a representative preparation are summarized in Table 1. The nuclease was fully active without any divalent cation, and even in the presence of 1 mM of EDTA. Maximal activity was observed at pH 5.2, 50% activity was at pH 6.0 and the enyzme was inactive above pH

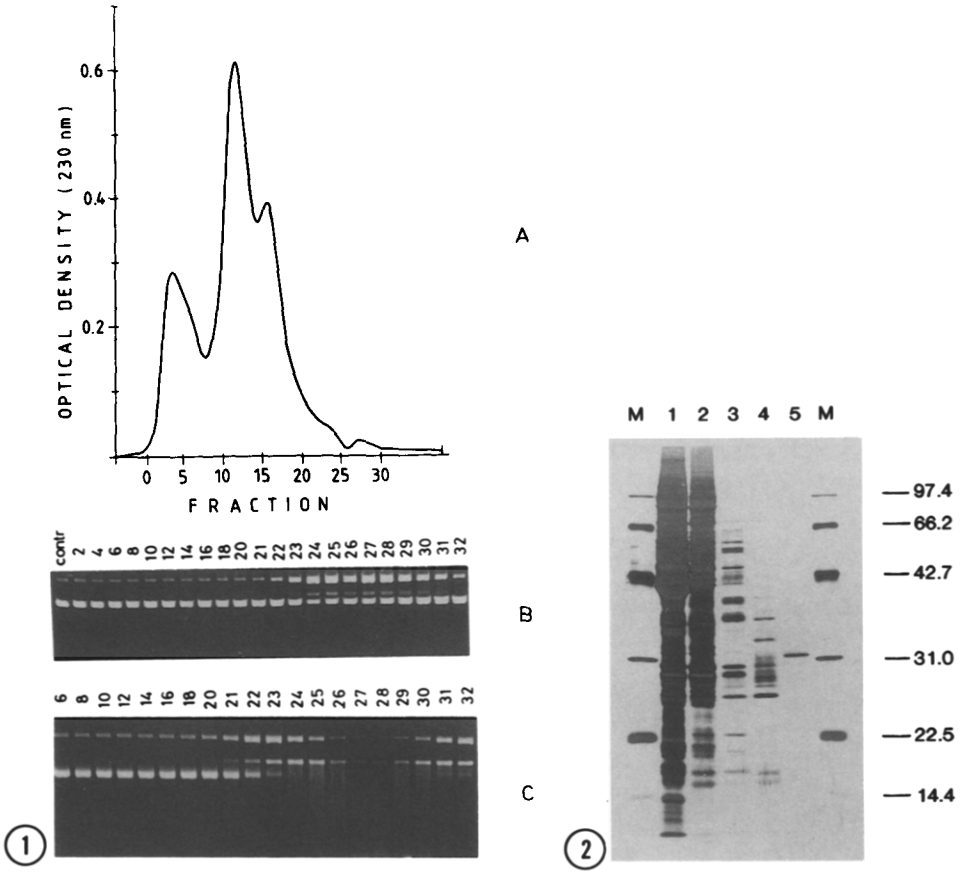

Fig. 1. FPLC separation of the acid nuclease on Superose 12 HR 10/30 column.
Panel A. The optical profile of the chromatography.
Panel B. Elution profile of the nuclease activity. pBR 322 plasmid DNA was digested with 1 µl aliquots for 5 min. The tested fractions of the Superose 12 chromatography are indicated at the top of the lanes. DNA incubated in the same reaction mixture without protein is shown in lane "contr".
Panel C. The same test with 2 µl aliquots and 20 min. incubation.

Fig. 2. SDS-polyacrylamide gel analysis: purification of the acid nuclease.
Aliquots from the different purification steps were separated on 14% SDS-polyacrylamide gel and visualized by silver staining. Embryonic extract (lane 1), hydroxylapatite fraction (lane 2), flowthrough of the Q-Sepharose, S-Sepharose and aminopentyl agarose columns (lane 3), Superose fraction (lane 4) and the Con-A Sepharose fraction (lane 5). The molecular weight (kDa) of the marker proteins (lane M) is marked on the figure.

7.0. 50 mM monovalent ion concentration was found to be optimal for the nuclease activity, at 200 mM Na concentration the enzyme activity was inhibited by 50%. The nuclease introduces single stranded nicks into the DNA, which causes the relaxa-

Table 1

Purification of a lysosomal acid DNase from Drosophila embryo

Fraction	Volume ml	Protein mg	Yield %	Specific activity units/mg
Embryonic extract	200	1150	100	170
Hydroxylapatite fraction	33	142	81	1120
Q-Sepharose, S-Sepharose, Aminopentyl agarose flowthrough	47	11.5	65	11000
Superose 12	4	0.33	13	77000
Con A-Sepharose	1	0.045	4.7	205000

tion of the supercoiled DNA (Fig. 1). After prolonged incubation, due to the accumulation of single stranded nicks the full length linear form, and later shorter linear fragments appear. The accumulation of single stranded nicks before the appearance of the linear form of the plasmid DNA can be detected on denaturing agarose gel (data not shown).

Although the acidic pH optimum and the glycosilated structure of this nuclease strongly suggested its lysosomal origin it is only the presence of mannose-6-phosphate residues in the sugar side chains which can be accepted as direct evidence for the lysosomal localization of the nuclease. The mannose-6-phosphate content of the purified nuclease was determined by an enzymatic method which converts mannose-6-phosphate liberated from the enzyme by acid hydrolysis into 6-phospho-gluconate and NADPH (5). In repeated assays the mannose-6-phosphate content of the nuclease was found to be 1.8-2.4 mol/mol enzyme. Thus, this acid nuclease is a lysosomal enzyme. Its localization in the high speed supernatant is probably due to a disruption of the lysosomes during the homogenization step.

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