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ISOLATION AND CHARACTERIZATION OF MULTIPLE FORMS OF MALT DEOXYRIBONUCLEASE*

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Key Word Index—*Hordeum vulgare*; barley; malt; deoxyribonuclease; enzyme purification; comparison; bovine pancreatic deoxyribonuclease.

Abstract—A deoxyribonuclease (DNase), similar to bovine pancreatic DNase, has been isolated from germinating barley. Commerically available malt was used as source of the enzyme. The purification procedure involves (a) ammonium sulfate fractionation (45–65% saturation), (b) CM-cellulose chromatography at pH 4.7 and (c) DEAE-cellulose chromatography at pH 8. DEAE-cellulose separates the enzyme into 4 distinct forms, designed as DNases A, B, C, and D. DNase A and B may be rechromatographed on DEAE-cellulose employing a $CaCl_2$ instead of Tris–HCl gradient. Both forms appear homogeneous on regular and sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. In addition, both forms have a sp. act. of *ca* 700 units per *A* unit at 280 nm, similar to the potency of the pancreatic enzyme. DNase C and D, which are present in relatively small quantities in malt, were not characterized. The MWs of DNases A and B, as estimated by the SDS gel electrophoresis techniques, are near 32000, slightly larger than that of the pancreatic enzyme. In the presence of either Mn^{2+} or Mg^{2+}, the pH-activity profile of the barley enzyme is similar to that obtained with the pancreatic enzyme. Like the pancreatic enzyme, barley DNase is protected by Ca^{2+} from inactivation. The amino acid compositions of the A and B forms are about the same; a comparison of the malt and pancreatic enzymes shows many similarities but major differences in the amounts of glutamic acid, proline and glycine. The hydrolysis products of DNA by malt DNase are indistinguishable from those obtained with pancreatic DNase. Further hydrolysis of these products by snake venom phosphodiesterase shows malt DNase to be a 5′-phosphate producer. Deoxythymidine 3′,5′-di-*p*-nitrophenyl phosphate, one of the synthetic substrates of pancreatic DNase, is also hydrolyzed by malt DNase.

INTRODUCTION

Two types of deoxyribonuclease (DNase) are known in animals [1]. One of these, designated DNase I, has been found only in secretory glands such as pancrease [2] and parotid [3]; the enzyme has a pH optimum near 7, requires metal ion and produces nucleotides with 5′-phosphate. The other type, designated DNase II, is a lysosomal enzyme occurring in almost all cells [4]; it has an acidic pH optimum and produces nucleotides with 3′-phosphate. A DNase I-type enzyme from bovine pancrease, readily available in crystalline form, has been studied in considerable detail both as an enzyme and as a protein [5]. Its primary structure is known [6, 7].

The occurrence of DNase in plants has been reported [8, 9] but very little information concerning its properties is available. The relatively high activity of DNase in various germinating seeds [9, 10] suggests an important role in seed sprouting. In addition, studies with germinating barley (commercially available as malt diastase) have shown that some of the enzymatic properties of malt DNase, such as the bivalent metal ion requirement and the pH optimum near 7, are very similar to those of DNase I. It is therefore of interest to isolate and compare this enzyme with pancreatic DNase, whose occurrence in animals is confined to secretory glands.

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RESULTS

Purification of the enzyme

The purification steps are summarized in Table 1. Elution profiles of DNase in CM- and DEAE-celluloses are shown in Figs. 1 and 2. It is noted that DEAE-cellulose chromatography separates the enzyme into 4 distinct forms, designated as A, B, C and D according to their order of elution. The multiple forms of the enzyme are not an artifact of poor performance of the ion-exchanger because rechromatographing each fraction under identical conditions did not change its elution position. The rechromatographic operations, however, did not yield any significant further purification. If, however, each fraction was chromatographed on DEAE-cellulose with a $CaCl_2$ gradient (Fig. 3) the purification was improved a great deal; many inert proteins were removed as shown in the monitored protein curve (280 nm). The change in chromatographic patterns may be due to the fact that the order of elution of proteins is now not based solely on ionic strength but rather partly dependent upon affinity of the proteins for Ca^{2+}. A similar experiment, in which elution of pancreatic DNase with a $CaCl_2$ gradient on DEAE-cellulose chromatography facilitated the removal of contaminating proteases, has also been demonstrated [11]. The DNases A and B thus purified have sp. act. of 600–700 U/A_{280} (Table 1) compared to 650–810 U/A_{280} for pancreatic DNases [11]. DNases C and D are not homogeneous: the contamination of other proteins is shown later in polyacrylamide gel electrophoresis.

Table 1. Summary of purification steps of malt DNase

Purification step		Total activity (units)	Activity recovery (%)	Specific activity (U/A_{280})	Purification (fold)
1. Crude extract		3240	100	0.09	1
2. $(NH_4)_2SO_4$ precipitate		1930	60	1.45	16
3. CM-cellulose chromatography		1450	45	33.5	372
	Fraction				
4. DEAE-cellulose	A	511	15.8	144	1600
chromatography	B	495	15.3	147	1630
—Tris–HCl	C	238	7.3	92	1020
gradient	D	109	3.4	40	440
	Total	1350	41.8		
	Fraction				
5. Chromatography of each of the	A	405	13.8	600	6600
fractions from Step 4 on	B	333	10.3	680	7600
DEAE-cellulose	C	118	3.7	410	4600
-$CaCl_2$ gradient	D	40	1.2	250	2800
	Total	896	29.0		

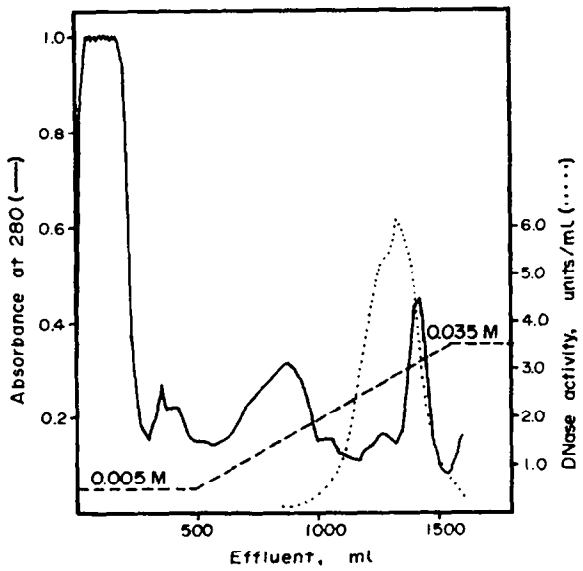

Fig. 1. Chromatography of malt DNase on CM-cellulose. Column dimensions, 2 × 13 cm; flow rate, 50 ml/hr. Dashed line indicates the concentration of the eluting buffer. Gradient from 7 chambers: 150 ml each of 5, 10, 15, 20, 25, 30, 35 mM calcium acetate, pH 4.7.

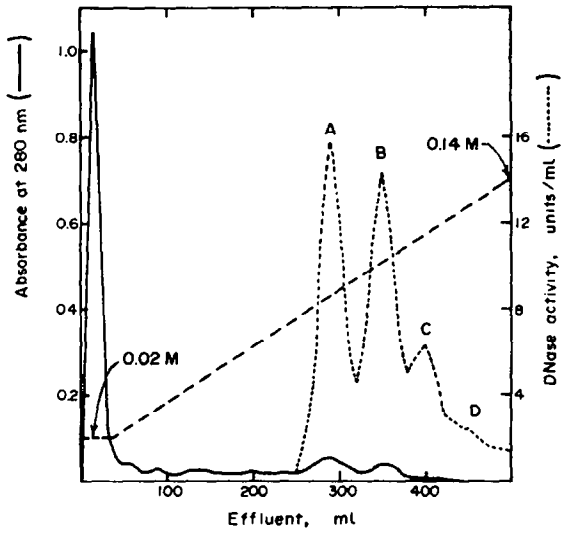

Fig. 2. Chromatography of malt DNase on DEAE-cellulose. Column dimensions, 0.9 × 14 cm; flow rate, 20 ml/hr. Dashed line indicates the concentration of the eluting buffer. Gradient: 150 ml each of 0.02, 0.08 and 0.14 M Tris–HCl, pH 8, 2 mM in $CaCl_2$.

Other enzymatic activities

The crude extract and the purified DNases A and B were assayed for 5'-nucleotidase, deaminase, phosphodiesterase and ribonuclease activities. Whereas the crude extract showed significant amounts of all four enzymatic activities, the purified DNases A and B had none of these activities, even when the quantity of sample assayed was 50 times that used in DNase assays. Because of lack of these enzymatic activities associated with nucleic acids, the preparations of DNases A and B may prove useful to the structural studies of DNA and other related areas of investigation.

Stability and the effects of Ca^{2+}

It has been shown that Ca^{2+} is required for stability against either chemical [12–16] or enzymatic [17, 18] inactivation of pancreatic DNase. For malt DNase, Ca^{2+} must also be present for stability, especially in an environment where a mixture of other proteins and impurities exists. As shown in Fig. 4, most of the enzymatic activity was recovered (90%) when crude extracts were chromatographed through Sephadex G-100 in the presence of $CaCl_2$. In a similar experiment, where only $CaCl_2$ was removed from the eluting buffer, fractions that contained protein had no DNase activity even

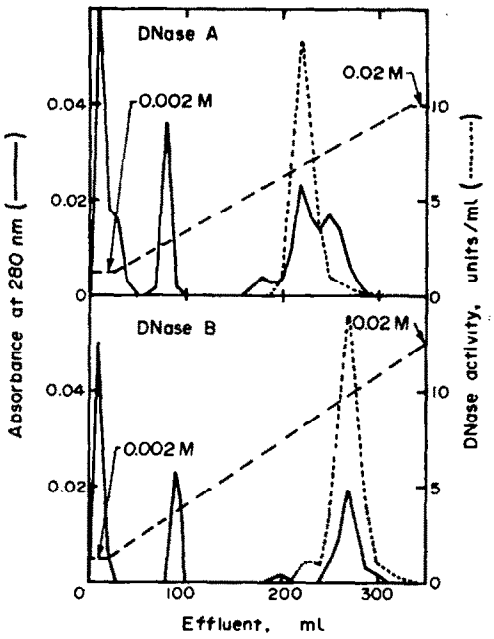

Fig. 3. Chromatography of malt DNase on DEAE-cellulose. Column dimensions, 0.9 × 14 cm; flow rate, 20 ml/hr. Dashed line indicates the concentration of $CaCl_2$ in the eluting buffer. Gradient: 150 ml each of 2 and 20 mM $CaCl_2$ in 0.02 M Tris–HCl, pH 8.

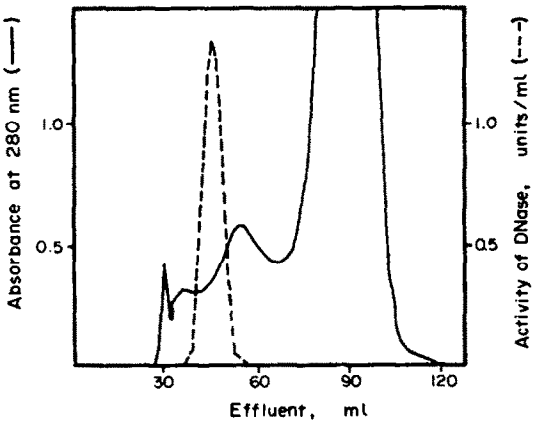

Fig. 4. Gel filtration of malt DNase on Sephadex G-100. Column dimension, 1.5 × 47 cm; flow rate, 23 ml/hr; eluting buffer, 0.05 M Tris–HCl, pH 7, 5 mM in $CaCl_2$. Supernatant (2.5 ml) from the crude extract were loaded on the column. The elution was carried out at 25°.

though the elution pattern monitored at 280 nm was identical to that of Fig. 4. Further additions of Ca^{2+} in the assay medium (10 mM) did not restore the activity. Thus, Ca^{2+} is loosely bound to the enzyme and can be removed by gel-filtration; once Ca^{2+} is removed, the inactivation that follows is due to an entirely different, yet unknown, mechanism.

Price *et al.* [12] have shown that Ca^{2+} protects pancreatic DNase from inactivation by proteolytic enzymes. Without calcium pancreatic DNase is completely inactivated by trypsin or chymotrypsin in 1 hr at 25°. However, the addition of trypsin or chymotrypsin in either the crude extract or the purified malt DNases A and B, in the absence of Ca^{2+}, did not result in significant inactivation in 8 hr at 25°. This indicates that instability of malt DNase is probably not due to trypsin or chymotrypsin-like protease in malt; this property, which differs from that of pancreatic DNase, is the only known difference between the two enzymes in the present study. Also without Ca^{2+} at pH 7 and 25°, the enzyme activity in the crude extract prior to gel-filtration retained 75% of its activity in 24 hr. Prolonged storage in the frozen state (in H_2O) also causes a gradual decrease in activity, the half life of the purified DNases A and B being *ca* 2 months. Addition of Ca^{2+} greatly improves the storage life of DNase in the frozen state with no significant inactivation after 6 months. It is therefore preferable to work up the $(NH_4)_2SO_4$ fractionation within the same day to the point where the proteins are dialyzed in buffers containing Ca^{2+}.

Metal ion requirements and pH-activity profile

The enzyme shows different pH optima, depending on the bivalent metal ions. As shown in Fig. 5a and 5b, the pH optimum of both DNases A and B is at pH 7.5 in the presence of Mn^{2+}. The pH-activity profile of malt DNases is almost identical with that of pancreatic DNase shown in Fig. 5c. The profile is also in good agreement with Brawerman and Chargaff [9] who showed that with Mg^{2+} present the pH optimum of malt DNase is near 6. It should be noted that Ca^{2+} was not included in the experiment in Fig. 5. Wiberg [19] showed that Ca^{2+} exerts a potent synergistic effect with Mg^{2+} in the activation of pancreatic DNase. The same effect is also observed in malt DNase. At pH 7, the enzymatic activity in Mg^{2+}-DNA as substrate with the addition of Ca^{2+} (10 mM) was *ca* four times that in Mg^{2+}-DNA alone.

Polyacrylamide gel electrophoresis and the MW of the enzyme.

On electrophoresis of DNases A and B in regular and SDS gels, a single band of protein stained with Coomassie Blue appears in each gel. DNases C and D give a major band and several contaminating bands. In regular gels, an unstained gel was cut into *ca* 1 mm sections; only the extracts (with 0.2 M Tris–HCl, pH 7, 10 mM in $CaCl_2$) of segments corresponding to the stained band (in DNases A and B) showed significant DNase activity.

Using the ratio of the migration distance of the protein band and the tracking dye and the plot given by Weber and Osborne [20] in SDS polyacrylamide gel electrophoresis, a MW of 32000 was calculated for the reduced form of this enzyme. Under the identical conditions, SDS polyacrylamide gel electrophoresis of chymotrypsinogen and pancreatic DNase A gave appropriate respective MWs of 25000 and 30000. From the elution position in Sephadex G-100 (Fig. 4), the MW of malt DNase in the native state, calculated according to the formula of Determann [21] is 34000 ± 2000. Because of the similar values in the intact and reduced forms, it is concluded that malt DNase consists of a single polypeptide chain per molecule as does pancreatic DNase [22].

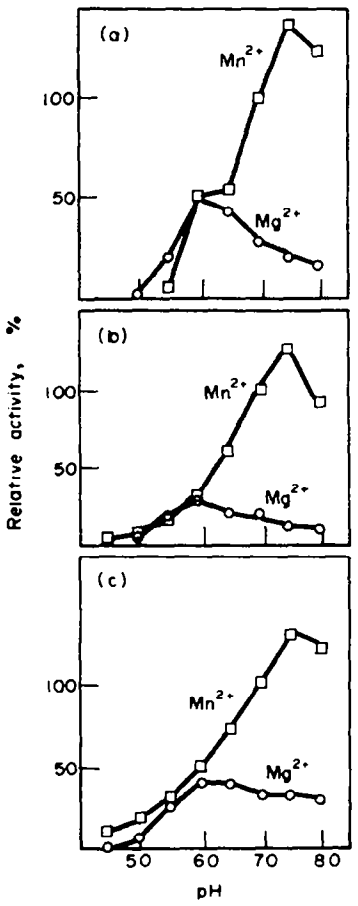

Fig. 5. The pH-activity profiles of malt DNases A and B and pancreatic DNase A. Concentrations of both metal ions, 10 mM; buffer, 0.1 M Tris acetate. Relative activity is set in such a way that the activity, in the presence of Mn^{2+}, at pH 7, is equal to 100. The enzymes used; (a) malt DNase A; (b) malt DNase B; (c) pancreatic DNase A.

Amino acid composition

Comparison of the amino acid compositions of malt and pancreatic DNases is shown in Table 2. Malt DNases A and B are almost the same in their composition; when the malt and pancreatic enzymes are compared there are many similarities but major differences in the amounts of glutamic acid, proline and glycine. Even though the malt and pancreatic enzymes originated from phylogenetically remote organisms, the great similarity in most amino acid residues, especially the content of half-cystine, suggests that malt DNase may contain the same two pairs of disulfide bridges, one of which is essential for activity as pancreatic DNase [12].

Examination of phosphodiester bonds cleaved by malt DNase

When DNA was hydrolyzed by malt DNase (1 mg DNA, 1 unit DNase in 1 ml of the assay buffer, 2 hr at 25°) and the products were separated by paper electrophoresis at pH 3.5, only oligonucleotides were detected; no mononucleotides were found under these conditions. To determine whether these oligonucleotides bear 3′ or 5′ phosphate groups, they were further hydrolyzed by snake venom phosphodiesterase [23]. The electrophoretic pattern of the venom hydrolysate proved that 5′-phospho oligonucleotides were produced by malt DNase because only 5′-mononucleotides were detected (Reaction 1). Nucleosides or nucleoside 3′,5′-diphosphates, which are the products expected if the oligonucleotides were 3′-phospho derivatives (Reaction 2), were not detected.

$$\underset{\text{5′-phospho oligonucleotides}}{pXpYpZ} \xrightarrow[\text{Phosphodiesterase}]{\text{Snake venom}} pX + pY + pZ \quad \text{(Reaction 1)}$$

$$\underset{\text{3′-phospho oligonucleotides}}{XpYpZp} \xrightarrow[\text{Phosphodiesterase}]{\text{Snake venom}} X + pY + pZp \quad \text{(Reaction 2)}$$

The results were identical for both purified malt DNases A and B. In a control experiment the same results were obtained when pancreatic DNase was substituted for malt DNase. Many investigators [1] have shown that pancreatic DNase produced 5′-phospho oligonucleotides.

Malt DNase also hydrolyzes the 3′-*p*-nitrophenyl group of deoxythymidine 3′,5′-di-*p*-nitrophenyl phosphate as does pancreatic DNase [24]. However, the rate of hydrolysis of this synthetic substrate is slightly higher with malt DNase than with bovine DNases, based on an equal ability to hydrolyze DNA.

DISCUSSION

Brawerman and Chargaff [9] noted that malt DNase is rather unstable. The instability thus limited their purification attempts to ammonium sulfate fractionation. In the present study, gel chromatography demonstrated that Ca^{2+} can be utilized to stabilize the enzyme. Ion-exchange chromatographic systems must therefore be adopted to include Ca^{2+} in order to carry out further purification of the enzyme. In general, bivalent ions are stronger binding ligands than monovalent for ion-exchange groups such as carboxyls. This strong binding affects the binding of the enzyme on CM-cellulose. Therefore, a low concentration of Ca^{2+} and a rather acidic pH were employed during CM-cellulose chromatography. In DEAE-cellulose chromatography, Ca^{2+} is not the ionic species exchanged and its presence in eluting buffers does not create ion exchange effects.

Malt DNase has 4 forms as does the pancreatic enzyme. Malt DNases A and B, by the procedure presented in this paper, have been purified to apparent homogeneity, as shown by their electrophoretic patterns in polyacrylamide gels and the increase in sp. act. to a value previously obtained with the purified pancreatic enzyme. Other information, such as the close resemblance of the amino acid compositions of malt and pancreatic enzymes and lack of related enzymatic activities, also supports this conclusion. However, malt DNases C and D, present in small amounts in malt, did not appear as homogeneous as A and B for characterization.

It is known [11, 25, 26] that the multiple forms of pancreatic DNase are due to the charge differences in which substitution of histidine by proline and sialylation of carbohydrate side-chain occur among the 4 forms of DNase. Information to distinguish 4 forms of malt DNase is not yet available. However, the amino acid

Table 2. Comparison of amino acid compositions of malt and bovine pancreatic DNases

Amino acid	Malt DNase A	Malt DNase B	Bovine pancreatic DNase A
Lysine	7.9 (8)	7.1 (7)	9
Histidine	6.8 (7)	7.0 (7)	6
Arginine	12.5 (13)	13.2 (13)	11
Aspartic acid	35.5 (36)	36.0 (36)	32
Threonine	15.9 (16)	15.6 (16)	15
Serine	29.8 (30)	29.6 (30)	30
Glutamic acid	30.4 (30)	30.0 (30)	19
Proline	16.2 (16)	14.0 (14)	9
Glycine	17.7 (18)	17.5 (18)	9
Alanine	25.9 (26)	25.8 (26)	22
1/2 Cystine*	3.7 (4)	3.6 (4)	4
Valine	19.4 (19)	18.0 (18)	24
Methionine*	3.2 (3)	2.8 (3)	4
Isoleucine	12.3 (12)	11.9 (12)	11
Leucine	24.0 (24)	23.6 (24)	23
Tyrosine	11.6 (12)	10.6 (11)	15
Phenylalanine	13.0 (13)	13.0 (13)	11
Tryptophan	N.D.†	N.D.†	3
Calculated MW	31300	30700	28200

Results are expressed as the calculated number of residues per enzyme molecule based on the average of duplicate analyses and compatible with a MW of 31000; the possible number of residues is in parenthesis. The values for bovine pancreatic DNase A are the numbers of residues established in the sequence study [7]. Corrections are made for destruction during acid hydrolysis: threonine, 5%, serine, 10%.

* Determined as cysteic acid or methionine sulfone after performic acid oxidation and hydrolysis.

† Not determined.

‡ Tryptophan and carbohydrates are not included in calculations.

analysis showed that A form contains 5 more residues than does B form, these being one residue each of lysine, valine and tyrosine and two of proline. The extra residue of lysine in A may account for its early elution on DEAE-cellulose.

There are striking similarities between bovine pancreatic and malt DNases. These include the metal requirement, the pH-activity profile, the Ca^{2+} stabilization of the enzyme, the presence of multiple forms, the reaction products, the MW and the presence of two disulfide bridges. The similarity in properties may suggest great similarity in structure and reaction mechanism.

EXPERIMENTAL

Materials. Calf thymus DNA and chymotrypsinogen were obtained from Worthington. Bovine pancreatic DNase A was isolated according to the procedure of ref. [25]. Malt diastase was obtained from National Biochemical Co. The celluloses were washed and regenerated according to the procedure of the manufacturer and equilibrated with the starting buffers before being packed into columns. Equilibration of CM-cellulose requires titration with $Ca(OH)_2$ to bring the pH to 4.7. Deoxythymidine 3',5'-di-*p*-nitrophenyl phosphate was obtained from Aldrich. Snake venom phosphodiesterase was obtained from Sigma and was purified by gel-filtration on Sephadex G-100. 5'-Adenylic acid, 5'-deoxyadenylic acid and di-*p*-nitrophenyl phosphate were also obtained from Sigma.

DNase assay. Activity was determined by modification [11] of the hyperchromicity assay of ref. [2]. The assay medium contains 0.05 mg of DNA in 1 ml of 0.1 M Tris–HCl, pH 7, 5 mM in $MnCl_2$. One unit of DNase is defined as the activity that causes the increase of one *A* unit at 260 nm per min per ml of assay medium at 25°. The sp. act. is designated as units of 1 ml enzyme soln per *A* unit of that soln at 280 nm (U/A_{280}). All of the photometric measurements were performed in 1-cm lightpath cells.

Other enzymatic activities. The activity of 5'-nucleotidase was assayed at pH 5.7 according to ref. [9] using 5'-adenylic acid as substrate and measuring Pi release [27]. The substrate for the deaminase assay was 5'-deoxyadenlyic acid; to 1 ml of assay soln containing 1 *A* unit (at 260 nm) of the substrate in 0.1 M Tris–HCl, pH 7, was added appropriate amounts of enzyme and the decrease of *A* at 260 nm was measured. Phosphodiesterase activity was assayed with di-*p*-nitrophenyl phosphate as substrate at pH 8.8 [23]. Ribonuclease activity was measured according to the procedure of ref. [28]. The enzymatic activity with deoxythymidine 3',5'-di-*p*-nitrophenyl phosphate as substrate was measured as before [24].

Electrophoresis. The 7% polyacrylamide gel electrophoresis was performed as described by ref. [29] with a buffer of pH 9.5 instead of 8.3 [30]. SDS polyacrylamide gel electrophoresis was according to ref. [20]. Paper electrophoresis for the separation of nucleotides was carried out on a flat plate as described previously [24]. The buffer was 0.05 M NH_4 formate, pH 3.5.

Amino acid analysis. Proteins were hydrolyzed in 100 µl of 6N HCl in evacuated, sealed tubes at 110° for 24 hr [6, 31]. For the determination of half-cystine and methionine, proteins were first oxidized with performic acid [7] and then hydrolyzed with 6N HCl in separate tubes. Amino acid analyses were performed on the nmol scale with an analyzer [32] modified for use with 2.8 mm bore columns [33].

Extraction and $(NH_4)_2SO_4$ fractionation of malt DNase. Unless

otherwise indicated, all of the following operations were at 4°. Solid malt diastase (120 g) was suspended in 600 ml of 0.1 M Tris–HCl pH 7 and stirred for 30 min. To the yellowish supernatant (520 ml) obtained by centrifugation, were added 145 g of $(NH_4)_2SO_4$ (to 45% satn), and the mixture was stirred for 30 min. After the ppt. was removed by centrifugation, 80 g of $(NH_4)_2SO_4$ (to 65% satn) were added to the supernatant (600 ml). The second ppt., which contained most of the activity, was recovered by centrifugation, dissolved in 80 ml of H_2O and dialyzed against H_2O for 1 hr and 5 mM Ca acetate, pH 4.7, for 6 hr. The small amount of ppt. which formed was removed by centrifugation. All of the above centrifugations were at 27000 *g* for 20 min.

CM-cellulose chromatography. The dialyzed sample was applied directly to a column of CM-cellulose, previously equilibrated with 5 mM Ca acetate, pH 4.7. The column was then washed with *ca* 500 ml of the same buffer before the application of a linear gradient of Ca acetate, pH 4.7 from 5 mM to 35 mM, with a multi-chamber gradient mixer. Fractions containing the activity were combined, dialyzed against H_2O (12 hr, 2 changes) and lyophilized. The freeze-dried material was dissolved in 10 ml of H_2O and dialyzed against a buffer of 0.02 M Tris–HCl, pH 8, 2 mM in $CaCl_2$.

DEAE-cellulose chromatography. The dialyzed sample from CM-cellulose chromatography was applied to a column of DEAE-cellulose, previously equilibrated with 0.02 M Tris–HCl, pH 8, 2 mM in $CaCl_2$. The elution profile is shown in Fig. 2. The active fractions were combined, dialyzed against H_2O (12 hr, 2 changes) and lyophilized. Each of the 4 DNases was rechromatographed on DEAE-cellulose using 20 mM Tris–HCl with a $CaCl_2$ gradient from 2 to 20 mM.

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