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PURIFICATION OF MULTIPLE FORMS OF ADENOSINE DEAMINASE FROM RABBIT INTESTINE

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Summary

Two forms of adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4), differing in molecular size, have been purified and obtained in homogeneous form from rabbit intestine. The purification procedures involved extraction with acetate buffer, pH 5.5, precipitation and fractional reextraction with $(NH_4)_2SO_4$, ion-exchange chromatography on DEAE-cellulose and gel filtration on Sephadex G-75 and Sephadex G-200.

Gel filtrations analysis gave molecular weight estimates of 265 000 and 32 000 for the large and small deaminases respectively.

The two enzyme forms had similar pH optima and pH stability ranges.

Introduction

Numerous investigations have revealed the presence of adenosine deaminase activity in a variety of mammalian tissues [1—6]. Column chromatographic and electrophoretic separations have indicated the presence of several multiple forms of this enzyme. Generally speaking, two types can be identified: multiple forms distinguished by differences in net charges of the protein molecules and multiple forms based on differences in molecular weight [7]. Tissues possessing deaminases of the former type include bovine intestine, lung and spleen, bovine serum, cat lung and human red blood cell. At least 5—6 isoenzymes (molecular weight 35 000) have been isolated and purified from bovine tissues [8—11], and three forms have been identified in the red blood cell [12].

Multiple forms of adenosine deaminase differing in molecular weight have also been shown to be widely distributed. Ma and Fisher [13—17] found three molecular species of the enzyme in the livers of a wide variety of amphibians and mammals, having molecular weights of the order of 200 000, 100 000 and 35 000 as estimated by gel filtration. The authors have designated these types

A, B and C, respectively. Recently, molecular weight forms of the enzyme, which appear to correspond with types A and C, have been demonstrated in human tissues [18].

Studies in this laboratory [19] have shown that rabbit intestine possesses two forms of adenosine deaminase having different molecular weights. The present investigation deals with the purification and partial characterization of the two enzyme from this tissue.

Materials and Methods

Determination of adenosine deaminase activity. Enzyme activity was measured by following the decrease in absorbance at 265 nm [20]. A unit of adenosine deaminase activity is defined as that amount of enzyme which catalyses the hydrolysis of 1 μmol of adenosine/min under standard conditions, i.e. with a 1-cm pathlength cell containing 3.0 ml $1.0 \cdot 10^{-4}$ M adenosine in 0.1 M phosphate buffer (pH 7.0) at 37°C.

Electrophoretic procedures. Polyacrylamide gel electrophoresis was performed in 7% gels at 5 mA and 20°C for 20 min as described by Davis [21]. Gels were stained for protein by immersion in 0.1% Amido black in 7% acetic acid. Adenosine deaminase activity was detected in the gels by a modification of the procedure of Spencer et al. [22]: 10 mg adenosine and 0.2 g agar were dissolved in 15 ml 0.1 M phosphate buffer (pH 7.5) and the mixture was heated to 90°C. On cooling to 40°C, 8 μl nucleoside phosphorylase (1 mg/ml; specific activity 25 units/mg), 8 μl xanthine oxidase (10 mg/ml; specific activity 0.4 unit/mg), 2 mg 3(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide and 2 mg phenazine methosulphate dissolved in 5 ml 0.1 M phosphate buffer (pH 7.5) were added to the agar gel. The acrylamide gels were placed in tubes containing this mixture and incubated in the dark at room temperature for 5 min. Sites of enzyme activity showed up as blue bands on a yellow background.

Thin-layer gel filtration chromatography on Sephadex G-75. Sephadex G-75 superfine was swollen for 24 h in 0.05 M KCl. A 0.5 mm thick layer was spread on a glass plate (20 × 10 cm) using a Miller-Kirchner type spreader (CAMAG, Switzerland). The plate was subsequently inclined to approx. 20° and contact between the gel layer and the KCl solution was made by filter paper wicks. The KCl solution was allowed to flow through the gel for at least 24 h.

10—50 μl of adenosine deaminase solution was then carefully applied to the gel surface. Blue Dextran 2000 was run simultaneously as a marker. Chromatography was stopped when the spot of Blue Dextran had migrated 15 cm from the start. Under our experimental conditions 6—8 h was sufficient for completion. After stopping the run, sites of adenosine deaminase activity were detected by covering the plate with a thin layer of solidified agar (1%) adenosine in saturated phenol violet.

Densitometry. Gels were scanned to determine relative positions and staining intensities with a Joyce Lobel Chromoscan.

Sephadex gel filtration. Sephadex gel filtration for the determination of molecular weights was carried out according to Andrews [23].

Determination of protein. The protein content of crude extracts was deter-

mined by the Biuret method [24]. Elution of proteins during column chromatography was monitored by measuring absorption at 280 nm.

Results

Gel filtration of adenosine deaminase in various rabbit tissues

When supernatant solutions of rabbit intestine were subjected to gel filtration with Sephadex G-75 two peaks of adenosine deaminase activity were observed suggesting the existence of two molecular species of the enzyme differing in molecular size. A preliminary study [25] using aqueous extracts gave estimated molecular weights in the order of 260 000 and 34 000 for the large and small enzymes, respectively. Distribution patterns of the two enzymes in various rabbit tissues were obtained using the thin-layer gel filtration technique. The large adenosine deaminase (mol. wt. 260 000) was present in intestine, liver and spleen, the contribution to the deaminase activity from this enzyme being 30, 100 and 5% in each tissue, respectively. Veriform appendix and brain possessed only the small adenosine deaminase (mol. wt. 34 000).

Purification of the two molecular forms of adenosine deaminase from rabbit intestine

The enzymes were purified using the following procedures. Both enzyme forms were simultaneously purified through Steps 1—4 and thereafter different procedures were used for each deaminase. All operations were carried out at 4°C.

Step 1: Preparations of crude extract. Both wild and domesticated rabbits (New Zealand white variety) were used. Having killed the animals by a sharp blow on the neck, the intestines were removed and flushed with a gentle stream of tap water. 300 g of whole intestine was then homogenized with five volumes of extraction buffer (0.2 M sodium acetate, pH 5.5) and the homogenate was clarified by centrifugation at 15 000 × *g* for 20 min.

Step 2: $(NH_4)_2SO_4$ precipitation and re-extraction. The supernatant (1820 ml) from Step 1 was brought to 60% saturation with solid $(NH_4)_2SO_4$ which precipitated all deaminase activity. The resulting precipate was collected by centrifugation at 15 000 × *g* for 20 min and was subjected to further fractionation. The fractionation was done by extracting the solid precipitate with a series of $(NH_4)_2SO_4$ solutions of decreasing saturation [26]. Most of the adenosine deaminase activity was re-extracted between 20 and 40% $(NH_4)_2SO_4$.

Step 3: Dialysis against distilled water. The 20—40% $(NH_4)_2SO_4$ extract from Step 2 was dialysed overnight against distilled water. This both removed the $(NH_4)_2SO_4$ and caused precipitation of a large amount of inactive protein. The inactive precipitated protein was removed by centrifugation giving a 5-fold purification of the deaminases.

To justify the choice of re-extraction with $(NH_4)_2SO_4$ rather than precipitation, enzyme solution from a separate batch of intestines was subjected to $(NH_4)_2SO_4$ fractionation in the conventional method, followed by dialysis. That this 'extraction method' is more efficient than the 'precipitation method' is evident from Table I.

Step 4: DEAE-cellulose chromatography. The enzyme solution from Step 3

TABLE I

SPECIFIC ACTIVITIES OF RABBIT INTESTINAL ADENOSINE DEAMINASE OBTAINED BY $(NH_4)_2SO_4$ FRACTIONATION

The precipitation method involved precipitation of the enzyme protein with increasing concentrations of $(NH_4)_2SO_4$, starting with the lowest one. The extraction method was initiated from the highest concentration of $(NH_4)_2SO_4$, as outlined by Ghosh and Fishman [26].

	Purification step	Specific activity (units/mg)
Precipitation method	40—60% $(NH_4)_2SO_4$	0.31
	Dialysis against water	0.90
Extraction method	20—40% $(NH_4)_2SO_4$	0.30
	Dialysis against water	1.80

(887 mg) was adsorbed to a DEAE-cellulose column (1.5 × 25 cm) equilibrated with 2.0 mM sodium citrate buffer (pH 5.8). Washing of the column overnight with the same buffer was followed by elution with a linear buffer gradient which formed with 300 ml of equilibration buffer in the mixing chamber and with 300 ml of 30 mM sodium citrate buffer (pH 5.8) in the reservoir.

Fig. 1 shows the elution pattern of adenosine deaminase activity from the column. Both forms of the enzyme eluted in the same position. However, it is evident from Table II that the relative amounts of the two deaminases differed in various regions, the small deaminase contributing to most of the activity in the mid region of the peak (β region) and the large deaminase contributing to most of the activity in the side regions (α and γ regions). From a knowledge of the total number of enzyme units and the relative amounts of the two forms in

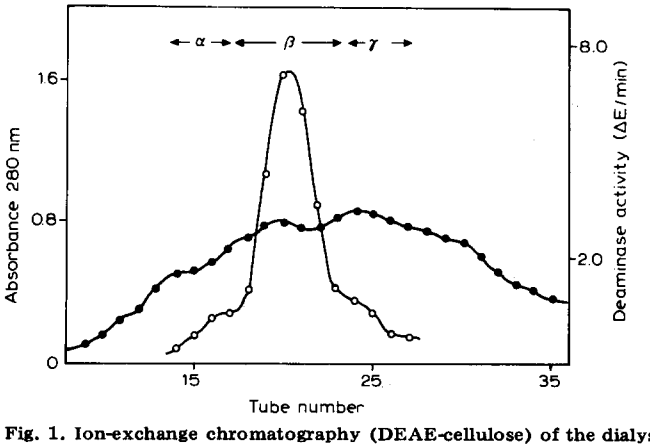

Fig. 1. Ion-exchange chromatography (DEAE-cellulose) of the dialysed 20—40% $(NH_4)_2SO_4$ extract. Elution was achieved with a linear gradient of citrate buffer ranging from 2.0 to 30 mM at a constant pH of 5.8. The eluate fractions were assayed for protein at 280 nm (●——●) and for adenosine deaminase activity (○——○). The enzyme peak divided into three regions (α, β and γ) when the eluate fractions were being pooled.

TABLE II

RELATIVE AMOUNTS OF THE LARGE AND SMALL ADENOSINE DEAMINASES IN VARIOUS REGIONS OF THE ENZYME PEAK AFTER DEAE-CELLULOSE CHROMATOGRAPHY

50-μl samples from each region (α, β and γ) of the enzyme elution curve were spotted on to a thin-layer Sephadex G-75 plate and after development the gel was scanned on a Joyce Label Chromoscan. The relative amounts of the two deaminases was obtained by measuring the areas under the peaks.

Region of enzyme peak	Percent adenosine deaminase activity *	
	Large deaminase	Small deaminase
α	100	0
β	40	60
γ	80	20

* 100% adenosine deaminase activity is taken to be equivalent to the total area under both peaks (large and small deaminases) obtained on the chromoscan chart.

each region, it was calculated that approx. 50% of the applied large deaminase eluted in the β region while the remaining 50% was distributed between the α and γ regions. This necessitated using all three regions of the peak for further purification of the large enzyme.

Gel filtration

The β region (tubes 18—24) after DEAE-cellulose chromatography was dialysed overnight against distilled water and concentrated to a volume of approx. 1 ml using a small DEAE-cellulose column (2.5 × 1.0 cm). 2 M NaCl was used to elute the enzyme from the concentration column. The concentrated enzyme solution was then applied to a Sephadex G-75 column (50 × 2.8 cm) equilibrated with 2 mM citrate buffer, pH 5.8. The elution pattern (Sephadex G-75 I_β) is shown in Fig. 2. This step completely separated the two forms of the deaminase.

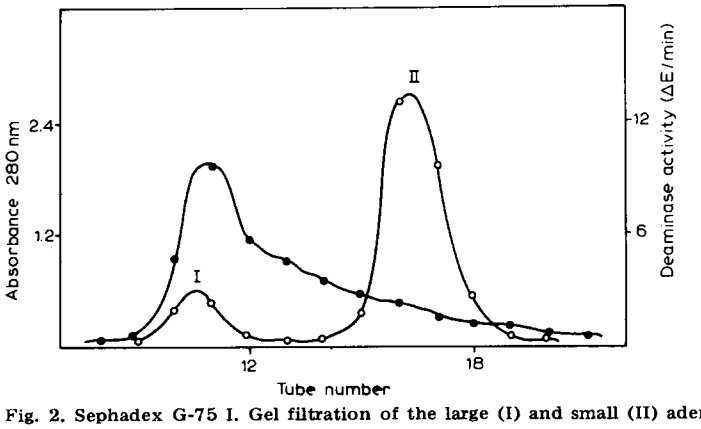

Fig. 2. Sephadex G-75 I. Gel filtration of the large (I) and small (II) adenosine deaminases on Sephadex G-75. The β region from the DEAE-cellulose step (specific activity 15.2 enzyme units) was concentrated and applied to the Sephadex column which was equilibrated with 2 mM citrate buffer, pH 5.8. Protein was determined by absorption at 280 nm (●——●) and enzyme activity was estimated spectrophotometrically at 265 nm (○——○).

Further purification of the small deaminase

Tubes 15—18 from the gel filtration step (which contained the small deaminase) were pooled, concentrated as before and re-chromatographed on the Sephadex G-75 column. Concentration followed by re-chromatography, which increased the specific activity of the enzyme, was repeated a third and fourth time. The final elution profile showed a peak of enzymic activity coincident with a single protein peak.

Further purification of the large deaminase

Tubes 10—11 from Sephadex G-75 I_β (which contained the large deaminase) were pooled, concentrated and applied to a Sephadex G-200 column (40 × 2.5 cm) equilibrated with 2 mM sodium citrate buffer (pH 5.8). Fractions containing adenosine deaminase activity were pooled and retained.

The combined α and γ regions from the DEAE-cellulose chromatography step (Step 4) were pooled, concentrated and applied to a Sephadex G-75 column. This step removed the residual amount of small deaminase. Tubes containing the large deaminase were pooled, concentrated and chromatographed on the Sephadex G-200 column.

The final step in the purification of the large deaminase involved combining fractions from the two Sephadex G-200 steps and re-chromatographing the resulting solution on the Sephadex G-200 column.

A summary of the purification procedures including the results of protein and enzyme assays is given in Table III.

Evidence for purity of the enzymes

The purity of the final products was examined by polyacrylamide gel elec-

TABLE III

SUMMARY OF PURIFICATION OF THE SMALL AND LARGE ADENOSINE DEAMINASES FROM RABBIT INTESTINE

	Volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Crude extract	1820	11375	1874	0.16	100
$(NH_4)_2SO_4$, 20—40%	248	8600	1609	0.19	86
Dialysis against water	606	887	1178	1.33	62.2
DEAE-cellulose (β region)	86	51.6	800	15.2	42
(α + γ regions)	80	67.2	212	3.2	12

Small deaminase				Large deaminase			
	Total protein (mg)	Specific activity (units/mg)	Yield (%)		Total protein (mg)	Specific activity (units/mg)	Yield (%)
Sephadex G-75 I_β	7.6	70	30	Sephadex G-75 I_β	10.6	7	4.2
Sephadex G-75 III_β	2.6	180	22	Sephadex G-200$_\beta$	4.5	14	3.4
Sephadex G-75 IV_β	1.8	210	19	Sephadex G-75$_{\alpha\gamma}$	15	5.8	4.8
				Sephadex G-200$_{\alpha\gamma}$	5	12.5	3.3
				Final Sephadex G-200	2.1	30	3.2

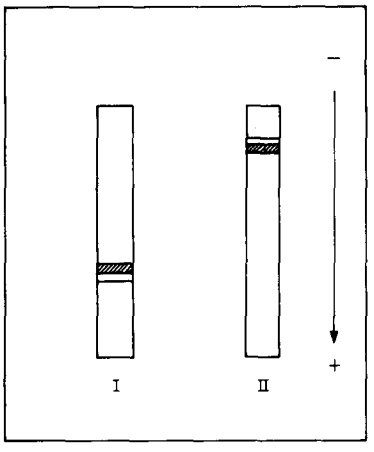

Fig. 3. Polyacrylamide gel electrophoretic patterns of (I) purified small and (II) purified large adenosine deaminases. 10 µl of each enzyme preparation was applied to the gels and electrophoresis performed as described in Materials and Methods. Protein bands were visualized with 0.1% Amido black.

trophoresis. A major and a minor protein band was obtained for each deaminase (Fig. 3). When separate gels were stained for adenosine deaminase activity both protein bands in each gel were enzymatically active.

Molecular weights

The molecular weights of the enzymes were estimated by analytical gel filtration [23] on a 2.5 × 50 cm column of Sephadex G-200 equilibrated with 2 mM sodium citrate buffer, pH 5.8, containing 0.1 M KCl and standardized with thyroglobulin, γ-globulin, pepsin and myoglobin. Molecular weights of 265 000 and 32 000 were obtained for the large and small deaminases, respectively.

pH optima and adenosine deaminase stability

Maximum activity of both forms of the enzyme was observed at pH 6.5 with adenosine as substrate. When deoxyadenosine was used as substrate the large enzyme was optimally active at pH 6.0. For examination of pH stability the deaminases were incubated at various pH values at 37°C for 24 h and on adjusting the pH to 6.5 residual activity was determined. Both forms of the enzyme retained almost 100% activity over the pH range 5.0—9.0.

The heat stability of each enzyme was examined by measuring the activity of enzyme solutions heated in a water bath at 20, 37, 50, 60 and 70°C for 15 min and then assayed at 37°C. The activity of both forms dropped to zero between 60 and 70°C.

Discussion

Adenosine deaminases have been purified from a variety of sources such as bovine intestine [8], lung and spleen [10], bovine serum [9] and chicken duodenum. All of these enzymes have been shown to possess molecular weights in the region of 35 000. Recently Ma and Fisher [13—17] have demonstrated

that adenosine deaminases of higher molecular weight exist and they classified the enzymes into types A, B and C having molecular weights in the order of 200 000, 110 000 and 35 000, respectively.

Interest in the type A deaminases has centered around the relationship between those enzymes and the lower molecular weight deaminases (type C). Akedo et al. [27] have demonstrated the presence of a type A adenosine deaminase (mol. wt. 200 000) in human tissues and they have suggested that the enzyme consists of a 'conversion factor' bound to a type-C deaminase (mol. wt. 50 000).

The present study demonstrates the existence of type A and C adenosine deaminases in rabbit intestine. These enzymes resemble the type A and C deaminases purified by Adedo et al. [27] in that the specific activity of the large type A enzyme (30 enzyme units/mg) is much lower than that of the small type C enzyme (210 enzyme units/mg). A pattern which appears to be emerging therefore is that the type A adenosine deaminases have very low specific activities. Preliminary studies on the properties of the two rabbit deaminases showed that they have similar pH optima, pH stabilities and heat stabilities. The pH optima of 6.5 is, however, lower than the pH optima of 7.0 found for the bovine enzyme.

A further report dealing with enzymological and structural properties of the two enzymes is in preparation.

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