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ADENOSINE DEAMINASE FROM PIG THYROID GLAND
PURIFICATION AND SOME PROPERTIES

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SUMMARY: Adenosine deaminase was isolated from the pig thyroid gland and purified over 900-fold using DEAE Sephadex A-50 column chromatography, Sephadex G-100 gel filtration and DEAE Sephadex A-50 rechromatography. The enzyme was specific towards adenosine. The Michaelis constant based on the Lineweaver-Burk plot was 5×10^{-5} M. The optimum pH was about 7.0, and molecular weight 44 700.

During the course of studies on thyroid adenylate deaminase in this laboratory, an appreciable and specific adenosine deaminase activity was detected in pig thyroid extracts (1). Adenosine deaminase (adenosine aminohydrolase EC 3.5.4.4), catalyzing irreversible hydrolytic deamination of adenosine to inosine and ammonia, was found in some mammalian tissues and has been studied intensively (2 - 15). Adenosine and adenine nucleotides affects three important biological processes: neurotransmission (16), blood platelet function (17), and vasodilatation (18). It was reported that adenosine, by adenylate cyclase activation or inhibition, modulated cAMP levels in many tissues according to the species, tissue source, and concentration used (19). Thus, adenosine deaminase by affecting the adenosine concentration may play an important role in the regulation of cellular metabolism. The enzyme from the thyroid has not until

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now been purified nor have its properties been studied. In the present paper, a method for its purification and some properties of the purified enzyme are described.

MATERIALS AND METHODS

Materials. Various nucleotides and nucleosides, cytochrome c, were obtained from Sigma Chemical Co., DEAE Sephadex A-50, Sephadex G-100 from Pharmacia, bovine serum albumin from Calbiochem, and other reagents from POCH Gliwice (Poland).
The pig thyroid glands were obtained from the slaughterhouse. After the removal of the outer membranes and fat, the thyroid glands were kept frozen at -20°C.

Enzyme assay. The activity of adenosine deaminase was assayed by the determination of ammonia liberated from adenosine (20). The incubation mixture contained, in a final volume of 0.5 ml, 0.1 M phosphate buffer, pH 7.0, 2 mM adenosine, and the enzyme. The reaction was usually carried out at 37°C for 10 min and stopped by adding 1.5 ml deproteinizing agent. In the control experiment a deproteinizing agent was added to the mixture at 0 time. Ammonia was assayed after centrifugation in the 0.5 ml samples. One unit of the adenosine deaminase activity was defined as that amount of enzyme catalyzing the formation of one micromole of ammonia per minute.
For kinetic assays, adenosine deaminase activity was also determined spectrophotometrically by following the decrease in absorbance at 265 nm resulting from the conversion of adenosine to inosine (21, 22). Measurements were made in cuvettes of 1 cm pathlenght containing 3.0 ml of 0.04 mM adenosine in phosphate buffer, pH 7.0. The cells were thermostated at 30°C. The reaction was initiated by introduction of 5 to 20 µl of the enzyme solution. The value of the change in molar absorbance for adenosine at pH 7.0 was taken to be 8100 M^{-1} cm^{-1} at 265 nm.

Protein concentration was estimated by the method of Lowry et al. (23) with bovine serum albumin as the standard.

Purification of the enzyme. The finely chopped thyroid glands were homogenized in 0.01 M acetate buffer, pH 6.4 (1 g tissue + 3.3 ml buffer) in a Potter homogenizer. The homogenate was centrifuged for 15 min at 15 000 g and the sediment was discarded. The supernatant, after being chilled in ice, was adjusted to pH 5.0 with 2 M acetic acid. After 1 hour, the suspension was centrifuged and the precipitate was discarded. The supernatant was adjusted with 2 M NaOH to pH 6.4 and was applied to DEAE Sephadex A-50 column (4 x 15 cm) equilibrated with 0.01 M acetate buffer, pH 6.4. After washing the column with the same buffer, the enzyme was eluted with 0.01 M acetate buffer, pH 6.4 containing 2 M NaCl. The collected enzyme solution was applied to Sephadex G-100 column (4.4 x 50 cm) that had been equilibrated previously with 0.01 M acetate buffer, pH 6.4. Elution was carried out with the same buffer. Fractions containing adenosine deaminase were pooled and dialyzed overnight against 0.01 M phosphate buffer, pH 7.4. The dialyzed enzyme was chromatographed on DEAE Sephadex A-50 column (1 x 15 cm) equilibrated with 0.01 M phosphate buffer, pH 7.4. After washing with 0.01 M phosphate buffer, pH 7.0, the enzyme was eluted with a linear gradient of 0 to 0.4 M NaCl in 0.01 M phosphate buffer, pH 7.0. The fractions containing

adenosine deaminase were pooled and concentrated by vacuum dialysis against 0.01 M phosphate buffer, pH 7.0.
All the buffers used for the preparation of the enzyme contained 0.02 % NaN_3.

Molecular weight was determined by the method of gel filtration described by Andrew (24). A column (1.2 x 62 cm) was packed with Sephadex G-100 in 0.05 M phosphate buffer, pH 7.0, and eluted with the same buffer. The reference proteins were: cytochrome c, bovine serum albumin and glucose oxidase.

Polyacrylamide gel electrophoresis was performed by the method of Davis (25).

RESULTS

The results of purification of pig thyroid adenosine deaminase at particular stages are summarized in Table I. By means of precipitation of the protein impurities at pH 5.0, chromatography on DEAE Sephadex A-50 column, gel filtration on Sephadex G-100 (Fig.1), and rechromatography on DEAE Sephadex

Table I PURIFICATION OF ADENOSINE DEAMINASE

Fraction	Volume (ml)	Protein (mg/ml)	Total activity (μmol/min)	Specific activity (μmol/min/mg protein)	Yield (%)	Purification (-fold)
Homogenate	490	34.0	583.1	0.035	100	1.0
Crude extract	420	26.5	534.2	0.048	91.6	1.3
pH 5.0 soluble fraction	395	22.6	526.7	0.059	90.3	1.6
DEAE Sephadex A-50 column eluate	125	8.2	348.5	0.34	59.7	9.7
G-100 Sephadex column filtrate	100	0.6	240.0	4.0	41.6	114.3
DEAE Sephadex A-50 column eluate (linear gradient)	40	0.09	123.1	34.2	21.1	977.1

For experimental details see the text. The starting material was 125 g of the frozen thyroid

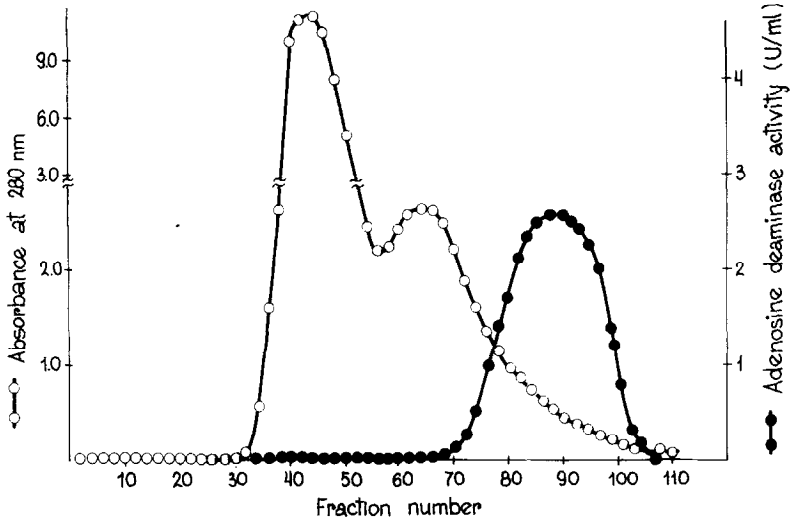

Fig.1 Sephadex G-100 chromatography column of adenosine deaminase. The enzyme solution (about 100 ml) was applied to the column (4.5 x 65 cm) equilibrated with 0.01 M acetate buffer, pH 6.4. The enzyme was eluted with the same buffer, and 5 ml fractions were collected.

A-50 (Fig.2), over 900-fold purification of the enzyme with a specific activity of approximately 34 μmole/min/mg protein was obtained. The application of such procedures as: ammonium

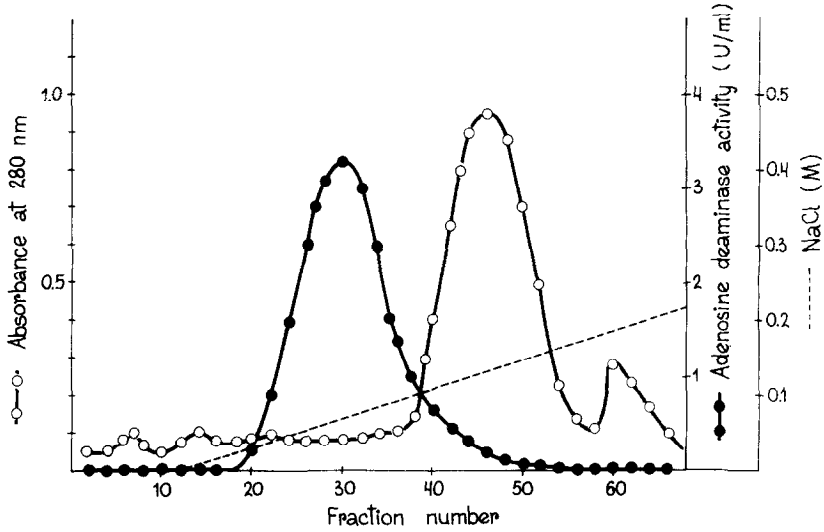

Fig.2 DEAE Sephadex column chromatography of adenosine deaminase. The dialyzed enzyme solution (about 90 ml) was applied to the DEAE Sephadex A-50 column (1 x 15 cm) equilibrated with 0.01 M phosphate buffer, pH 7.4. After washing with 0.01 M phosphate buffer, pH 7.0, the enzyme was eluted with a linear gradient of 0 - 0.4 M NaCl in 0.01 M phosphate buffer, pH 7.0. 5 ml fractions were collected.

Table II SUBSTRATE SPECIFICITY OF ADENOSINE DEAMINASE

Substrate	Relative velocity
Adenosine	1.0
Adenine	0.0
5'-AMP	0.0
2'-AMP	0.0
3'-AMP	0.0
ADP	0.0
ATP	0.0
Guanosine	0.0
Cytosine	0.0
Guanine	0.0

Assays were carried out by colorimetric method in the presence 2 mM concentrations of the substrate in 0.05 M phosphate buffer, pH 7.0.

sulphate fractionation, precipitation with ethanol and acetone, the use of an Octyl-Sepharose 4B column did not give satisfactory results.

Properties of the enzyme.

Substrate specifity. The thyroid adenosine deaminase catalyzed the deamination adenosine as the preferred substrate and had no activity towards adenine, 5'-AMP, 2'-AMP, 3'-AMP, ADP, ATP, guanosine, cytosine and guanine (table II).

The enzyme was free of detectable 5'-nucleotidase activity.

Kinetic properties. The Michaelis constant according to the Lineweaver-Burk plot was 5×10^{-5} M. The determination were made by the spectrophotometric method in 0.05 M phosphate buffer, pH 7.0.

Effect of pH. The optimum activity of pig thyroid adenosine deaminase was about pH 7.0 (Fig.3).

Molecular weight. The molecular weight was 44 700 determined by gel filtration technique.

Adenosine deaminase from pig thyroid gland was inhibited 100 % by $HgCl_2$ in a concentration of 1 mM, and 98 % in a concentration of 1 uM. No inhibition was observed in the presence of 100 uM dipyridamole.

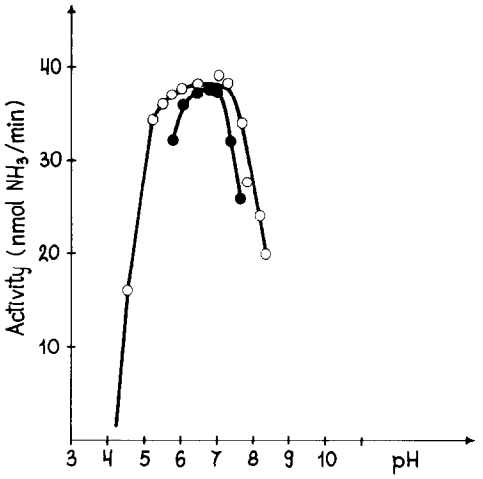

Fig.3 The pH optimum of adenosine deaminase. The reaction mixture contained 2 mM adenosine, the enzyme, and buffer in a final volume of 0.5 ml. Time of incubation 10 min. -o—o- 0.143 M veronal buffer. -•—•- 0.1 M phosphate buffer. Each point represents the mean from three experiments.

The enzyme was not homogenous in electrophoresis on polyacrylamide gel. It gave 3 protein bands.

DISCUSSION

It was previously shown that in the pig thyroid gland both AMP deaminase and adenosine deaminase occur, the specific activity of the latter being considerably higher (1, 26). In the paper presented here, a method by which it is possible to purify the enzyme over 900-fold with a yield of approximately 20 % is described. The enzyme obtained is specific in relation to adenosine. It does not deaminate either adenine or AMP. Adenosine deaminase from the thyroid is inhibited by Hg^{2+} ions, like the deaminases from other sources (6, 8, 10, 11) thus indicating the presence of the -SH groups essential for the enzymatic activity. The enzyme from the thyroid gland is not inhibited by dipyridamole, a known adenosine uptake inhibitor.

The molecular weight of thyroid adenosine deaminase is approximately 44 700. It appears from the data in the litera-

ture that adenosine deaminase is an enzyme which occure in the tissues in various molecular forms. Akedo et al. (3) reported the separation of high and low molecular forms of adenosine deaminase from extracts of human lung and stomach with a molecular weight of 47 000 and 230 000. Van den Weyden and Kelley (27), on the other hand, showed the presence in human tissues of as many as four molecular species with estimated molecular weights of >20 000 000, 298 000, 114 000 and 36 000 (designated particulate, large, intermediate and small forms respectively). Furthermore, the small form of the enzyme regardless of tissue source exhibits at least three electrophoretic variants. The results of the present investigations indicate that in the thyroid gland, adenosine deaminase of a low molecular weight is to be found. The presence of forms of a higher molecular weight was not noted but it is possible that such a form may have been removed during the preparation of the enzyme. The preliminary experiments showed that the thyroid adenosine deaminase under certain conditions probably associate, resulting in the fall in the enzyme activity. The association and dissociation of deaminase molecules resulting in changes of the enzyme activity may be of great physiological significance. Studies on this problem are in progress.

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