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RESOLUTION OF ENZYMES THAT HYDROLYSE PHOSPHATE ESTERS FROM *AGAVE CANTALA* ON AMBERLITE IR-120 (Al^{3+})

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SUMMARY

A technique for the resolution of functionally similar enzymes is described. Enzymes that hydrolyse phosphate esters, *viz.*, 3′-nucleotidase, pyrophosphatase, phosphatase, and ribonuclease, from *Agave cantala* have been resolved on an IR-120 (Al^{3+}) column free from each other, and their eventual purification thereby obtained is described.

INTRODUCTION

Although several chromatographic procedures exist for the separation of enzymes and proteins, often they do not provide adequate resolution of complex mixtures of functionally similar proteins and enzymes necessary for their precise individual study. In addition, they are not free from operational limitations such as clogging of the column, slow flow-rates leading to irreversible adsorption and consequent loss of biological activity, denaturation and low recoveries.

Proteins, being macromolecules with an infinite variety of molecular species that are very closely related, can be visualised theoretically. When any single criterion of separation is applied, groups of molecules will be segregated. Such an apparently homogeneous material can still be a mixture of very closely related molecules, which may differ marginally with respect to one applied criterion but widely by another. It is evident, therefore, that a number of distinct separation procedures based on different properties of the molecules must be applied in order to achieve their ultimate resolution. For this reason, new methods for the fractionation of proteins and other macromolecules have some value beyond whatever advantages and convenience they may offer.

A method of column chromatography on Amberlite IR-120 (Na^+) equilibrated with aluminium ions developed in this laboratory for the fractionation of DNA[1,2] and RNA[3–6] has already been reported. The separation of chymopapain from papaya latex[7] on IR-120 (Hg^{2+}) has been described.

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This paper reports the resolution of enzymes (3′-nucleotidase, pyrophosphatase, phosphatase and ribonuclease) from *Agave cantala* using Amberlite IR-120 (Al^{3+}).

EXPERIMENTAL

Preparation of Amberlite IR-120 (Al^{3+}) column

Ten grams of dry regenerated Amberlite IR-120 (Na^+) were converted into the Al^{3+} form as described earlier[3] and equilibrated to pH 4.8 with 0.05 *M* acetate buffer. The amounts of aluminium in the influent, effluent and buffer washings were determined colorimetrically by the 8-hydroxyquinoline method[8]. It was observed that 1 mequiv. of Al^{3+} per gram of the resin was retained under these conditions.

Extraction of the enzyme

Leaves of *Agave cantala* preserved at −10° were cut and homogenized in ice-cold saline in a Waring blender for 5 min. A 90-ml volume of 0.14 *M* saline was added per 100 g of *Agave cantala* leaves taken for extraction. The extract was allowed to stand for 1 h at 0° for the insoluble matter to settle.

The supernatant was passed through cheese-cloth and the filtrate was centrifuged at 5000 *g* for 10 min. The resultant pale yellow-green extract was used as the source of enzyme.

Activity and protein determination

Enzyme activities were determined by determining the inorganic phosphorus[9] (P_i) liberated from the specific substrates and conditions as shown in Table I.

After terminating the reaction with trichloroacetic acid (TCA), aliquots were centrifuged at 5000 *g* and P_i determined by Fiske and Subbarow's method[9].

TABLE I

CONDITIONS FOR ENZYME ASSAY

Enzyme	*Samples (suitably diluted) (ml)*	*Substrate*	*Acetate buffer, pH 4.8, 0.05 M (ml)*	*Incubation time*	*10% TCA (ml)*
Phosphatase	1.0	Sodium β-glycerophosphate (0.01 *M*), 1.0 ml	1.0	15 min	2.0
Pyrophosphatase	1.0	Sodium pyrophosphate (0.01 *M*), 1.0 ml	1.0	15 min	2.0
3′-Nucleotidase	0.5	Adenylic acid (0.04 *M*), 1.0 ml	5.0	1 h	1.0
Ribonuclease	1.0	RNA (1 mg/ml), 2.0 ml	2.0 + 3.0 ml of water* or 3′-nucleotidase	24 h	2.0

* Except for the crude extract which contains adequate 3′-nucleotidase, external addition of *Agave cantala* 3′-nucleotidase (free from RNAse) ensured P_i liberation from the nucleotides formed by RNAse action. The P_i liberated wzs taken as a measure of RNAse.

Definition of enzyme unit

One unit of phosphatase, pyrophosphase, 3′-nucleotidase and ribonuclease is defined as the activity that liberates 0.1 mg of P_i under the above special conditions at 37° and with corresponding substrates.

Protein contents of various fractions were determined by the Folin–Ciocalteu method[10]. Specific activities are expressed as units of enzymes per milligram of protein in the fraction.

Chromatographic studies

A 25.0-ml volume of the above extract was loaded on the column of Amberlite IR-120 (Al^{3+}) buffered at pH 4.8. A flow-rate of 2.0 ml/min was maintained throughout the operation. After complete passage of the extract, the column was flushed with 0.05 *M* acetate buffer (pH 4.8) to remove tailing material. Elution of adsorbed enzymes was achieved with a discontinuous gradient of ammonium acetate. The influent, effluent, subsequent buffer washings and the different fractions obtained were assayed for phosphatase, pyrophosphatase, 3′-nucleotidase and ribonuclease activities and protein content.

RESULTS

A typical elution profile, showing the clear separation of the four enzymes phosphatase, pyrophosphatase, 3′-nucleotidase and ribonuclease, is presented in Fig. 1. The enzymes were eluted in the order pyrophosphatase, phosphatase and ribonuclease with recoveries of 98.7, 89.9 and 76.5% and purifications of 3.97-, 14.5- and 4.38-fold, respectively. 3′-Nucleotidase was not adsorbed and was contained in the effluent free from pyrophosphatase, phosphatase and ribonuclease activities. Its recovery was 97.1% with 4.5-fold purification (see Table II).

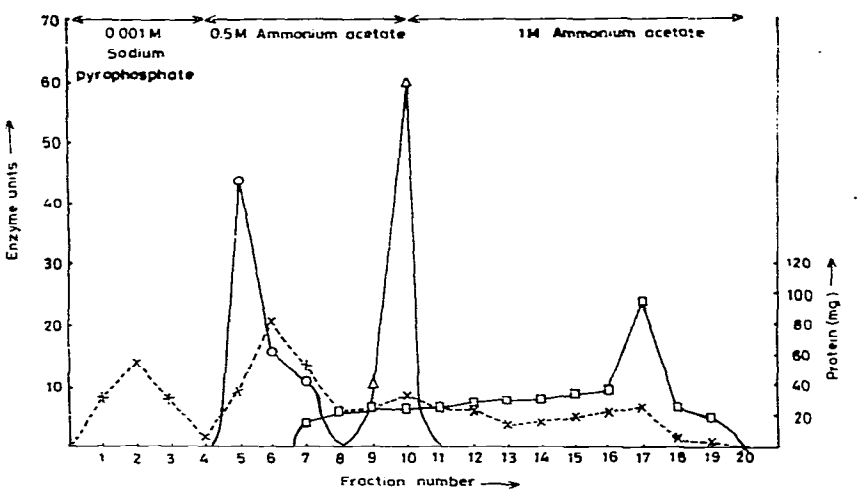

Fig. 1. Elution pattern of enzymes that hydrolyse phosphate esters from *Agave cantala* adsorbed on an IR-120 (Al^{3+}) column. ○, Pyrophosphatase; △, phosphatase; □, ribonuclease; ×, protein (mg).

TABLE II

PURIFICATION OF *AGAVE CANTALA* ENZYMES ON AN IR-120 (Al^{3+}) COLUMN

Enzyme	*Fraction*	*Volume (ml)*	*Total enzyme (units)*	*Total protein (mg)*	*Specific activity*	*Enrichment ratio*	*Recovery (%)*
3′-Nucleotidase	(1) Original extract	25	87.5	950	0.092	—	100
	(2) Effluent and first washing	50	84.88	205	0.414	4.5	97.1
	(3) Ammonium sulphate precipitation	5.0*	79	7.5	10.53	114	90.2
Pyrophosphatase	(1) Original extract	25	71.57	950	0.075	—	100
	(2) Combined eluate (5–7)	75	70.67	176	0.401	5.3	98.74
	(3) Ammonium sulphate precipitation	5.0*	50	20.5	2.439	32.4	69.87
Phosphatase	(1) Original extract	25	78.4	950	0.082	—	100
	(2) Combined eluate (9–10)	50	59	1.196	14.5	14.5	90
	(3) Ammonium sulphate precipitation	5.0*	60.33	5.5	10.96	132.9	76.95
Ribonuclease	(1) Original extract	25	109.1	950	0.114	—	100
	(2) Combined eluate (11–19)	225	83.59	165.57	0.503	4.38	76.58
	(3) Ammonium sulphate precipitation	7.0*	71.2	16.5	4.315	37.6	65.31

* Precipitate of the protein obtained with 2.3 *M* ammonium sulphate concentration was dissolved in acetate buffer (pH 4.8, 0.05 *M*).

The method gives a substantial increase in the specific activities of these enzymes and the pooled fractions can be further purified by ammonium sulphate precipitation. The pooled fractions of the different enzymes were fairly free from each other. With the application of only two general methods of purification, *viz.*, passage through an IR-120 (Al^{3+}) column and ammonium sulphate precipitation, one would not expect all of the above enzymes to separate into discrete pure fractions. The enzymes are, in fact, only semi-purified and have not reached a homogeneous state of purity. The significant point is that they are fairly free from each other.

DISCUSSION

The results indicate that an IR-120 (Al^{3+}) column can clearly resolve the enzymes pyrophosphatase, phosphatase, ribonuclease and 3'-nucleotidase from *Agave cantala* leaves. A close examination of the recoveries indicates that there are no significant losses of these enzymes on the IR-120 (Al^{3+}) column under the conditions used.

There are certain inherent advantages that Al^{3+}-equilibrated resins offer. Flow-rates through the IR-120 (Al^{3+}) column are fast and no choking of the column results. This makes the operation very easy and the whole column operation can be finished in a short period with little danger of inactivation of the enzymes.

It should be further noted that Amberlite IR-120 cannot be used in either the H^+ form for enzyme fractionation. With the highly acidic H^+ form, the enzymes are denatured and inactivated. Further, proteins have very little affinity towards the Na^+ form of the resin. Therefore, it is interesting that IR-120 (Al^{3+}) columns can be very useful for enzyme fractionation; they retain 75 mg of *Agave cantala* proteins per gram of the Al^{3+} form of the resin.

Although the affinity of heavy metal ions such as Pb^{2+}, Hg^{2+}, Ag^{2+}, Zn^{2+} and Cu^{2+} towards active groups such as COOH, NH_2, SH, imidazole, phenolic OH and indole in proteins is known, these metal ions cannot be applied with enzymes as many o them are inhibitors. In this respect, Al^{3+} is sae.

It must be noted, however, that aluminium ions are eluted together with the eluting agents used and are distributed in all of the fractions, but they do not interfere in the activity determinations.

REFERENCES

1 P. N. Joshi, S. Joshi and R. M. Kothari, *Abstracts, International Convention of Biochemists, Bangalore, 1967: Ind. J. Biochem.*, 4 (1967) 41
2 P. N. Joshi, *IX International Congress of Biochemistry, Stockholm, 1973*, Abstracts p. 30.
3 V. Shankar and P. N. Joshi, *J. Chromatogr.*, 90 (1974) 99.
4 V. Shankar and P. N. Joshi, *J. Chromatogr.*, 95 (1974) 65.
5 V. Shankar and P. N. Joshi, *J. Chromatogr.*, 104 (1975) 443.
6 V. Shankar and P. N. Joshi, *J. Chromatogr.*, 104 (1975) 449.
7 P. N. Joshi, V. Shankar, K. I. Abraham and K. Srinivasan, *J. Chromatogr.*, 121 (1976) 65.
8 E. B. Sandel, *Colorimetric Determination of Traces of Metals*, Interscience, New York, 2nd ed., 1950, p. 143.
9 C. H. Fiske and Y. Subbarow, *J. Biol. Chem.*, 66 (1925) 375.
10 O. Folin and V. Ciocalteu, *J. Biol. Chem.*, 73 (1927) 627.