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Note

Sephadex columns equilibrated with NaCl to purify invertase, acid phosphatase and glycosidases from plants

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During purification of invertase[1], acid phosphatase[2] and β-glucosidase from wheat leaves, forms of these enzymes were encountered that eluted from Sephadex G-200 gels in the void volumes. Other forms of β-glucosidase, β-galactosidase and invertase were found that eluted as if they were quite small proteins. These enzymatically active small proteins could not always be recovered if rechromatographed on the same columns of Sephadex G-200.

The enzymes eluting in the void volume contained soluble polysaccharides (unpublished, *cf.* ref. 3). There are numerous reports that some enzymes bound to cell walls[4–9] and other insoluble cell components[10,11] may be eluted from the debris in homogenates with 1 *M* NaCl solutions. Other enzymes may be disaggregated by high concentrations of monovalent cations[12,13]. Solutions of NaCl can prevent the interactions between glycosaminoglycans and basic polypeptides in some cases[14].

Since the form of invertase that behaves as a small protein on Sephadex G-200, is a basic protein[1], it might adsorb on the few acidic groups of Sephadex G-200 and, consequently, be eluted from the column with 0.01 *M* sodium maleate buffer, pH 6.5, much later than it would if molecular size were the only factor controlling elution volume. This behavior has been observed with several small basic proteins on Sephadex G-50[15–17].

Experiments with Sephadex G-200 columns, equilibrated with NaCl, were done to try to separate those enzymes that were eluted in the void volume of Sephadex G-200 columns from the soluble carbohydrate materials, and to prevent the adsorption of small basic proteins on Sephadex G-200.

EXPERIMENTAL

Juice from the leaves of Kharkov 22 MC winter wheat was clarified, concentrated and chromatographed on a Sephadex G-200 column, eluted with 0.01 *M* sodium maleate buffer pH 6.5[1]. The fractions obtained were assayed for invertase[1], acid phosphatase hydrolyzing adenosine-5-phosphate[18], β-glucosidase, and β-galactosidase to identify those fractions containing activity. The glycosidases were assayed by incubating 0.5 ml of the unknown with 0.5 ml of McIlvaine's buffer[19] containing 2.4 mg/ml of either *p*-nitrophenyl β-D-glucopyranoside or *p*-nitrophenyl β-D-galactopyranoside. The liberated *p*-nitrophenol was assayed colorimetrically (No. 42 filter,

Klett Summerson) after the addition of 4 ml 1 *M* Na_2CO_3. For rechromatography, the appropriate fractions were combined and concentrated by dialysis against 30% aqueous polyethylene glycol (MW 20,000). The concentrate was applied to the same column, equilibrated either with 0.01 *M* sodium maleate buffer, pH 6.5, or maleate buffer containing 1 *M* NaCl, and eluted with the equilibrating solution. Fractions were assayed for invertase, acid phosphatase, and glycosidases.

RESULTS AND DISCUSSION

Disaggregation of large-molecular complexes

The fractions representing the void volume from the first chromatographing on Sephadex G-200, eluted with buffer only, contained "large-molecular" forms of invertase, acid phosphatase, and β-glucosidase. When these fractions were concentrated and rechromatographed under the same conditions, the invertase was eluted again as a single peak in the void volume[1], the bulk of the acid phosphatase was eluted in the void volume[2], and the β-glucosidase was eluted partly in the void volume and partly as two distinct peaks of lower molecular weight (unpublished).

The conditions required to disaggregate the high-molecular-weight form of invertase were explored in experiments using small-bore columns of Sephadex G-50 and G-200 connected in series[20]. Disaggregation was greater in columns equilibrated and eluted with 1 *M* NaCl in buffer than in columns equilibrated and eluted with 0.1 *M* NaCl in buffer. However, even when 1 *M* NaCl was used, aggregated enzyme was sometimes detectable.

When the fraction of wheat-leaf enzymes that was eluted in the void volume from Sephadex G-200 with 0.01 *M* sodium maleate buffer, pH 6.5, was rechromatographed on a similar column previously equilibrated and eluted with 1 *M* NaCl in buffer, the bulk of the invertase, β-glucosidase, and acid phosphatase was eluted as if these enzymes were now smaller molecules than originally (Fig. 1). Invertase consistently gave one peak when eluted with 1 *M* NaCl in buffer, whereas β-glucosidase has sometimes yielded two peaks, one eluting in the same volume as fractions 65–70 and the other in fractions 95–125 as shown in Fig. 1. These results indicate that chromatography on Sephadex G-200 equilibrated and eluted with buffer containing 1 *M* NaCl can disaggregate some of the large molecular complexes present in the crude preparations.

Enzymes present as large aggregates in the crude preparations can be freed of smaller unaggregated proteins by chromatography on Sephadex G-200 in the absence of 1 *M* NaCl. The aggregates may then be broken down during rechromatography in the presence of 1 *M* NaCl. This step also removes any adsorbed polysaccharide that may have been present initially.

Desorption of "small" proteins from Sephadex

During the initial chromatography of processed wheat-leaf juice on Sephadex G-200 columns eluted with 0.01 *M* maleate, pH 6.5, forms of β-glucosidase and β-galactosidase were present in fractions (145–159) that were expected to contain "small" proteins. When these fractions were concentrated and rechromatographed on a Sephadex G-200 column equilibrated and eluted with buffer containing 1 *M* NaCl, the two glycosidases eluted sooner (fractions 95–125) than they did originally (Fig. 2).

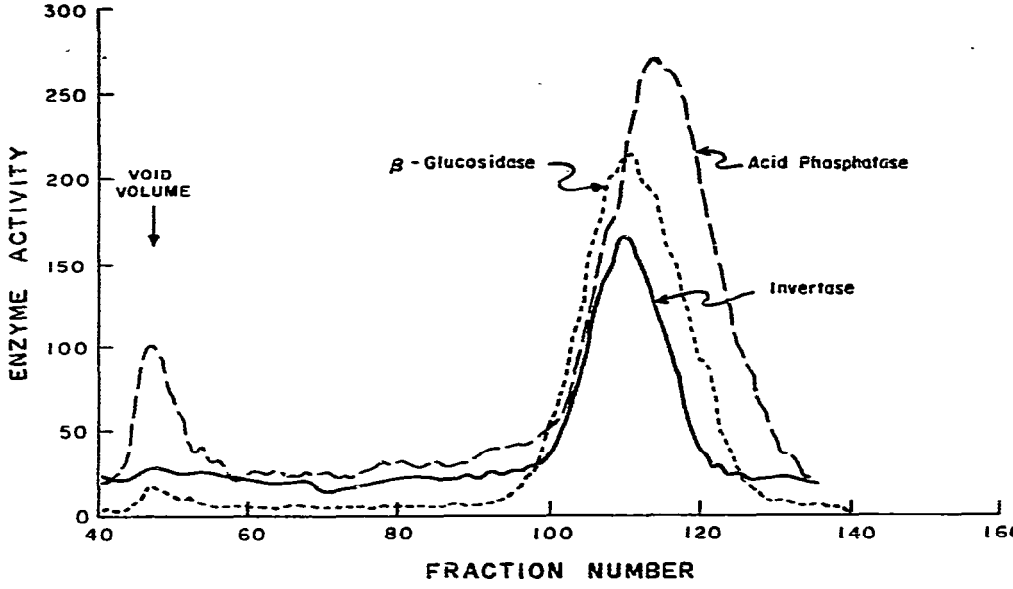

Fig. 1. Rechromatography of high-molecular-weight enzyme fraction from wheat leaves on a column equilibrated and eluted with 0.01 *M* maleate buffer, pH 6.5, containing 1 *M* NaCl. The sample was derived from a wheat-leaf preparation that had been chromatographed on the identical Sephadex G-200 column under identical conditions, except that NaCl had been omitted from equilibrating and eluting buffer. During the initial chromatography, the sample eluted in the void volume.

This behavior probably resulted from the adsorption of these forms of β-glycosidases on Sephadex G-200 during the first chromatography and their failure to adsorb on Sephadex G-200 in the presence of an adequate concentration of NaCl. During initial chromatography, these forms of β-glycosidases are probably eluted by ionic impurities of low molecular weight present in the crude preparations. Enzymes that behave like this give spurious values for their molecular weights when chromatographed on Sephadex G-200 in the absence of sufficiently high salt concentrations.

The differential ability of these enzymes to adsorb on Sephadex under different conditions may be used for their purification. If crude, partially deionized, preparations of wheat-leaf juice are chromatographed on Sephadex G-200, enzymes such as some forms of β-glucosidase and β-galactosidase will be eluted by a large volume of buffer. If such samples are concentrated and rechromatographed in the presence of 1 *M* NaCl, these enzymes will be eluted sooner than during the initial chromatography in the absence of 1 *M* NaCl. This procedure will separate these enzymes from impurities (including small proteins) that do not adsorb on Sephadex G-200. A modification of this procedure has been used successfully on Peak III invertase, a basic protein[1]. In this case, the active eluate from the chromatography of the crude preparation was thoroughly dialyzed to remove ionic impurities during its concentration. This concentrated deionized sample was applied to a Sephadex G-200 column equilibrated and later eluted with 0.01 *M* maleate buffer, pH 6.5. The Peak III invertase was not recovered. Then the column was eluted with 1 *M* NaCl in maleate buffer and the Peak III invertase was recovered in the salt front.

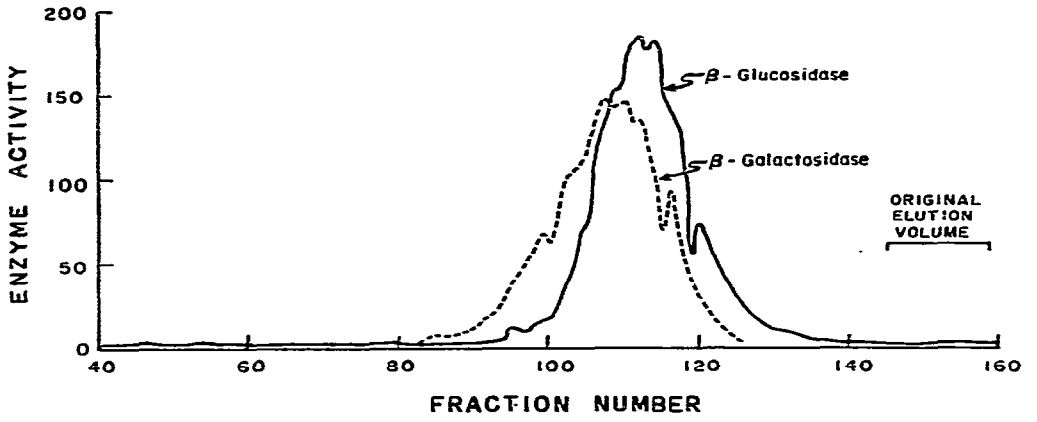

Fig. 2. Rechromatography of low-molecular-weight enzyme fraction from wheat leaves on a column equilibrated and eluted with 0.01 *M* maleate buffer, pH 6.5, containing 1 *M* NaCl. The sample was derived from a wheat-leaf preparation that had been chromatographed on the identical Sephadex G-200 column under identical conditions, except that NaCl had been omitted from equilibrating and eluting buffer. During the initial chromatography, the sample eluted in fractions 145–159.

Regeneration of Sephadex columns

When dealing with enzymes that adsorb on carbohydrates, Sephadex columns should be regenerated before re-use. I have found flushing with buffer containing 1 *M* NaCl useful for this purpose when dealing with wheat-leaf hydrolases. This procedure apparently desorbs the adsorbed enzymes.

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REFERENCES

1 D. W. A. Roberts, *Biochim. Biophys. Acta*, 321 (1973) 220.
2 D. W. A. Roberts, *Enzymologia*, 39 (1970) 151.
3 G. N. Festenstein, *J. Sci. Food Agr.*, 27 (1976) 849.
4 T. Suzuki and S. Sato, *Plant Cell Physiol.*, 17 (1976) 847.
5 T. G. Watson and L. Novellie, *Phytochemistry*, 13 (1974) 1037.
6 Y. Ueda, H. Ishiyama, M. Fukui and A. Nishi, *Phytochemistry*, 13 (1974) 383.
7 G. Little and J. Edelman, *Phytochemistry*, 12 (1973) 67.
8 J. L. Wray and R. E. Brice, *Phytochemistry*, 12 (1973) 1917.
9 T. T. Lee, *Physiol. Plant.*, 29 (1973) 198.
10 G. M. Polya and A. T. Jagendorf, *Arch. Biochem. Biophys.*, 146 (1971) 635.
11 T. A. Smith, *Phytochemistry*, 11 (1972) 899.
12 I. Caras and B. Shapiro, *Biochim. Biophys. Acta*, 409 (1975) 201.
13 A. Guranowski and Z. Schneider, *Biochim. Biophys. Acta*, 482 (1977) 145.
14 K. P. Schodt, R. A. Gelman and J. Blackwell, *Biopolymers*, 15 (1976) 1965.
15 J.-C. Janson, *J. Chromatogr.*, 28 (1967) 12.
16 F. Miranda, H. Rochat and S. Lissitzky, *J. Chromatogr.*, 7 (1962) 142.
17 A. N. Glazer and D. Wellner, *Nature (London)*, 194 (1962) 862.
18 D. W. A. Roberts, *Can. J. Biochem.*, 45 (1967) 401.
19 T. C. McIlvaine, *J. Biol. Chem.*, 49 (1921) 183.
20 D. W. A. Roberts, *Can. J. Bot.*, 54 (1976) 1509.