Gel Filtration Studies of Sugarcane Acid Phosphatases

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INTRODUCTION

Strong phosphatase activity has been recognized in sugarcane leaves for several years (*1,2*).[2] This is believed to be the work of several distinct enzymes, making possible the rapid hydrolysis of organic phosphates, and posing a biochemical threat to phosphorylated intermediates of sugar synthesis and metabolism. Unfortunately, good separation of these catalysts from contaminant protein has not been achieved.

More refined methods were made available for enzyme resolution by the recent development of a material described as Sephadex (*10*). Added to water or buffer, it forms a true gel which is readily packed into a column. It is composed of cross-linked dextran which serves as a kind of molecular sieve. Mixtures passing through these columns are fractionated according to constituent size. The material was first used to desalt and to concentrate proteins, the proteins being generally unabsorbed while salts were retained on the column. Further studies have shown that by varying the degree of cross-linking, a variety of sieving potentials become available (*4,5,6*).

Gel filtration techniques were recently applied to sugarcane leaf preparations containing high phosphatase activity. Our objectives were twofold: 1, To separate phosphatases from the bulk of contaminant protein; 2, to separate the phosphatases from one another.

MATERIALS AND METHODS

PREPARATION OF ENZYME SAMPLES

Phosphatases were extracted from leaves +1 to +4 of 10-month-old sugarcane which had been frozen, lyophilized, and ground to a fine powder in accordance with procedures described earlier (*1*). The bulk of protein was precipitated by bringing clarified solutions to 95-percent saturation with solid ammonium sulfate, at pH 5.5 and 22°C. Protein was taken up in distilled water and dialyzed a minimum of 10 hours before passage through Sephadex.

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[2] Italic numbers in parentheses refer to Literature Cited, pp. 301–2.

PREPARATION OF COLUMNS

The dextran gel employed was Sephadex G-200[3]. A small amount of the powder was added to distilled water or buffer maintained in a state of slow agitation on a magnetic stirrer. When thoroughly soaked, excess fluid was decanted and the gel was poured into a 1.5 × 25-cm. column. The material was added slowly in small increments with care being taken to avoid entrapment of air bubbles. A glass-wool disk was fitted near the top of the column to retard turbulence of added sample and eluting fluids.

Four milliliters of phosphatase preparation were added to the column. Ordinarily, about 10 minutes were allowed for passage of the void volume, but at times effluent collection was begun immediately. One-milliliter fractions were collected manually until all of the descending protein was eluted. Columns could be reused without altering the flow patterns of constituent proteins, but a fresh column was invariably packed for each separation.

ENZYME ASSAY

The standard phosphatase digest was composed as follows: 1.0 ml. of acetate buffer (pH 5.5), 0.5 ml. of substrate (0.5 M[4] β-glycerophosphate or glucose-1-phosphate, 0.25 M ATP, disodium salt), 0.25 ml. of distilled water or additive solution, and 0.25 ml. of phosphatase preparation. Control tubes received water in place of enzyme. The digest vessels were allowed to stand for 1 hour at 30°C. in a constant-temperature water bath. One milliliter of 10-percent trichloroacetic acid was used to terminate the reaction, and color development was carried out by the phosphomolybdic acid technique described earlier (*1*).

Protein content of phosphatase preparations was measured by the technique of Sutherland *et al.* (*12*). One activity unit was arbitrarily defined as the amount of enzyme catalyzing the formation of 0.10 mg. of inorganic phosphorus under the prescribed conditions of the assay. Phosphatase action was expressed as specific activity (units per milligram of protein).

ELECTROPHORESIS ANALYSES

Some of the phosphatase peaks obtained by gel filtration were concentrated by freeze-drying and examined electrophoretically. A Beckman Model R paper electrophoresis system was used. Standard separations were run for 4 to 12 hours with 0.02-M phosphate buffer (pH 8.0 or 7.2) and a constant current supply of 2.5 MA. Paper strips were air-dried at the completion of a given run and then cut into 1-cm. sections. These were eluted

[3] Supplied by the Parmacia of Fine Chemicals, Rochester, Minn.

[4] The letter M designates "molar" herein.

with distilled water, and enzyme assays were conducted as usual with the protein samples.

RESULTS AND DISCUSSION

SOLUBILITY PROPERTIES AND PH OPTIMA OF SUGARCANE PHOSPHATASE

Attempts were made to isolate phosphatase from other leaf protein prior to gel filtration. Figure 1 illustrates the general precipitation of phosphatase between 38- and 62-percent saturation by ammonium sulfate. When glucose-

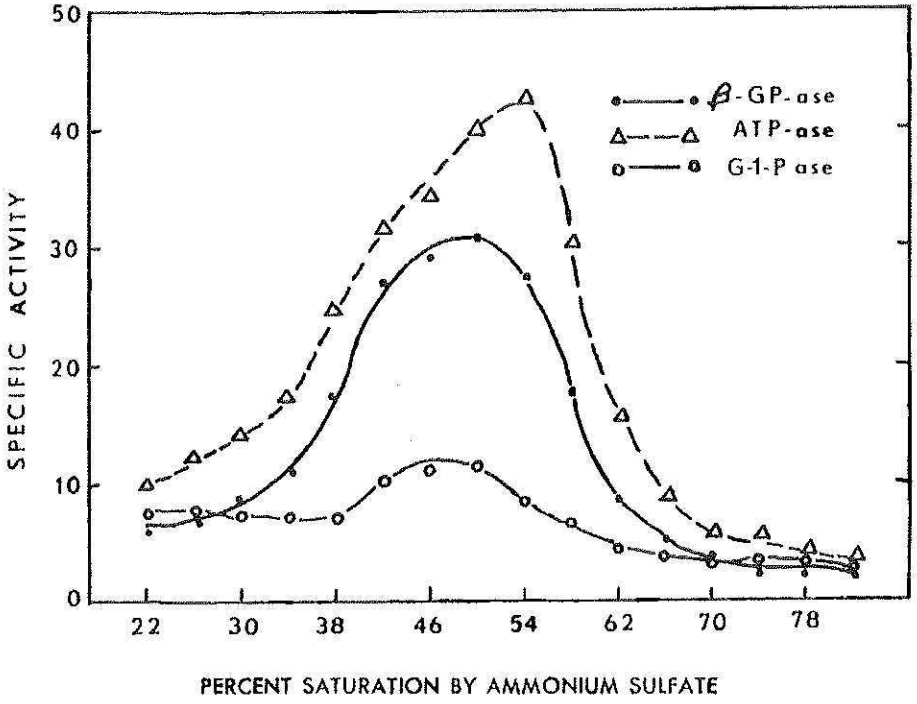

FIG. 1.—Phosphatase activity among protein increments precipitated from sugarcane leaf extracts by ammonium sulfate.

1-phosphate was used as substrate the range of maximum precipitation was somewhat narrower. Nevertheless, the 38- to 62-percent fraction was accepted for gel filtration study, and this included most of the leaf protein originally extracted.

Optimum pH lay between 4.5 and 5.5 when β-glycerophosphate was used as substrate (fig. 2). Both ATP and glucose-1-phosphate were readily hydrolyzed under basic conditions, as well as between pH 4.5 and 6.0. This is precisely the sort of data that suggest distinct phosphatases within an otherwise concise solubility group.

The enzyme attacking ATP at pH 7.5 to 8.0 is particularly interesting. Alkaline phosphatases, generally most active around pH 9.0, are widely

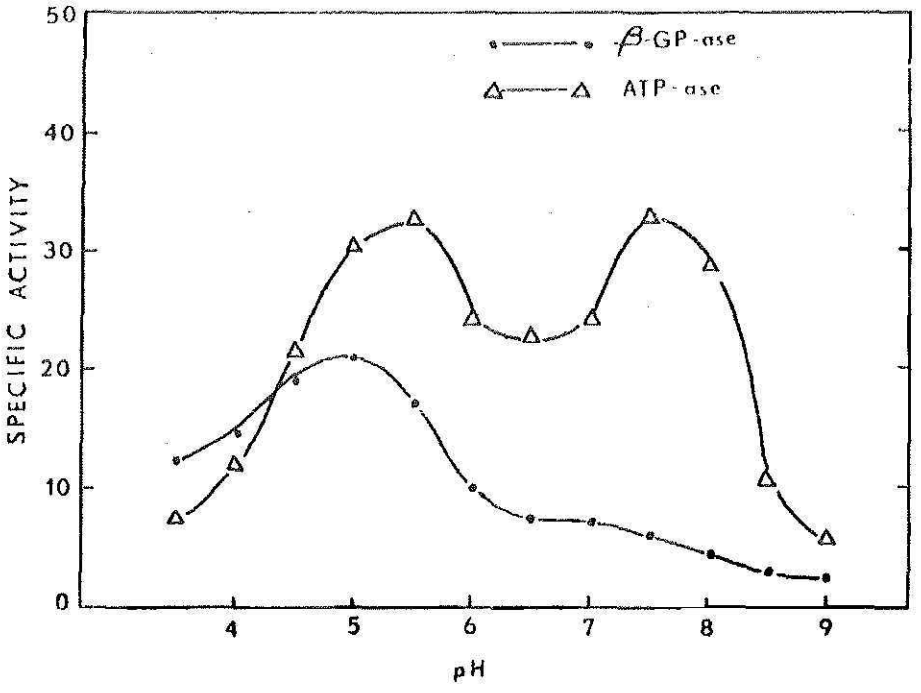

FIG. 2.—Effects of variable pH on phosphatase activity in sugarcane leaf preparations. The following buffers were employed: Acetate, pH 3.5–5.5; succinate, pH 6.0–7.0; and trishydroxymethylamino methane, pH 7.5–9.0.

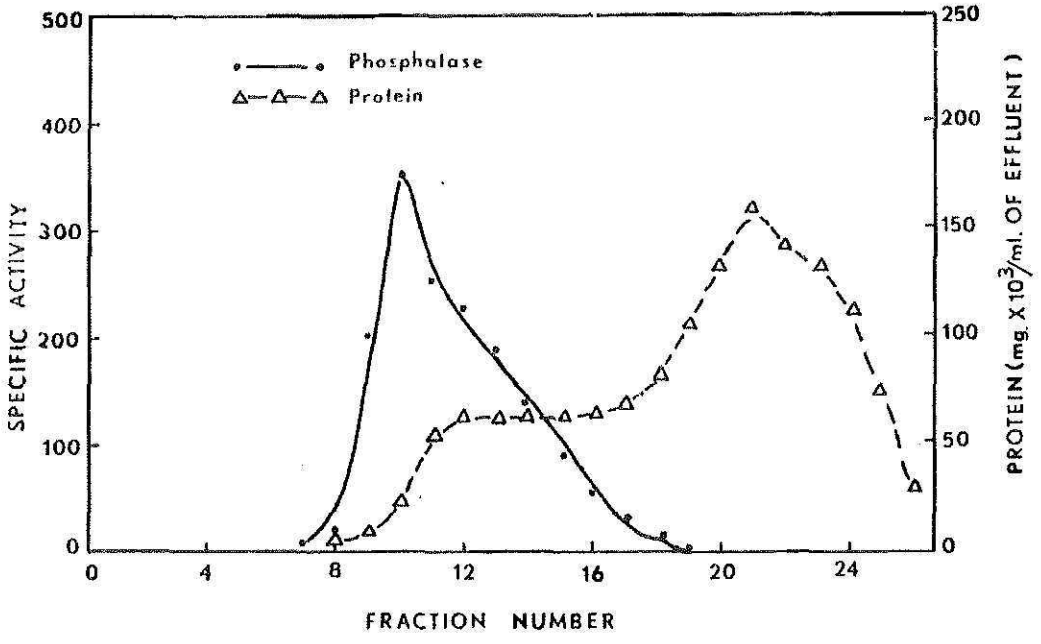

FIG. 3.—Separation of phosphatases from contaminant protein by passage of a sugarcane-leaf preparation through G-200 Sephadex. β-glycerophosphate was used as substrate with the standard phosphatase reaction.

known and have been implicated in bone formation for more than 40 years (*8*). They are also known in intestinal mucosa and milk (*9*), and in snake venom (*7*). Shuster and Kaplan (*1*) have described an alkaline nucleotidase from ryegrass but no alkaline phosphatase has been reported in sugarcane.

RESOLUTION OF A PHOSPHATASE-PROTEIN COMPLEX

Figure 3, which represents a typical filtration run with Sephadex, shows that the first traces of phosphatase activity appeared in fraction 7, and that maximum activity was obtained in fraction 10. No appreciable protein could be detected prior to fraction 11, and the mass of protein comprising the original sample was retained in fractions 19 to 24. Thus, two distinct peaks could be plotted for enzyme and protein, and for the first time during our studies a strong phosphatase activity was isolated from the bulk of other protein constituents. Similar separation of phosphatase from protein was obtained when ATP and glucose-1-phosphate were employed as substrates.

The high degree of enzyme purification is illustrated by figure 4. It can be seen that salt fractionation of phosphatase plus the subsequent dialysis step each caused marked purity increases, yet the gel filtration step alone made possible a tremendous activity increase over the second most favorable treatment.

PARTIAL RESOLUTION OF PHOSPHATASE-INHIBITOR COMPLEXES

It was learned earlier that traces of either molybdenum or tungsten cause almost complete inhibition of sugarcane-leaf phosphatases (*2*). This inhibition is competitive (*i.e.* reversible by high substrate concentrations). It was concluded that phosphatase is not in itself damaged by inhibition and, in theory, could be reactivated by cleaning away the inhibitor. Yet prolonged dialysis of the enzyme-inhibitor complex consistently failed to do this.

During the present studies the inhibited enzyme was partly reactivated by passage through a Sephadex column. Sufficient molybdenum or tungsten was added to dialyzed, nonfiltrated phosphatase to bring about complete inactivation (0.10 μmol of inhibitor per milliliter of enzyme solution). Filtration through Sephadex apparently left much of each inhibitor absorbed upon the gel (fig. 5). Gel filtration is thereby vindicated as an enzyme cleaning process which, at least in this instance, is superior to dialysis.

FAILURE TO SEPARATE PHOSPHATASES FROM ONE ANOTHER

Although phosphatases were generally well separated from contaminant protein, the enzymes themselves passed through Sephadex in a group (fig. 6). No satisfactory resolution of individual enzymes was obtained. This may indicate that all leaf phosphatases are of identical size. It is also probable that a high degree of protein-protein binding exists among the phosphatases.

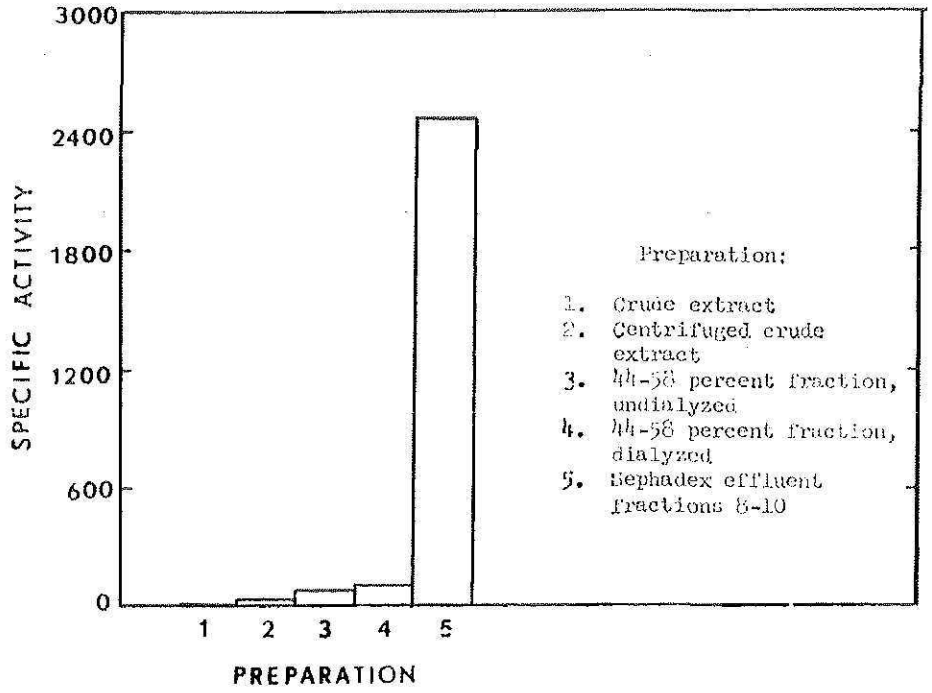

FIG. 4.—Specific activity increases for sugarcane leaf phosphatase during progressive purification steps. β-glycerophosphate was used as substrate with the standard reaction.

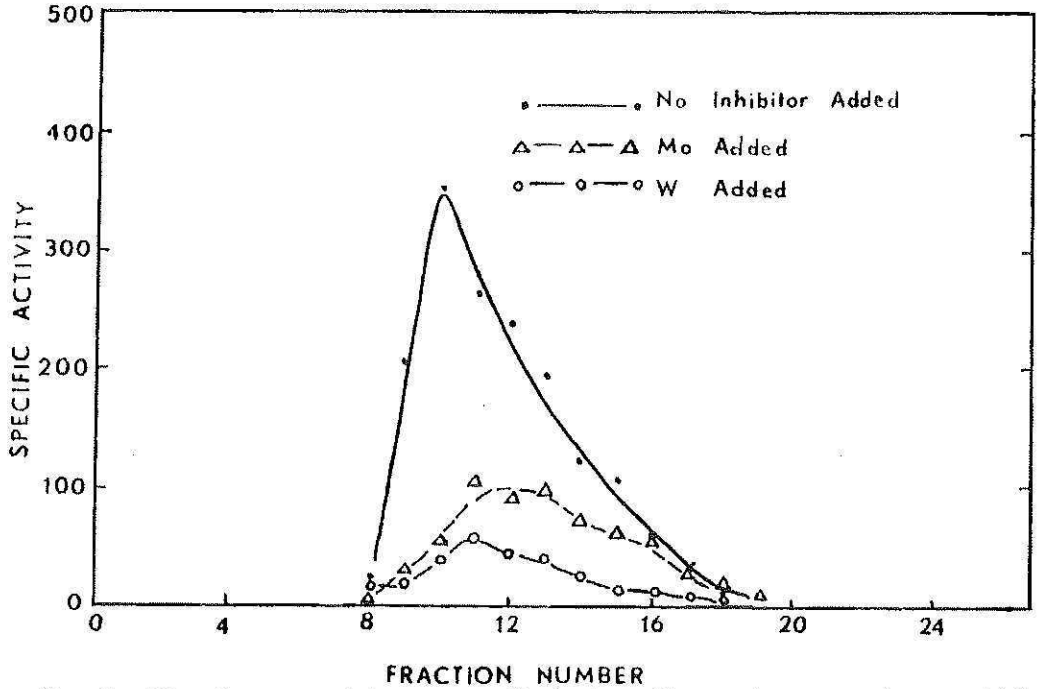

FIG. 5.—Phosphatase activity among Sephadex effluents from samples to which the inhibitors molybdenum and tungsten had previously been added. Inhibitor concentration was 0.01 μmol per milliliter of enzyme preparation. β-glycerophosphate was used as substrate.

Sephadex studies with sugarcane invertases[5] and amylases (*3*) revealed different mobility patterns when buffer rather than water was used to pack and elute columns. Concise peaks obtained with water tended to slur together with buffer in the area of major protein passage. For invertase this was attributed to increased ionic strength due to the buffer. Later work with amylase showed that NaCl solutions of ionic strength equal to the buffer permitted mobility patterns identical to those obtained with water, and it was concluded that a pH effect had been exerted by the buffer. Thus, during the present investigation, we considered that altering either the

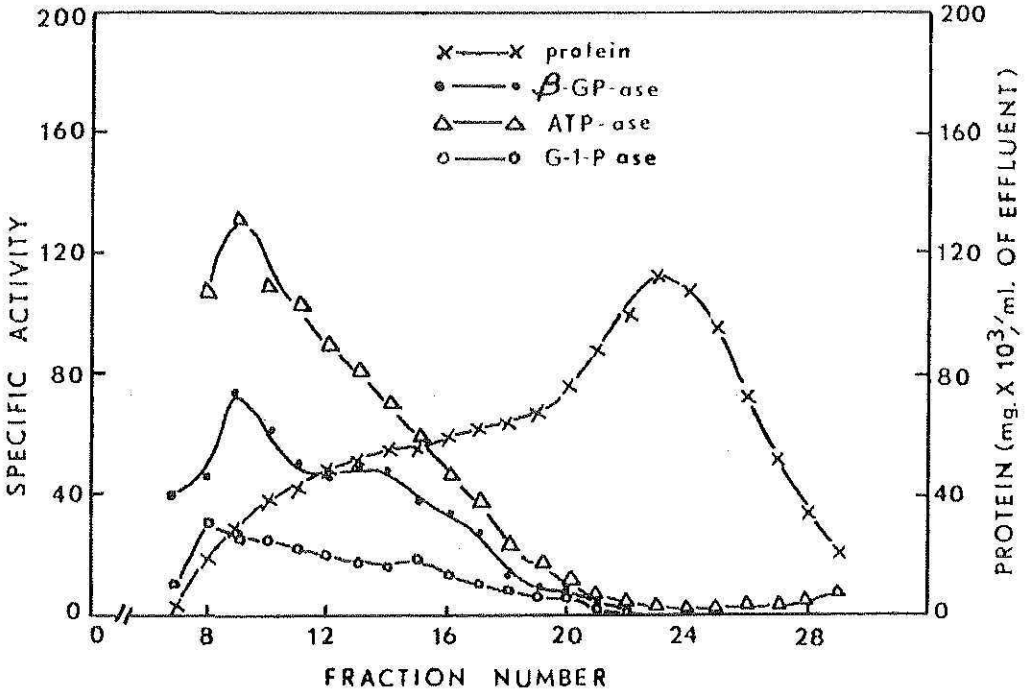

FIG. 6.—Phosphatase activity and protein content of Sephadex effluents from columns packed and eluted with water. One-milliliter fractions were collected. Reaction time was 20 minutes rather than 1 hour.

ionic strength or pH of the packing media might cause separation of phosphatases into distinct peaks. All such attempts proved negative.

Earlier work had also shown that amylase peaks obtained by gel filtration could be separated electrophoretically into distinct positively and negatively charged components. Similar attempts with sugarcane phosphatases were less successful (fig. 7). Virtually all phosphatase activity remained at the point of application during a typical 2-hour run, and only slight migration toward the negative pole was achieved after 7 hours. Only a single phosphatase peak was evident after each run and enzyme activity was markedly curtailed as the electrophoretic period was lengthened. Similar results were

[5] Unpublished experiments.

obtained with undialyzed preparations passed through Sephadex and with preparations not subjected to gel filtration at all.

We conclude that sugarcane leaf phosphatases comprise a group of enzymes with almost identical solubility properties, and are nearly identical with regard to molecular size. They apparently experience strong protein-protein binding, and, as a group, they must bear a virtually neutral charge.

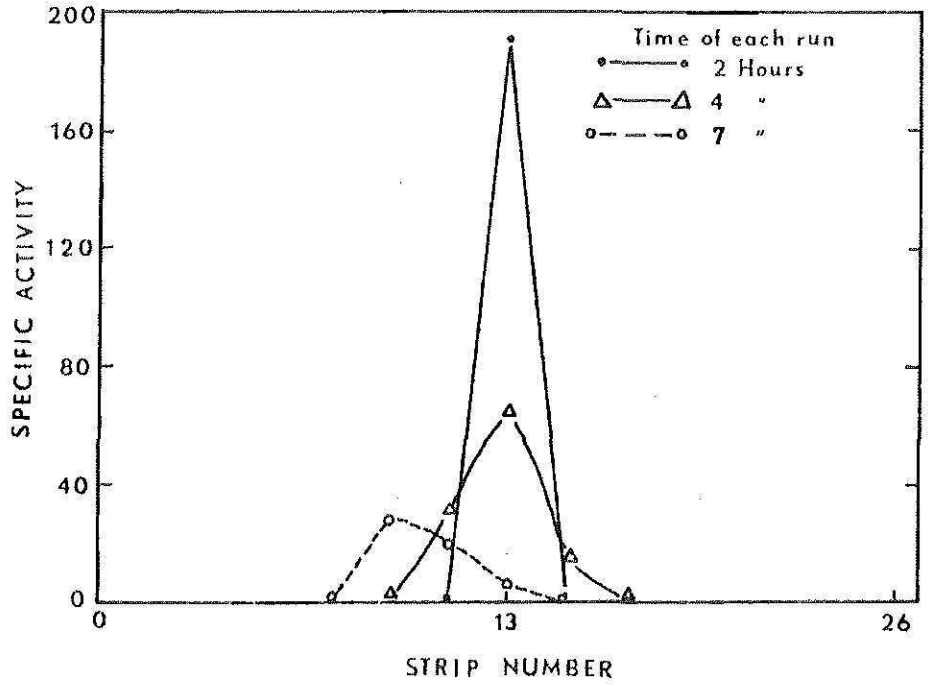

FIG. 7.—Phosphatase activity of Sephadex peak areas subjected to electrophoresis for varying periods of time. One-centimeter strips were cut and eluted with water. Strip 13 includes the area of sample application.

SUMMARY

Gel filtration studies were conducted with sugarcane leaf-protein preparations bearing strong phosphatase activity. The objectives were to isolate phosphatases from contaminant protein, and from one another. Sephadex, a dextran gel possessing variable sieving properties, was employed in 1.5 × 25-cm. columns packed and eluted with water or buffer.

Good resolution of enzyme and protein constituents was obtained. Collecting 1-ml. fractions during a typical filtration, major phosphatase activity was gathered in fractions 8 to 10, while the mass of noncatalytic protein trailed behind in fractions 19 to 24. The filtration process also served to remove such inhibitors as molybdenum and tungsten, permitting partial reactivation of inhibited enzyme.

Attempts to separate phosphatases from one another by gel filtration and paper electrophoresis were negative. It was concluded that sugarcane leaf phosphatases have nearly identical properties with regard to solubility, molecular size and type of charge. A powerful protein-protein binding is also suspected among different phosphatases.

RESUMEN

Se hicieron estudios de filtración de gel, con preparaciones de proteínas extraídas de las hojas de la caña de azúcar en las que la fosfatasa se mostró muy activa. Los objetivos eran aislar las fosfatasas para evitar que se contaminaran con proteína, y separar las unas de las otras. Para este fin se usó Sephadex, gel de dextran que al actuar de colador posee propiedades variables, en columnas de 1.5 × 25 cm., diluído con agua o con un agente amortiguador.

Se logró una buena separación de la enzima y las proteínas. Al recoger fracciones de 1 ml. durante una filtración típica, la actividad mayor de la fosfatasa se obtuvo en las fracciones del 8 al 10, mientras que la masa de la proteína no-catalítica quedó rezagada en las fracciones del 19 al 24. El proceso de filtración también sirvió para remover inhibidores, tales como el molibdeno y el tungsteno, permitiendo así la reactivación de la enzima inhibida.

Fueron negativos los esfuerzos por separar las fosfatasas, una de la otra, mediante la filtración de gel y por electroforesis de papel. Se llegó a la conclusión de que las fosfatasas de la hoja de la caña de azúcar poseen propiedades casi idénticas respecto a solubilidad, tamaño de las moléculas y tipo de carga. Se sospecha, además, que entre las diversas fosfatasas existe una extraordinaria fuerza de vinculación que une proteína a proteína.

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