ISOLATION OF ANTITHYROID PHYTOPRECIPITIN BY PREPARATIVE ELECTROPHORESIS AND GEL-FILTRATION

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A method of isolating a phytoprecipitin reacting specifically with thyroglobulin from an extract of Pisum sativum is described. The method is based on preparative electrophoresis and subsequent gel-filtration. The resulting specimen of phytoprecipitin was homogeneous when tested by electrophoresis on cellulose acetate and by gel-filtration on Sephadex G-100. Its mobility in veronal-acetate buffer corresponded to the mobility of human blood serum beta-globulin. The molecular weight of the phytoprecipitin, determined by the gel-filtration method, was approximately 26,800 daltons.

The development of methods for isolating hemaglutinating and mitogenic fractions from plant extracts has been a subject of numerous investigations. Nevertheless, the possibility of isolating phytoprecipitins has been studied only in connection with the isolation of anti-A lectin from lima beans [6] and of concanavalin A from the seeds of Canavalia gladiate [4].

The object of the present investigation was to isolate the antithyroid phytoprecipitin discovered by one of us (V. S. Ts.) from the seeds of Pisum sativum L.

EXPERIMENTAL METHOD

The pea extract and thyroid gland extract were prepared by methods described previously [3].

The number of protein fractions of the extract was determined by analytical electrophoresis in agar [2] for 3h at a voltage of 30 V, with a current of 22-26 mA.

The fraction of the extract possessing antithyroid activity was isolated by preparative electrophoresis in agar [2]. Electrophoresis was conducted for 6 h in veronal-medinal buffer, pH 8.6, $\mu = 0.025$, at a voltage of 120 V, current 200 mA. After the end of electrophoresis the agar was cut into strips 10 mm wide (nine strips altogether). Proteins were eluted from the agar by freezing it to $-20°C$ and subsequently centrifuging at 18° (60 min at 5000 rpm). The resulting solution was poured into cellophane bags, concentrated 10 times by evaporation under a fan at 4°C, and then dialyzed for 24 h against distilled water. The protein concentration was determined by the biuret reaction, using Benedict's reagent, and the subsequent spectrophotometric measurements were made on a type SF-4A spectrophotometer at $\lambda = 330$ nm. The serologically active fraction of the extract was lyophilized.

Gel-filtration was carried out on a Sephadex G-100 column (height 96 cm, diameter 1.5 cm), equilibrated with 0.1 M sodium phosphate buffer, pH 7.5. The solution of phytoprecipitin, in which the protein concentration was 14.3 mg/ml, was applied to the column in a volume of 1 ml. Elution was carried out with the same buffer at the rate of 25 ml/h, maintained by an LKB (Sweden) peristaltic pump. The protein

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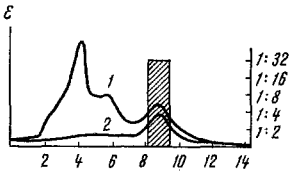

Fig. 1

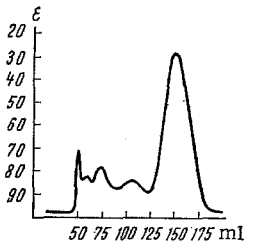

Fig. 2

Fig. 1. Electrophoresis in agar gel: 1) "crude" extract from seeds; 2) phytoprecipitin isolated by preparative electrophoresis. Shaded column shows fraction possessing precipitating activity (here and in Fig. 3, titer along ordinate on the right).

Fig. 2. Gel-filtration of phytoprecipitin on Sephadex G-100 column.

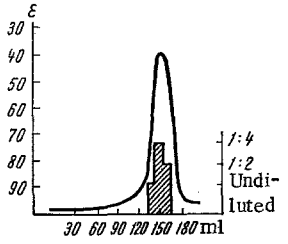

Fig. 3. Rechromatography of phytoprecipitin on Sephadex G-100 column. Shaded column shows titer in precipitation test.

concentration in the eluted fractions was determined by means of a Uvicord II (LKB, Sweden) continuous-flow ultraviolet photometer or the optical density of the fractions (10 ml) was measured at 262 nm on the SF-4A spectrophotometer. To determine the antithyroid activity, identical fractions obtained in four parallel experiments were pooled and concentrated 10-23 times, depending on their volume.

Antithyroid activity of fractions isolated by preparative electrophoresis and gel-filtration were investigated by Ouchterlony's double diffusion method in agar in the micromodification of Gusev and Tsvetkov [1], using extract from the thyroid gland diluted with 0.14 M NaCl solution in the ratio of 1 : 64 as antigen.

The sedimentation constant of the active fraction was determined by ultracentrifugation on a model E analytical ultracentrifuge (Beckman) at 60,000 rpm (solvent veronal-medinal buffer, protein concentration 14.3 mg/ml).

The electrophoretic mobility of the component possessing antithyroid activity was studied by microelectrophoresis on cellulose acetate strips in the Microphor electrophoretic apparatus (Boskamp). Samples measuring 0.04 μl were applied with a doser to cellulose acetate strips measuring 25 × 145 mm. Electrophoresis was carried out in veronal-acetate buffer, pH 8.6, for 40 min at 250 V, current 16-25 mA. After the end of electrophoresis, the strips were stained for 4 min with amidoblack 10B, washed three times, in a mixture of methanol and acetic acid, spread out on slides, decolorized with dioxan and isobutanol, and dried in a drying cupboard at 90°C. The resulting specimens were examined with an Opton (West Germany) integrating densitometer.

EXPERIMENTAL RESULTS

The pea extract obtained by the previous method [3] contained 27.6 mg/ml protein, and had a titer of 1 : 32 in the precipitation test with thyroid extract diluted 1 : 64. This extract was first subjected to analytical electrophoresis in agar (Fig. 1). The initial extract can be seen to contain at least three different components. Determination of the titers of the fractions isolated by preparative electrophoresis in the precipitation test showed that phytoprecipitin was contained only in the fraction with least mobility on electrophoresis in this particular buffer solution. This fraction was present in only 1 of the 9 zones of agar. For subsequent use, only the eluate of this zone was used and the remaining fractions were discarded. After concentration, the volume of this eluate was 5 ml, its protein content 14.3 mg/ml, and its titer in the precipitation test 1 : 32.

The resulting solution was next subjected to gel-filtration on Sephadex G-100 (Fig. 2). The material of this fraction can be seen to be heterogeneous, and it separated into at least five compounds on gel-filtration. The results of determination of the activity of these components after their concentration showed that phytoprecipitin was present in only one of them (Fig. 2 : 1). The volume of solution containing this component after concentration was 2 ml, the protein concentration was 9.9 mg/ml, and the titer of phytoprecipitin 1 : 16.

To determine the molecular weight of the phytoprecipitin semiquantitatively, it was again subjected to gel-filtration on the same column. The column was first calibrated, using trypsin (East Germany) and human serum albumin (Hungary) as standards. The results of rechromatography, with simultaneous determination of the titer of the corresponding (concentrated 33 times) fractions in the precipitation test are shown in Fig. 3. The clear correlation between the protein concentration and its activity in the precipitation test (Fig. 3) enabled the molecular weight of the phytoprecipitin to be determined from the position of the maximum of the curve reflecting the protein concentration in the particular fraction. To judge from the results of gel-filtration of the phytoprecipitin through the calibrated G-100 column, its molecular weight is 26,800 daltons. This figure agrees with the sedimentation constant of 2.6 S found for the main fraction of the phytoprecipitin by analytical ultracentrifugation.

A sample of phytoprecipitin which appeared homogeneous during rechromatography on the G-100 columns (Fig. 3) was subjected to electrophoresis on cellulose acetate strips. The results showed that this preparation was also homogeneous during electrophoresis. Its mobility in this buffer corresponded to the mobility of human serum β-globulin.

The methods of isolation and purification of the serologically active fractions of certain plant extracts are based on adsorption and elution on Sephadex columns [4, 7]. It was impossible to obtain purified phytoagglutinin from pea seeds by these methods, for the extract was not adsorbed on Sephadex [5]. The results of the present investigation indicate that gel-filtration on Sephadex G-100 can be used to isolate antithyroid phytoprecipitin from pea seeds.

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