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PURIFICATION OF MITOGENIC PROTEINS FROM HURA CREPITANS AND ROBINIA PSEUDACCACIA

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SUMMARY: The lectin-mitogens from Hura crepitans and Robinia pseudacaccia have been purified by affinity chromatography and compared to that from Abrus precatorius by sodium lauryl sulfate gel electrophoresis. Robinia lectin is quite similar to that from Abrus precatorius in that it consists of two distinct polypeptide chains of 32,000 and 30,000 daltons but unlike abrus lectin the chains are not joined by disulphide bonds. Hura lectin is composed of only a single polypeptide chain which migrates identically with the heavy chain of the abrus lectin. This heavy chain is likely responsible for binding to galactose residues on cell surfaces. The lectin from Robinia pseudaccacia has been obtained in crystalline form.

INTRODUCTION

Lectins stimulate lymphocytes that may normally be in the quiescent state, i.e., presumably in a prolonged G_1 or G_0 stage of their cell cycle, into an active metabolic state. Once activated, these cells undergo what appears to be a normal sequence of events which culminates in mitosis. These compounds have been termed mitogens.

Lectins have been relatively easy to obtain in quantities which have permitted extensive biological and chemical characterization. Relatively few of these, however, are potent lymphocyte mitogens. The activity resides in the lectins ability to interact with particular receptors in the cell membrane which are presumably polysaccharide or glycoprotein in nature.

Kauffman and McPherson (1) reported the discovery of two mitogens, abrin and hurin, lectins derived from the toxic seeds of the plants Abrus precatorius and Hura crepitans. While abrus lectin was isolated in pure form and characterized, hura lectin, which had by far the greatest stimulatory effect, was used in their experiments only as a crude seed extract. We wish to report here the puri-

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fication of a lectin from Hura crepitans. In addition, we can describe the purification of a third lectin-mitogen derived from the seeds of another toxic plant, Robinia pseudacaccia. This lectin has also been reported to possess potent ability to induce the transformation of human peripheral lymphocytes (2). We have attempted to compare and further characterize these three proteins with regard to molecular and biological properties.

MATERIALS AND METHODS

Seeds of Abrus precatorius were collected in the environs of Stuart, Florida; seeds from Hura crepitans were supplied us through the efforts of Dr. Julia Morton of the University of Miami, Miami, Florida; and seeds of Robinia pseudacaccia were purchased from F. W. Schumacher of Sandwich, Massachusetts. Fetuin and hog gastric intestinal mucosa were purchased from Sigma Biochemicals, Sepharose 4B from Pharmacia Ltd., Vibrio cholera sialidase was purchased from Worthington Biochemicals and (DPBS)[1] from Gibco Scientific Co. All materials for polyacrylamide gel electrophoresis were from Bio Rad. Galactose and lactose were from Fisher Co.

Assays for the lectins were based on their ability to agglutinate erythrocytes and this proved of sufficient sensitivity to allow us to monitor their appearance in column eluents even when little or no increase in optical density at 280 nm was observed. The erythrocytes employed were, for robinia and hura lectin, rabbit erythrocytes treated with trypsin and washed with saline according to the procedure of Lis and Sharon (3). We found the trypsinization essential in the case of these two lectins. For abrin, either untreated human, rabbit, or sheep erythrocytes could be used and gave clear positive results even at extremely low concentrations of lectin.

Blood group substance was prepared from hog intestinal mucosa according to the procedure of Aminoff, et al. (4) and linked to Sepharose 4B using the cyanogen bromide procedure of Cuatrecacus and Anfinsen (5). Fetuin was linked to Sepharose 4B according to Sela et al. (6) and the terminal sialic acid residues removed by treatment with commercial sialidase according to the procedure of Den and Malinzak (7). The columns were washed thoroughly with phosphate buffered saline before protein was applied.

Tests for mitogenicity on spleen lymphocytes from Balb C mice were as described by Kauffman and McPherson (1) using tritiated thymidine to measure DNA production. Five hour pulses with the isotopic thymidine were applied to the cells sixty hours after initial exposure to the mitogens and followed by extensive washes with unlabelled thymidine prior to harvest.

Preparation of Hura Lectin: Fifty grams of seeds from Hura crepitans were shelled and ground to a paste with a mortar and pestle and mixed with 400 ml of DPBS. To this was added 1 ml of toluene and the slurry allowed to stand with occasional stirring for about 12 hours. The heavy residue was centrifuged off at 2500 g, reextracted for 6 hours with 300 ml DPBS and centrifuged again. The supernatants were pooled, combined with 100 ml of chloroform and shaken thoroughly. After standing for an hour, the chloroform and extracted oils were removed with a separating funnel.

[1] Dulbecco's phosphate buffered saline.

The aqueous phase was made 20% saturated with ammonium sulfate and insoluble residue removed by centrifugation at 12,000 g for 45 minutes. The supernatant was increased in ammonium sulfate to 50% saturation and left at 4°C overnight. The precipitate was collected by centrifugation and redissolved in 100 ml DPBS and then dialyzed against DPBS for 12 hours. Before application to the column, the protein solution was clarified once more by centrifugation.

To a column 30 cm X 1 cm packed with the blood group substance-Sepharose and thoroughly equilibrated with DPBS the protein solution containing the crude hura lectin was applied. The protein solution was followed with DPBS until 100 ml of eluant that gave an A_{280} reading of background level was observed. At this point the column was eluted with DPBS saturated with lactose and fractions were collected. The absorbance and agglutinating ability of each fraction was recorded, and those containing agglutinating activity were pooled. The active material was thoroughly dialyzed against distilled water at 4°C for 24 hours in the presence of toluene.

Preparation of Robinia Lectin: Fifty grams of seeds from Robinia pseudacaccia were crushed and ground with a mortar and pestle and doubly extracted as above with a total of 600 ml of DPBS. No chloroform treatment was given the extract but a low speed centrifugation step followed by a 12,000 g high speed spin for 45 minutes was used for clarification. Several filtrations through glass wool were also used to remove some insoluble material prior to the centrifugations.

The extract was made 50% saturated with ammonium sulfate and allowed to stand at 4°C for 12 hours. The pricipitate was collected by centrifugation and the pellet appeared to contain virtually all of the agglutinating activity of the extract. The pellet was suspended in DPBS, dialyzed against DPBS for at least 12 hours, and then clarified by centrifugation.

The crude lectin solution was applied to a column 30 cm X 1 cm packed with Sepharose linked fetuin. The protein was followed with DPBS until 100 ml of background absorbancy of eluant was observed, and elution with 0.2 M galactose was initiated. A considerable amount of black colored material remained bound to the column and could not be removed, however, the agglutinating protein was liberated and collected in fractions.

The active fractions from the fetuin column were pooled and dialyzed against H_2O in the presence of toluene for 24 hours and lyophilized. The total yield of protein was about 10 mg.

Gradient slab gel electrophoresis was performed using an apparatus made by ourselves. The discontinuous buffer system was that of Laemmli (8) and the polyacrylamide gradients were 5% and 15% and run at 150 V and 30 mA. Tests for neutral carbohydrate were by the phenol-sulfuric acid procedure (9).

The abrin used for comparison was from previous affinity column preparations based on its capacity to bind directly to the galactose residues on Sepharose 4B. This procedure was described by McPherson and Rich (10). For crystallization, 1 mg/ml samples of both the hura and robinia lectin were concentrated in minicon ultra filtration devices (Amicon, Lexington, MA) to 100 µl volumes. These were then subjected to vapor diffusion techniques (11) using a range of polyethylene glycol sizes and concentrations. No buffer was included in the samples and all attempts were made at 25°C over a period of three months. The sample volumes were 10 µl consisting of 5 µl of the protein solution (10 mg/ml) and 5 µl of the reservoir solution. Trials were conducted using Corning glass depression plates (12).

RESULTS

Unlike abrin, we found that we could adsorb neither hura lectin nor robinia lectin directly to Sepharose, although galactose inhibited the agglutinating ability of both. In addition, although abrin was effective in agglutinating native erythrocytes from several species at very low concentrations, both hura lectin and robinia lectin were effective only with erythrocytes that had been treated with trypsin. These results indicated that, although the three lectins shared a common affinity for galactose, hura and robinia lectin were more discriminating and probably bound specific di- or oligosaccharides. We also attempted to isolate both hura and robinia lectin by adsorbing them to galactosamine linked to Sepharose through a caproic acid stem and N-acetylgalactosamine linked in the same fashion (13). These attempts were unsuccessful.

Analysis of robinia and hura lectin for carbohydrate yielded positive results in both cases implying that both are glycoproteins. Abrin was known from other studies to be so (10).

When run on adjacent tracks of a gradient slab gel in the presence of excess β-mercaptoethanol and SDS, the results seen in Fig. 1 were obtained. Abrus lectin, which has a monomer molecular weight of 65,000, yields two bands which correspond about 33,000 and 31,000 daltons. Robinia lectin shows a similar band pattern, reproducibly yielding in equal proportion two polypeptide chains of about 31,000 and 30,000 daltons. In the case of abrus lectin, the two chains are joined by disulfide linkages. If the gel is run in the absence of reducing agent, however, the pattern seen in Fig. 1 persists. Thus the two chains of robinia lectin are not joined by disulfide bonds but are held together only by noncovalent linkages.

Hura lectin is different from both abrus or robinia lectin in that it yields only a single band upon electrophoresis. This polypeptide chain, however, runs identically with the heavy chain of the abrus lectin. The similarity between the two chains may be more than coincidence since it is

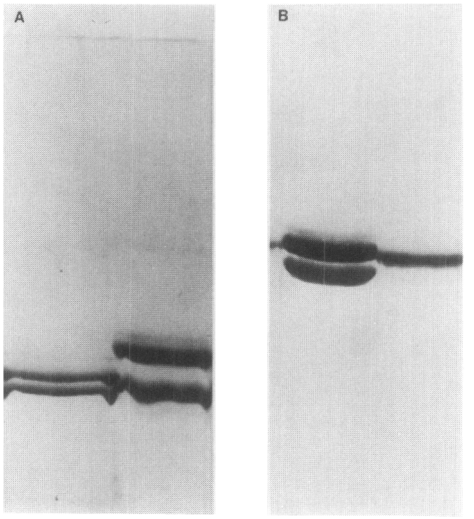

Figure 1. In (A) is a 5% to 15% gradient SDS polyacrylamide gel of the lectin from Robinia pseudaccacia on the left and abrin on the right. In (B) is seen an equivalent gel of abrin on the left and purified lectin from Hura crepitans on the right. In neither case could contaminating proteins be observed on the gels that totaled more than 1% of the protein sample applied.

the heavy chain of the abrus lectin that is responsible for binding of the protein to cell membranes and by inference that responsible for its mitogenic activity.

Although no success can be reported as yet in the case of the hura lectin, we have obtained in several trials crystals of the lectin from Robinia pseudaccacia. In figure 2 is seen a typical cluster of the spike shaped crystals grown from 18% polyethylene glycol 4000 after six weeks. A second form of crystals can frequently be seen coexisting with those of figure 2 and they have the shape of rectanglular prisms. At present, the crystals are too small for X-ray diffraction analysis, but work is continuing to increase their dimensions to the point where such techniques can be applied.

Since the hura and robinia lectin, like abrus lectin, agglutinate blood cells, they must be multi-valent and, therefore, exist as either dimers or tetramers. Abrus lectin has been reported to be both a dimer of 130,000 and a tetramer of 260,000 daltons (10,15). Thus, we could we expect that hura

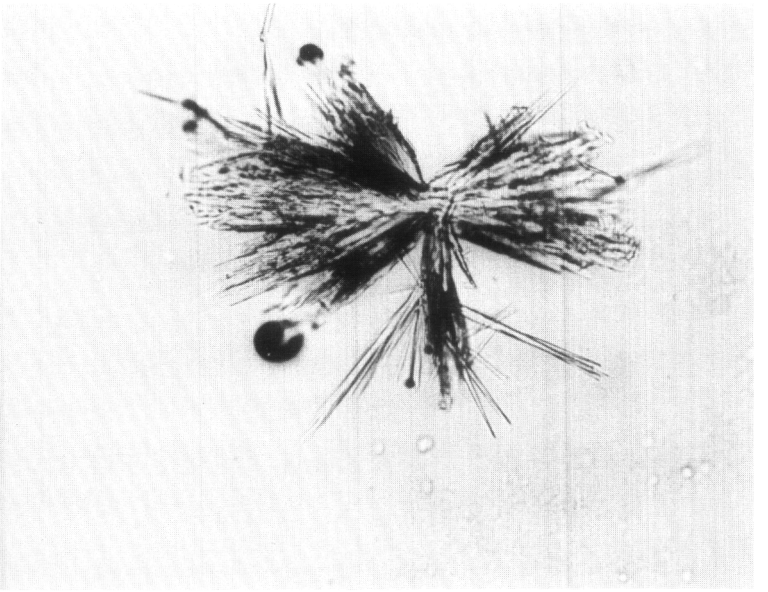

Figure 2. A needle cluster of crystals of the lectin from Robinia pseudaccacia seen under a low power light microscope. The crystals were grown from 18% polyethylene glycol 4000 using the vapor diffusion technique.

lectin will exist as either a dimeric species of 70,000 or a tetramer of 140,000, and similarly robinia lectin as a dimeric molecule of 122,000 or a tetramer of twice that weight.

It was previously shown that both abrus lectin and the crude protein extract from Hura crepitans possessed powerful mitogenic capacity when tested on mouse spleen T lymphocytes, and human peripheral lymphocytes (1). We proceeded to test the purified lectins from Robinia pseudacaccia and Hura crepitans on Balb C mouse spleen lymphocytes using the procedures described by McPherson and Kauffman (1). In both cases we found that the mitogens produce approximately the same level of stimulation as does kidney bean phytohemagglutinin. In no instance could stimulation comparable to that reported for the crude extract of Hura crepitans be found for the purified lectin.

DISCUSSION

Robinia lectin failed to adsorb to the blood group substance-Sepharose column while hura lectin failed to bind to the fetuin-Sepharose column. This

suggests that the specificities of the three lectins, although similar, are significantly different. It should be noted that neither hura nor robinia lectin agglutinated even trypsinized erythrocytes at anywhere near the low level that abrin proved effective. This could reflect the greater number of available receptor sites on the cells surface due to abrin's lower binding specificity.

There are two likely explanations for the dramatic reduction in activity observed here for the hura lectin. It may well be that there are additional mitogenic proteins present in the seeds which act synergistically with the mitogen we have isolated, but which evade our affinity column procedure because of a different specificity or simply because they are not lectins at all. Such a situation has been observed by Waxdahl (16) for certain of the pokeweed mitogens. A second possibility is that since hura lectin only produces substantial agglutination with cells that have been trypsin treated, the mitogenic effect may also be dependent on pre-digestion of the cell surface with a proteolytic agent. Such a protein called hurain is, in fact, known to be an endogenous constituent of the seeds from Hura crepitans (17,18). Both of these possibilities are currently under investigation.

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