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ABSTRACT

Plant agglutinins or lectins are sugar binding proteins or glycoproteins which agglutinate cells and/or precipitate glycoconjugates. The physiological role of plant agglutinins distributed on the surface of leaf and root remain unclear, but the hypothesis that these lectins might protect the plants from fungal and bacterial pathogens is being extensively investigated. In this research, epicuticular plant surface agglutinins (EPSA) were screened in various economic crops, namely Cucumber surface agglutinins (CSA), EPSA of rice (*Oryza sativa*), Loofah (*Luffa acutangula*, *Luffa cylindrica Roem.*) and legumes. It has been concluded that CSA binds specifically to chitin and its derivatives, thermostable and inhibits formation of conidiospore in powdery mildew, necrotic spot of TNV and spore germination of some fungi. CSA also contain superoxide dismutase (SOD) activity. Purified CSA has been determined for the sequence of 30 terminal amino acids, and a number of synthetic 60-mer deoxyoligonucleotides were synthesized. Hopefully, the CSA gene would be cloned and used as a probe for larger scale production of CSA. Screening of EPSA in various cucumber related crops in Thailand was performed and *Luffa acutangula* or angle Luffah (LSA) were selected for further studies because of their high agglutinating activity, which also showed SOD activity. Surface extract from various legumes and other economic crops were screened for EPSAs and partially purified fractions of surface agglutinins contained SOD activity and were found to inhibit spore-germination and development of some agriculturally important fungi. EPSA from rice (RSA) is similar to purified embryo and bran lectins, with respect to biochemical and immunological properties. RSAs are thermostable and located at the potential infection sites such as stoma, hydathodes, root tips and root hair tips of rice seedlings. Purified rice lectin inhibits spore germination of *Trichoderma viride*. Since rice and EPSA gene has been cloned, further study at the molecular level is possible.

The terminal 30 amino acids of cucumber and rice EPSAs have been sequenced. Three 60mer oligodeoxyribonucleotide probes have been synthesized and a λ_{10} library established. Since the rice lectin gene has been cloned, further studies of EPSA as plant protectants can be undertaken in the near future at the molecular level.

INTRODUCTION

A recently discovered epicuticular protein (EPSA) has been the focus of investigation of this project. EPSA was found to be (1) located on plant surfaces and (2) functioned as agglutinin, superoxide dismutase and peroxidase. Its hemagglutinating (but neither its peroxidative nor its superoxide neutralizing) activities became specifically inhibited by N, N', N"-triacetylchitoriose, chitin and chitosan (96). Chitin is absent in higher plants but is a common wall constituents in many fungi (74). Chitosan, which is a deacetylated derivative of chitin, is also a component of many fungi (3), and has fungicidal activity. Therefore, EPSA is a possible candidate involved in the protection of plants against pathogens. Preliminary assays indicated that EPSA indeed inhibits germination and development of some pathogenic fungi.

PLANT SURFACES

The surface structure

The outer part of the plant epidermis consists of five layers: 1) surface was layer; 2) cutin layer embedded in wax; 3) a mixed layer containing some cutin, wax and carbohydrate polymers mixed possibly with traces of proteins; 4) pectin and 5) the cellulose wall of the epidermal cells (38,52). According to Sitte (94), the term "cuticle" refers to the outermost (oldest) layer which contains cutin as a major component and never contains cellulose. The other cutin-containing layers should be classified as a "cuticular layer". A cuticular layer is observed not only on the aerial surface of plants, but also even on mesophyll and palisade cells (38).

The surface of the plant cuticle is usually covered with a wax layer (50). The epicuticular wax was observed in many species (89). The diversified structure of wax projection has been clearly revealed by scanning electron microscopy (6,95,98). The wax consists of pure alkanes, ester, ketones, primary and secondary alcohols. Cutin as the structural component of the cuticle is described as a polyester composed of hydroxy fatty acids (53). It is attached to the outside of the epidermal cell wall in the aerial organs of all plants, such as: stem, petiole, leaf and stomata, flower, fruit, etc. Underground parts

of plants, such as roots, as well as wound surfaces and a variety of internal organs, are covered by a different type of polymer, namely, suberin (53).

The outer tangential wall of the epidermis is thicker than all the other walls of the cells (50). The walls of epidermal cells are thought to have a relatively normal architecture and to consist of cellulose embedded in a pectin and hemicellulose matrix (34). Generally, they can be considered as thickened primary walls similar to those of the collenchyma (50).

Hairs and surface modifications

The plant surface does not only consist of simple cuticle covered epidermal cells. From the surface emerges a whole range of trichomes (hairs of various sorts) whose cuticular structure may vary greatly from that of the simple epidermal cells (50). The trichomes are epidermal appendages of diverse forms and functions. They are represented by protective, supporting and granular hairs, scales, various papillae and the absorbing hairs of the roots (39).

Ectodesmata

The ectodesmata pass through the epidermal cell layers, but not through the cuticle itself. They are not specific wall structures (39,50) and may be important as regions of differential permeability in the whole cuticular complex.

Water-soluble materials

The occurrence of substances which may be removed from healthy leaves by washing with water has been reported by a number of workers (16,114). Water droplets retained on leaf surface contain both organic and inorganic substances released from leaf tissues, fungal spores, or pollen (16). A large number of substances have been recorded in leachate from leaves including simple sugars, amino acids, organic acids and phenols (16). Besides the low molecular substances which could be washed off from leaves there are a few reports on the appearance of lectins in washings of plant organs (35,78).

The studies of proteins in the cuticular layer or in the epidermal cell walls are yet relatively limited. One of the few reports is that of Leach *et al.* (55) and Morrow (73) on lectins localized in epidermal cell walls. Recently we described a novel development-

dependent lectin (agglutinin) which some plants excrete to their epicuticular surfaces (EPSA -96).

Functions

The major function of the surface components is probably their being biological barriers which, because of their metabolic inertness, do not require constant rebuilding. The waxy components of the cuticle create a highly effective barrier to water loss from plants. In addition to the influence on cuticular transpiration, wax structure and chemical composition are known to effect the wetting and retention of water droplets. Since the wax layer is also the first contacting site between pathogens and plants, wax probably plays an important role in the prevention of fungal infection. It is known that some fractions of wax inhibit fugal germination (32,114), while others stimulate it (15).

There are reports of positive correlationships between the resistance of plants and their well-developed cuticle. In these cases, the role of the cuticle has been ascribed solely to its action as a mechanical barrier, because of its extraordinary chemical and physical stability. But, the possibility that cutin might also be enzymatically decomposed by pathogens must be considered (38). Many studies have been conducted on the involvement of cellulose, hemicellulose, pectic substances and polysaccharide-degrading enzymes of pathogens in the infection process (2).

Finally, the trichomes themselves may play a role in plant defense, especially with regard to insects (57). The major function of the surface components is probably their being biological barriers which, because of their metabolic inertness, do not require constant rebuilding.

LECTINS

Lectins have been reported to exist in the walls of epidermal cells (55,70,71,103) and have been considered to be involved in the defense of plants against pathogens (22,90). Their properties and biological effects have been discussed in many recent reviews (9,59,60-62,87).

Definition

Lectins are best defined as sugar-binding and cell-agglutinating proteins or glycoproteins of non-immune origin (43). In contrast to the structural similarity of the antibodies, lectins vary in composition, molecular weight, subunit structure and number of sugar binding sites.

Distribution and localization

The lectins are widespread in all living organisms, but plants serve as the main source (60,62,68,72,80), the legumes being one of the richest sources (104). The best studied group, the legume lectins, are preferentially localized in the cotyledons (31,104). After germination, their content gradually decreases in the cotyledons, and the newly developing organs - leaves stems or roots - contain much less lectin (18,49,80,102). Special attention has been paid to the localization of lectins in legume roots (29,35), and it has been hypothesized that lectins may play a role in legume-rhizobium symbiosis. Studies on lectin appearance in other plants than legumes have also been carried out, i.e., with wheat germ agglutinin (68,69) fungi (8,41) and bacteria (7,33).

Physicochemical properties

Most lectins are glycoproteins with carbohydrate contents that can be as high as 50% as in the lectin from potato. A few lectins, such as Con A, WGA and PNA, are devoid of covalently bound sugars (62). The S-S bond in WGA stabilizes the protein to heat, to proteolytic enzymes and to denaturing agents. The lectins from potato and *Datura stroimonium* are rich in hydroxyproline (62). Their molecular weights range from 13,000 for *Pseudomonas aeruginosa* lectin (42) to 265,000 for lima bean lectin (67) and to 500,000 for a rare lectin from rabbit (61). All consist of subunits, which vary in plants between 2 or 4. In most lectins, the subunits are identical in structure and specificity of the sugar-binding sites. However, exceptions are known in which subunits of lectins differ in structure and sugar specificity.

With a few exceptions, all lectins reported in the literature contained Mn^{2+} and/or Ca^{2+}. Removal of the metals inactivated the lectins (103,110). The lectins have a high degree of structural stability against heat and proteolytic enzymes (19,58).

The lectins often occur as a group of closely related proteins or isolectins. But there exist lectins that should not be considered as isolectins because they differ markedly in their biological and molecular properties (58), although they are isolated from one plant.

Biological activities

Lectins as a group exert a variety of effects on cells, but not every lectin exhibits all of them. Agglutination is taken as evidence for binding to cells, but lack of agglutination does not mean that binding does not occur. Lectins agglutinate different cells and bind, among others, to fungi (90,66), bacteria (54,113,101) and viruses (77). Its agglutination activity can be inhibited by simple sugars, i.e., glucose and mannose (9,42) or by complex ones, such as chitin (4). Some lectins were found to react with plant cells. The binding of Con A to the stigma surface of Gladiolus flowers could be inhibited by sugars. Another lectin in the primrose flower promotes pollen growth.

Lectin in wheat, barley, rye and rice seeds have been found to be a group of closely related proteins occurring in embryos. Purified lectins from wheat germ, rye, and barley embryo possess the same molecular structure, sugar-binding specificity to N-acetyl-D-glucosamine, and serological characteristics (99). *De novo* synthesis of these lectins in germinating and developing wheat and rye embryo (79) and continual synthesis of lectins in roots and leaves of adult wheat and barley plants were reported (27,84).

Role in Nature

Although the chemical, physicochemical and biological properties of lectins are well documented, their physiological role is still a matter of controversy (2,9,62,87,90). Among their possible functions in higher plants, their participation in the binding of nitrogen-fixing bacteria to roots of legumes received by the most attention. Much work has been published dealing with this subject (12,13,29). Unambiguous and conclusive evidence that they play a major role in the recognition or binding of bacteria has not yet been presented.

Another hypothesis suggest that lectins may play an important role in the protection of plants against pathogens (2,90). Thus, WGA, because of its specific

binding to N-acetylglucosamine and its oligomers, is able to bind to fungal cell walls and inhibit *in vitro* fungal growth (22,54,66,101). Furthermore, other lectins, as Con A (36), also bind to bacterial walls.

Some other functions were also suggested. Lectins have been considered as enzymes which have lost their catalytic sites (48). They may be involved in protein synthesis (1), in plant cell growth and elongation (29) or in sugar transport and storage (51).

In bacteria some possible physiological roles are presently considered such as mediation of the bacterial adhesion to cells which could prelude to infection (11) and cellular interactions in developmental stages (33). The slime mold attracted much interest as a simple model system for the study of the molecular basis of development. It is believed that lectins mediate the specific cell-cell associations of slime mold (93).

PEROXIDASES

General characteristics and distribution

Peroxidase is one of the key enzymes which participates in the lignification of cell walls (45) and is also involved in defense mechanisms against invading pathogens (106). Like lectins, it can be found in epidermal cells (28).

Horseradish peroxidase (HRP) from *Armoracia lapathi* is the most investigated peroxidase enzyme. It catalyses a redox reaction between H_2O_2 as electron acceptor and many kinds of substances (phenolic substances, aromatic amines, ascorbic acid, ferrocytochrome C, NADH, etc.). Under certain circumstances, peroxidase also shows oxidase activity besides its peroxidative activity, i.e., it catalyzes the oxidation of different substances by atmospheric oxygen in the absence of exogenous peroxide (76).

The mechanism of peroxidative activity is characterized by a one-electron oxidation of donor molecules. The following reactions have been confirmed (26)

$$\text{Peroxidase} + H_2O_2 \longrightarrow \text{Compound I} \quad (1)$$

$$\text{Compound I} + AH_2 \longrightarrow \text{Compound II} + AH\bullet \quad (2)$$

$$\text{Compound II} + AH_2 \longrightarrow \text{Peroxidase} + AH\bullet \quad (3)$$

$$2AH\bullet + A + AH_2 \text{ (or AH -AH)} \quad (4)$$

Compounds I and II are thus found to be obligatory enzyme intermediates in the overall peroxidase reaction. The free radicals formed during this reaction sequence are very reactive and will result in a variety of peroxidase functions.

There is no unique reaction for measuring the peroxidative activity. Furthermore, not one test of activity is of special merit in rating the catalytic efficiency of all peroxidases (14,40).

Peroxidases are among the most extensively studied enzymes because of their wide distribution, especially in plant tissues. In plants, they were found in all parts (21,37,44,46). Peroxidases have also been found in fungi (64,91).

Physicochemical properties

The peroxidases as hemproteins are close to hemoglobin, myoglobin and the cytochrome (23). The prostatic groups of many peroxidases are known to be ferriprotoporphyrin IX. The heme group in plant peroxidases is less tightly bound to the protein than the heme in animal peroxidases (108). The enzymes have one heme group (one of the myeloperoxidases is said to have tow heme groups). Isoenzyme C of horseradish peroxidase consists of protein residue, prostatic group, 2 Ca^{2+} ions (92) and eight sites of carbohydrate attachment (107).

Plant peroxidases are characterized by the large number of isoenzymes present in most plant tissues (88). The isoenzymes may differ in their biochemical properties, such as specific activity, substance specificity, etc. (14).

Metabolic roles

Peroxidases have been involved in some important biological systems, such as: (a) lignin synthesis (45); (b) breakdown of flavonoids and benzoic acids; (c) degradation of auxin (75); (d) ethylene biosynthesis (65); (e) hydroxylation of proline (85); (f) participation in wound healing and (g) defense against pathogens (106). Peroxidases are

probably involved in defense mechanism against invading pathogens (87,92176) and, like lectins (103,73,86) have been found in epidermal cells.

METHODS

Growth conditions

(a) *Non-sterile conditions*

Cucumber and melon seeds were germinated on two layers of moistened filter paper (Whatman No. 1) and placed on plastic screens that were covered with transparent plastic bags for 3 days. Thereafter, they were transferred to polyvinyl rings floated on fresh water. Care was taken that the roots be covered with water. The seedlings were grown at 28°C in a controlled environmental chamber in darkness or under a 12-h photoperiod, respectively. Fluorescent standard cool-white lamps (120 μE, m^{-2}, sec^{-1} at the plant level) served as the light source.

Cucumber surface bacteria were isolated from 3-10-day-old seedlings which were grown as has been described above. The surface area of the seedlings were gently pressed on sterile bactoagar. The developing bacteria were grown in a sterile chamber at 28°C for 4 days.

Tobacco plants were grown in a greenhouse and cotyledons and 2-10-week-old leaves were tested for EPSA proteins.

(b) *Sterile conditions*

A series of experiments was carried out with sterile seedlings. Cucumber seeds were sterilized by soaking in 4% $NaHClO_4$ for 30 min. After washings, the seeds were sown in sterile bottles with plastic caps and germinated under the same conditions as described above.

Dry weight determination

After determination of the fresh weight, samples of the different plant parts were dried at 60°C for 48 hr during which they reached constant weight.

Chlorophyll assay

Chlorophyll was extracted with 80% acetone containing 0.1% Mg CO_3. The plant samples were weighed and homogenized at a ratio of 1 g fresh weight per 10 ml ethanol. After centrifugation at 12,000 g for 10 min, the supernatant was collected and measured at 645 nm and 663 nm, following essentially the method of Bruinsma (6). Arnon's formula (7) was used for the calculation of the chlorophyll contents, as follows:

$$20.2 \times O.D._{645} + 8.02 \times O.D._{663} = \mu g\ Ch/ml$$

Procedure of surface washings and homogenation for CSA assay

Samples of cotyledons, first leaves and hypocotyls were immersed, 4 times successively, in distilled H_2O at ambient temperature, each time at a surface area/water volume of 2.5. The first wash (fraction I) was from a 1 min immersion, and the 3 following washes (fractions II-IV) from immersions of 30 min each. After each wash the water was changed. Concurrently, an additional sample was immersed in water for 91 min (fraction V). Each one of the extracts (fractions I-V) was filtered and lyophilized to dryness. The dried material was dissolved in distilled H_20 at a ratio of 1 ml H_20 per 40 cm^2 original surface area (unless indicated differently in the text) to yield the crude CSA. After the 4th wash, the plant samples were homogenized in distilled H_20 (1 g fresh weight/10 ml) with a pestle and mortar. The homogenate was centrifuged at 12,000 g for 10 min. The supernatant (fraction VI) was examined for residual HA.

Surface excretions were also carried out for 1 hr at elevated temperature up to 100°C. The solutions were concentrated by lyophilization, brought to a volume of 1 ml and tested for HA. The heat treatments were carried out in a constant temperature bath (Temp-Block, Lab-line Instrument Co.).

Characterization of CSA was carried out with 1 min surface extracts from 3-day-old cotyledons containing 15 μg/ml of proteins which yielded a titer of 16.

Three-day-old hypocotyls and roots were homogenized in water as were the cotyledons and examined for HA.

Procedure of surface washings and homogenation for cucumber surface peroxidase assay

The procedure of surface washings and homogenation was similar to that described in the former paragraph. The lyophilized extracts were dissolved, unless indicated differently in the text, at a ratio of 1 ml water per 160 cm^2 original surface area to yield a crude fraction of surface peroxidase.

For isoelectric focusing, 4-day-old hypocotyls and cotyledons were immersed into water for 4 hr.

Treatments of human erythrocytes

(a) *Papain*

Human erythrocytes (5% cell suspension) were treated with 1% papain (crude preparation, Sigma Chemical Co.) and 0.1% cysteine at a 1:9 ratio of the enzyme/cell solutions. This mixture was incubated for 30 min. at 35°C and then the cells were washed three times with saline (8).

(b) *Sialidase*

Nine vols of a 5% human erythrocyte suspension was incubated with 1 vol of a sialidase solution in 0.05 M phosphate-buffered saline, pH 5.5. The enzyme-treated cells were washed 3 times in saline (9).

(c) *Trypsin*

A 4% erythrocyte suspension of rabbit or human cells containing 1 mg/ml of trypsin was incubated at 37°C for 1 hr. The trypsin-treated cells were washed 3 times with saline (10).

Hemagglutination assay

Hemagglutination titer was determined by the addition of 0.05 ml of a 5% suspension of papain-treated erythrocytes to a series of tubes containing 0.2 ml of serial two-fold dilutions of CSA dissolved in saline. 0.2 ml of each concentrated solution was added to the first tube in each series.

After 1 hr at 35°C, the tubes were centrifuged in a CW-1 Srovall centrifuge for 30 sec at 1000 g to allow the erythrocytes to sediment and then agglutination was examined. The degree of agglutination was determined after visual and microscopic examination.

Agglutination titer was expressed as the reciprocal of the highest dilution that yielded positive hemagglutination. Specific HA was expressed as titer per mg protein per ml of undiluted extract. The relative hemagglutination index expresses the number of tubes yielding a positive HA above or below the hemagglutination titer of 16, which was taken as 100%.

Effect of sugars and proteins on HA

Hemagglutinatin inhibition by sugars was tested with solutions of fraction I of 3-day-old seedlings that had a titer of 4 (3 μg protein/ml). Replicate serial two-fold dilutions of each sugar solution were made. Equal volumes (0.1 ml) of the CSA and sugar solutions were added to each tube. After an incubation of 1 hr at room temperature, a 5% solution (v/v) of papain-treated cells (0.05 ml) was added to each tube. The minimum amount of each sugar necessary to completely prevent hemagglutination was determined. Aqueous solutions of chitosan were prepared according to Young *et al.* (11).

Hemagglutination inhibition by proteins was tested with solutions of partially purified EPSA that had a titer of 32 (2 μg protein/ml). The inhibition was only determined qualitatively.

Effect of proteolytic enzymes on HA

1 ml of EPSA preparation was preincubated with 1 ml of 1% pronase in buffer tris-HCl, 0.1 M, pH 8), or with 1 ml of 1% trypsin and 1% papain in PBS, respectively. After incubation for 1 hr at 32°C, HA was examined.

pH resistance of CSA

EPSA stability over a wide range of pH was examined by mixing CSA with 0.1 M buffers composed, respectively, of solutions of citrate (pH 3.0, 5.0), phosphate (pH 6.0, 7.0, 8.0) tris-HCl (pH 8.0, 9.0) and glycine-NaOH (pH 10.0). After 1 hr of incubation at the indicated pH values at 20°C or at 98°C, the solutions were dialyzed overnight against 100 volumes of saline and tested for HA.

Removal of metals from EPSA

Removal of metals was attempted by four different methods:

(a) Dialysis for 24 hr at 4°C against 0.5 N NaCl adjusted to pH 1.5 - 2.0 with HCl (12)

(b) EDTA at a concentration of 0.2% in saline was included in the HA reaction mixture

(c) CSA was dialyzed overnight against a 1% EDTA solution at pH 3.0, 6.0 and 10.0, respectively. The dialysis against EDTA was followed by a 2 hr dialysis against saline

(d) CSA in saline, containing 0.2% EDTA, was heated at 98°C and then dialyzed against saline for 2 hr.

Effect of pretreatments of seeds on HA

Before sowing, seeds were pretreated for 6 hr with the following solutions, respectively: Ten-fold dilutions of 100ppm solutions of a protein inhibitor (cycloheximide), an inhibitor of RNA synthesis (actinomycin D) and an inhibitor of DNA synthesis (cytosine arabinose).

Enzyme assays

(a) *Peroxidase*

Determination of peroxidase was based on the oxidation of pyrogallol in the presence of H_2O_2 which was quanitified spectrophotometrically at 420 nm at 25°C (13). The reaction medium consisted of 5 ml 5 mM pyrogallol, 0.5 ml 0.29 M H_2O_2, plant surface extracts or homogenates and 50 mM Na/K phosphate buffer pH 6.0. The total volume of the reaction mixture was 6.5 ml. The reaction was continued until an O.D. of 0.4 was reached. The peroxidase activity was expressed as units of activity which were calculated according to the formula:

Units of peroxidase = 0.4 (O.D.) x $10^4/t_{(sec)}$ x w, where w = fresh weight, mg protein or cm^2 surface area, respectively, and t = time required to reach an O.D. of 0.4.

(b) *Catalase*

Catalase was determined polarographically by the oxygen electrode method (14). The reaction was carried out in a YSI oxygen monitor model 53. The reaction medium consisted of 0.1 ml 59 mM H_2O_2, 0.2 ml cotyledon surface extract and 2.7 ml 0.1 M, Na/K phosphate buffer pH 7.8.

(c) *Superoxide dismutase (SOD)*

Superoxide dismutase was determined by assaying the inhibition of autooxidation of epinephrine to adenochrome at alkaline pH (15). This was followed spectrophotometrically at 480 nm. The reaction mixture contained 1.33 mM 1-epinephrine, 33.3 mM Na_2CO_3 (pH 10.2), EPSA and 0.13 mM EDTA. SOD activity was also assayed by using the reduction of cytochrome C via the xanthine - xanthine oxidase system (16). The reaction mixture contained 167 μg/ml cytochrome C, 47 mM NaP buffer (pH 6.5), 0.1 mM xanthine and 40 μg/ml xanthine oxidase. The reaction was measured at 550 nm.

Protein assay and hydrolysis

Protein was determined according to Bradford (17) with bovine serum albumin as standard. Coomassie brilliant blue G-250 (100 mg) was dissolved in 50 ml 95% ethanol. To this solution 100 ml 85% (w/v) phosphoric acid was added. The resulting solution was diluted to a final volume of 1 liter.

Standard curve: Protein solutions containing 10 to 30 μg protein in a volume up to 0.1 ml were pipetted into glass tubes. The volume was adjusted to 0.1 ml with saline, 5 ml of the reagent were added and the content was mixed. After 2 minutes, the absorbance at 595 nm was measured. Purified EPSA was hydrolyzed with 2 M HCl for 6 hours. Tryptophan EPSA was also hydrolyzed with base (2 M NaOH, under N_3, 4 hrs).

Polyacrylamide gel electrophoresis (PAGE)

Electrophoresis was carried out in a discontinuous buffer system according to Davis (18). The stock running buffer was composed of 6.0 g Tris-HCl, 28.8 g glycine and distilled H_2O to 1000 ml (pH 8.3). Before use, the buffer was diluted 10 times with H_2O.

Samples were diluted with saline containing 60% sucrose to give a solution of about 1 mg/ml protein. The gels were run at 100V for 30 min; thereafter the voltage was increased to 200V for another 3-4 hr. Bromophenol blue was used as tracking dye during the electrophoretic run. The gels were stained overnight with 0.05% Coomassie brilliant blue R in a fixing solution composed of 450 ml ethanol, 100 ml glacial acetic acid and 450 ml distilled H_2O. Destaining was accomplished after several changes of the fixing solution diluted 1:1 with distilled H_2O. A comparison unfixed gel was sliced into 2.5 mm slices, extracted with saline and the agglutinating activity in the various sections of the gel was examined. Protein standards (Pharmacia, HMW) were used routinely in all runnings.

SDS-polyacrylamide gel electrophoresis

Molecular weight determinations, using 10% polyacrylamide gel in the presence of 0.1% SDS, was carried out according to King and Laemmli (19). For denaturation, the protein standard (Pharmacia, HMW) and CSA (partially purified, 0.1 mg protein) were dissolved in 100 μl saline containing 2.5% SDS and 5% ß-mercaptoethanol and heated for 10 min. Also, the running buffer contained 0.1% SDS. The staining and destaining procedures were the same as for the native gels.

Isoelectric focusing in polyacrylamide gel

Proteins of surface extracts at various concentrations were applied to polyacrylamide gel plates containing ampholines in a linear pH gradient at a range of 3.5 - 9.5 obtained from LKB (Bromma, Sweden). Samples of 10 μl were applied to 5 x 10 mm sheets. The isoelectric focusing was performed at 8°C for 1.5 hr at 100V and 20 mA at the start and was terminated at 400V, 5 mA. Then the gel was sliced longitudinally, one slice serving for protein staining with Coomassie brilliant blue R, according to the instructions of LKB. The other comparison unfixed slices were stained for peroxidase activity by incubating the slices in two different substrate solutions, either in a solution containing 15 mM guaicol, 5 mM H_2O_2 and 50 mM Na/K phosphate buffer pH 7.0, or in a solution of 5 mM pyrogallol, 0.29 M H_2O_2 and 50 mM Na/K phosphate buffer pH 6.0 (20). An additional slice was stained for superoxide dismutase activity. The remaining gel was sliced in the width into 2.5 mm slices, extracted with 2 ml saline in a glass-glass

homogenizer and the HA of the extracts was determined. Hemoglobin lysate was used as an internal pH marker (21).

Purification procedure of EPSA

In preliminary experiments we tried to purify EPSA by acetone precipitation (22) and by affinity chromatography on chitin columns. The columns were eluted with varying pH (23,24) in the presence or absence of agents such as 4N guanidine. Finally, the following precedure was adopted:

Cotyledons and leaves were harvested and heated at 90°C in distilled H_2O at a ratio of 1 g fresh weight per 5 ml H_2O. After 2 hr, the solution was filtered and cooled to room temperature. The solution was concentrated by filtration in a Diaflo ultrafiltration cell provided with PM-30 filters (American Corp.). The solution remaining on the filter (about 50 ml) was further concentrated by Centricon 30 filtration and then centrifuged at 26,500 g for 10 min. The clear supernatant was collected. Acetone was added to precipitate EPSA from the solution. The precipitate was left to settle overnight and then collected by centrifugation at 26,500 g for 15 min, dissolved in 4 ml of saline and recentrifuged in order to clarify the solution. To the clear solution, urea was added to a final concentration of 1.5 N and then applied to a column (2.5 x 80 cm) of Sephadex G-150 which was preequilibrated with saline containing 1.5 N urea. Urea improved EPSA elution from the column. Three mnl fractions of the eluate were collected and tested for HA. The fractions containing HA were combined, dialyzed against water, concentrated by lyophilization and redissolved in 1 ml saline.

Immunoblotting

Antibodies were raised against purified EPSA. Proteins were extracted in Tris-HCl buffer pH 7.4. EPSA and the extracted proteins were immunoblotted after Davis *et al.*

Assays for fungal infections

Effects of EPSA on fungal development and infection were tested with a variety of fungi and methods, as follows.

Preparation of spore suspensions

Spores of infected plants were collected into glass distilled water at 4°C. they were then filtered first through cheese cloth for the removal of conidiophores and then through 8 μ millipore filters for the removal of possible inhibitors. the filtered spores were resuspended in glass distilled water at a concentration of 5 x 10^4 spores per ml.

Infection procedures

1. For the examination of colonization and sporulation spores were dusted onto intact leaves by shaking of sporulating leaves above uninfected test plants.
2. For microscopic examination the infection was carried out by touch - infected leaves were slightly pressed on uninfected leaves.
3. *Sprays.* Spore suspensions were sprayed onto leaves or leaves were placed onto paper discs (5 mm dia.) soaked with spore suspensions. In some experiments leaf discs (3 cm dia.) were infected by a 10 μl drop of spore suspension. The leaf discs were placed on filter paper in covered Petri dishes and kept in the dark for one week at 20°C. Whole seedlings were kept in Percival fog cells at 20°C and a light intensity of 130 $\mu E \cdot m^{-2} \cdot sec^{-1}$ at a photoperiod of 12 h.

Germination tests

In vitro

Germination of spores under *in vitro* conditions was conducted with 2% agar discs (0.6 mm dia.) which contained 1-5 μg EPSA and which were placed on microscope slides. The slides were placed on wetted filter paper in Petri dishes. To each agar disc a drop of 10 μl spore suspension, containing about 500 spores, was applied. In other experiments the agar dishes were infected by touch with diseased leaves (see above).

In vivo

(1) Leaves of different ages were sprayed with a solution containing 5 μg EPSA and Tween 80. The leaves were then infected by touch. After 48 h collodium (7% collodium E950, Merck, in ether:ethanol, 3:1) prints were taken, treated with lactophenol trypan blue 0.1 % and germinating spores counted microscopically.

(2) Leaf discs (2.0 cm dia.) were excised, sprayed with 5% EPSA and Tween 80, and placed on wetted filter paper in Petri dishes. The discs were infected with 5-10 μl spore suspension (100 to 200 spores per drop). The Petri dishes were incubated for 5-18 hrs at 15°C. For estimation of spore germination, 5 μl of 0.01% calcafluor were put on the infected leaf disc and examined under a fluorescent microscope. The germinating spores fluoresced with a blue color while the plant tissue was black or red.

Colonization and sporulation

6 to 8 days after infection the plants were kept for 20 hrs in humid chambers at 18-20°C. Thereafter the spores were carefully brushed off the leaf surfaces into beakers containing a known volume of acetic acid:formalin:ethanol (5:5:90), counted in a haemacytometer and their number calculated per unit leaf area.

Penetration into leaves

48 hrs after infection, leaf discs were treated with alcoholic lactophenol blue: 95% ethanol (1:95 v/v), cleared by chloral hydrate and examined.

The fungal inhibitory effect of pure EPSA or partially purified EPSA was tested by spore germination inhibition and observed under a light microscope by cellophane-transfer technique or an epifluorescence microscope by calcofluor staining and by FITC-

conjugated antibody (18). Rice agglutinin content was assayed by indirect ELISA tests developed in our laboratory (18).

RESULTS

Proteins associated with cucumber seedlings

In the course of preliminary studies of plant surfaces we observed the occurrence of proteins which could be released from surfaces by short washes with water.

The first series of experiments was carried out under sterile growth conditions in order to eliminate the possibility that surface-bacteria are responsible or contribute to the surface-extracted proteins from sterile and non-sterile seedlings. Furthermore, bacteria isolated from non-sterile seedlings were devoid of any of the activities described in the following.

In a second series of experiments, we determined the protein amount released from plant surfaces into water by a 1 min wash. In light-grown cotyledons (Fig. 1), this protein level reached a peak level on the 2nd day after germination, declining thereafter. A quite similar pattern was obtained for etiolated cotyledons (Fig. 2).

CUCUMBER SURFACE LECTINS

During efforts to characterize the surface-located proteins, we found a lectin (hemagglutinin) which was termed "Epicuticular plant surface agglutinin" or (EPSA). Agglutinating activities were found in surface extracts of light- and dark-grown cotyledons, hypocotyls and first leaves. No activity could be detected in dry seeds or in seedlings up to 2 days after germination. The agglutination titer increased linearly with increasing surface area. This linear relationship enables the expression of hemagglutination titer per cm^2 of surface area. In surface extracts and homogenized cotyledons, EPSA could not be detected until the second day after germination. EPSA reached maximum activity on the 4th day after germination and gradually declined with progressing age (Table 1).

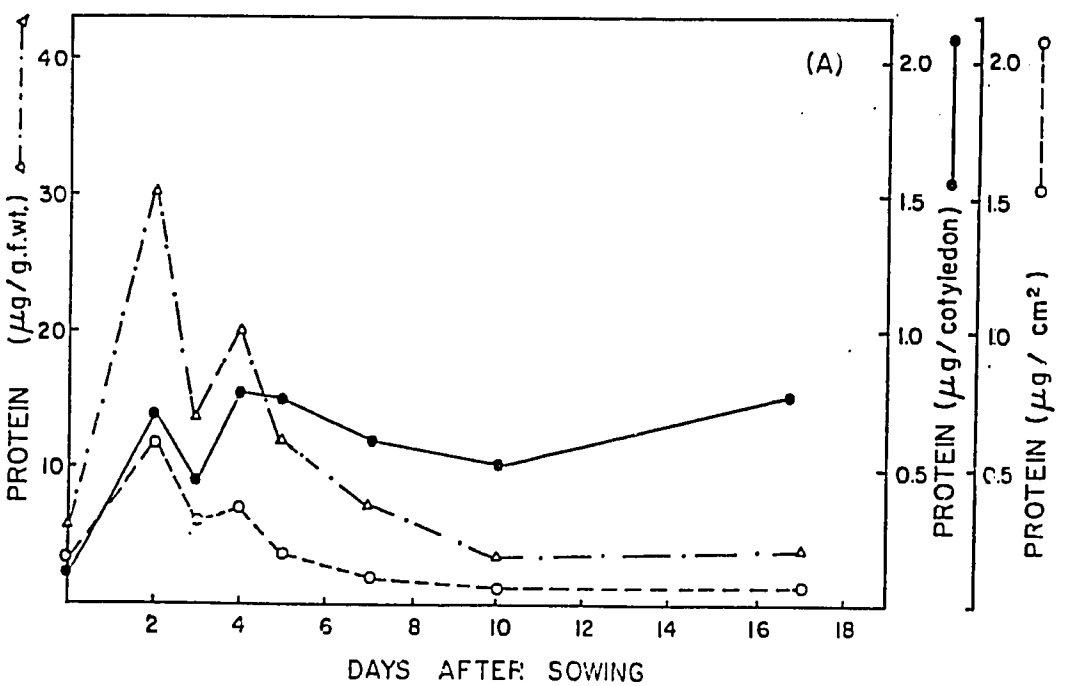

Fig. 1. Protein pattern in surface extracts of cucumber cotyledons

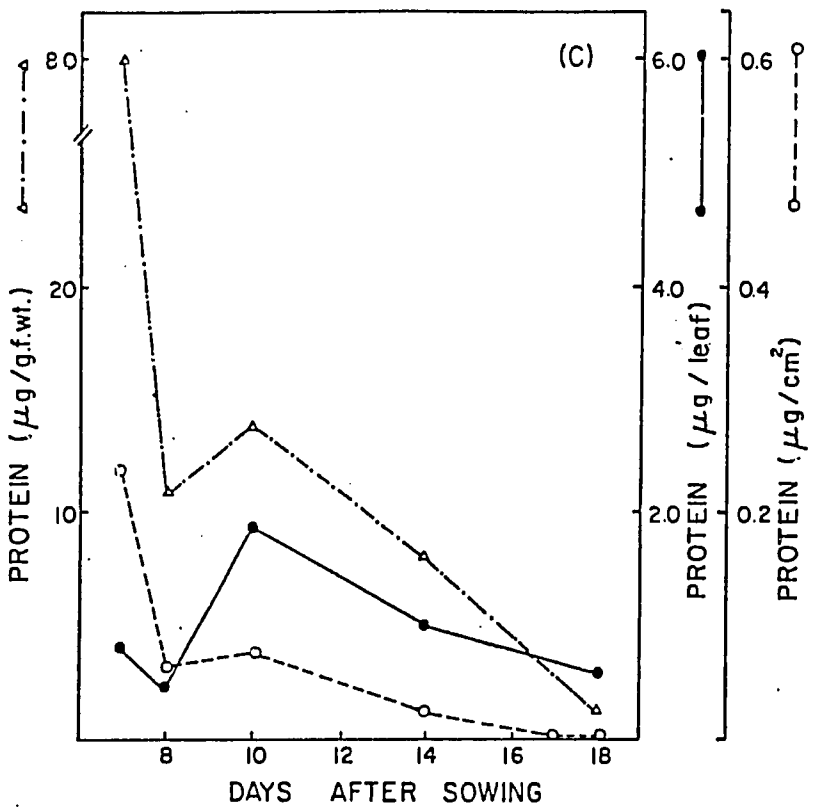

Fig. 2. Protein pattern in surface extracts of first leaves of cucumbers

Table 1. Specific hemagglutinating activity of EPSA x 10^2 from cucumber cotyledons and first leaves

Days from sowing	Cotyledons	First leaves
2	0	-
3	12	-
4	18	-
5	9	-
7	14	8
8	-	4
9	-	2
10	5	1
12	-	1
14	-	0
17	1	0

The specific activity of the surface extracts was 200 times higher than that of the homogenate of 4-day-old cotyledons. The specific activity of the homogenate of the etiolated cotgyledons was lower than that of the green cotyledons. The release of free agglutinin from the surface area of light- and dark-grown cotyledons was almost complete within 1 min; only an additional low activity was released when immersing the cotyledons for longer times. Homogenation of washed cotyledons liberated some residual agglutinin. The decrease of HA with age was much slower in dark-grown cotyledons.

Surface extracts and homogenates of first leaves exhibited high specific activities on the 7th day after sowing. This activity quickly declined and became undetectable with

further growth of the leaves. The kinetics of CSA release was similar to that of the other examined organs.

HA in surface extracts of melon and tobacco seedlings

Preliminary experiments with surface extracts of melon seedlings showed that this species also exhibits HA on its epicuticular surface similar to the cucumbers. Tobacco exhibited epicuticular activities only after increasing the pH to basic values (see later).

Characterization of EPSA

Effect of sugars and proteins on HA

Glucose, galactose, mannose, fructose, rhamnose, fucose, ribose, arabinose, xylose, maltose, lactose, sucrose, cellobiose, melibiose, glucosamine, galactosamine, mannosamine, N-acetyl-D-glucosamine $(GlcNAc)_2$, N-acetyl-D-galactosamine, polyglucuronic acid and carboxymethyl-cellulose, all at 1 mg/ml, did not inhibit agglutination. On the other hand, the complex sugars chitotriose $(GlcNAc)_3$, chitin, and chitosan entirely inhibited the agglutination reaction. The minimum concentration of $(GlcNAc)_3$ that inhibited HA of standard extract was 37.5 μg/ml and the minimum concentration of chitosan was 18.5 μg/ml. The inhibition by chitin at 1 mg/ml was determined only qualitatively because of its insolubility.

The following proteins, catalase, lipoxygenase, α-amylase, hexokinase and BSA, all at a concentration of 0.1 mg/ml, did not inhibit CSA activity. Only horseradish peroxidase, at the same conditions, entirely inhibited its agglutinating activity. This inhibition was determined only qualitatively.

Human erythrocyte specificity

A solution of 12 μg/ml of surface-derived EPSA was examined for specificity for blood groups. CSA agglutinated all three blood group types (A, B) (Table 2). EPSA activity was only four-fold higher for papain-treated erythrocytes as compared with untreated ones; Con A reacted more strongly with the papain-treated cells.

Table 2. Agglutination of papain-treated and untreated human erythrocytes by CSA and Con A

Agglutinin type	Blood group	Specific HA	
		Untreated cells	Papain-treated cells
CSA	A, B, O	333	1333
Con A	A, B, O	83	2666

Specific hemagglutinating activity was calculated as described in Materials and Methods. The initial concentration was 12 μmg/ml for crude EPSA and for Con A.

Effect of proteolytic enzymes

EPSA was differentially inactivated by proteolytic enzymes. CSA was resistant to pronase and less to trypsin which inactivated HA at an extent of 20-30%. HA was totally destroyed by papain.

Thermostability of EPSA

EPSA was found to be highly resistant to heat. Boiling of CSA inactivated HA within 100 min and autoclaving at 120°C within 15 min (Table 3).

Table 3. Effect of temperature on EPSA activity.

EPSA preparation of 12 μg/ml proteins derived from cotyledons were heated at 100°C or autoclaved, respectively The relative hemagglutination index was determined as in Table 4.

Temperature	Minutes 15	60	100
20	100	100	100
100	100	80	0
120*	0	0	0

* Autoclave

pH stability

At 20°C, EPSA was stable over a wide pH range (3.0 - 10.0). At 88°C, pH 10.0 EPSA was completely inactivated, while at pH 3.0, 10-20% of HA remained active.

Temperature effects on EPSA release

The high thermostability of EPSA could be used for improving its release from the surface. Elevated temperature increased EPSA release. Maximum release from young cotyledon surfaces was obtained at 88°C (a titer of 512 as compared to a titer of 16). Heating at 88°C of 16-day-old cotyledons had no influence on the release of EPSA. At 100°C, EPSA became inactivated.

EPSA activity in tobacco leaves

Water extracts of tobacco leaves were totally inactive. EPSA activity could be revealed at pH 8.4 and above (Table 4).

Survey of different plants for EPSA activity

EPSA activities could be detected in leaves of alfalfa, clover, vinca (Catharantus), persimmon and additional plants. Details of these plants will be represented in a further report.

Table 4 The effect of pH on the specific activity of EPSA in 2-week-old tobacco leaves

pH	HA
5.5	0
6.4	0
7.0	0
8.4	8
9.0	18
10.0	16
11.1	11
12.5	2

Cucumber surface peroxidase

Peroxidase may be released from the epidermis (e.g. by air pollution). Therefore, we examined its possible existence in the surface extracts. In parallel, we also tested catalase and superoxide dismutase activities. The surface extracts exhibited strong peroxidative activity. Maximum activity of surface-located peroxidase was observed 3-6 days after germination, at a period when the green cotyledons had just opened. This activity declined and disappeared entirely on day 10 (Fig. 3). In homogenates, about 14 days after germination, a second peak appeared as the cotyledons aged.

Catalase and superoxide dismutase activities in surface extracts

Catalase activity could not be detected in cotyledon surface extracts. Superoxide dismutase activity was found in surface extracts from all plant parts (Fig. 4). Slab gel electrophoresis yielded a number of weak SOD bands. In addition, large achromatic areas appeared at the anodic site which were not identical with the protein bands. This achromatic area appeared even after dialysis against GDTA.

Partial characterization of surface peroxidase

Peroxidase isoenzymes were separated by isoelectric focusing chromatography and made visible by guaicol and pyragallol stainings. Their pattern is presented in Fig. 5. Six bands of isoenzymes were separated: three, of basic properties (IV, V, VI) and the others were acidic isoenzymes (I, II, III). Of the six peroxidase bands revealed with pyrogallol as a donor, only three (I, II, III) became visible using guaicol. Two bands reacted intensively both with pyrogallol and gualicol (II, III). Fig. 5 further demonstrates that the lectin activity was electrofocused in parallel to peroxidase III.

Effects of EPSA on plant pathogens

Preliminary observations indicated that EPSA from cucumber cotyledons is able to inhibit the germination of spores on agar of a number of plant pathogens. In the report period we studied the development of Spherotheca fuliginea which induces powdery mildew in Cucurbitacea. For these tests we used cucumbers cv Bet-Alpha, Poinsett and Bush Champion. Plant cotyledons and leaves were brushed with an EPSA solution and then infected with the fungal spores. At various time intervals thereafter leave discs were cut out into beakers containing a fixative (formalin: acetic acid: ethanol, at a ratio of 5:5:90). The conidia were brushed into the fixative and then counted in a hemacytometer. Table 5 demonstrates that EPSA reduced markedly the sporulation of powdery mildew on the first leaf of cucumbers as well as the number of colonies. The small amount of EPSA available made it impossible to conduct larger and more detailed experiments. Such experiments will hopefully be done in the following year.

We further tested the effects of EPSA on spore germination of (1) Trichoderma viride, (2) Trichoderma lignorum, (3) Pseudomonas lacrimans, (4) Uromyces phaseoli,

(5) Fusarium solanii, (6) Fursarium oxysporum, (7) Sclerotium rolfsii and (8) Rhizocotonia solanii. Detailed results will be presented in the next report.

Experiments were initiated on the effects of EPSA on the infectivity of tobacco mosaic virus (TMV) in tobacco plants and tobacco necrosis virus (TNV) in cucumbers. Preliminary observations showed negative results for TMV and in about 50% inhibition of the formation of TNV lesions.

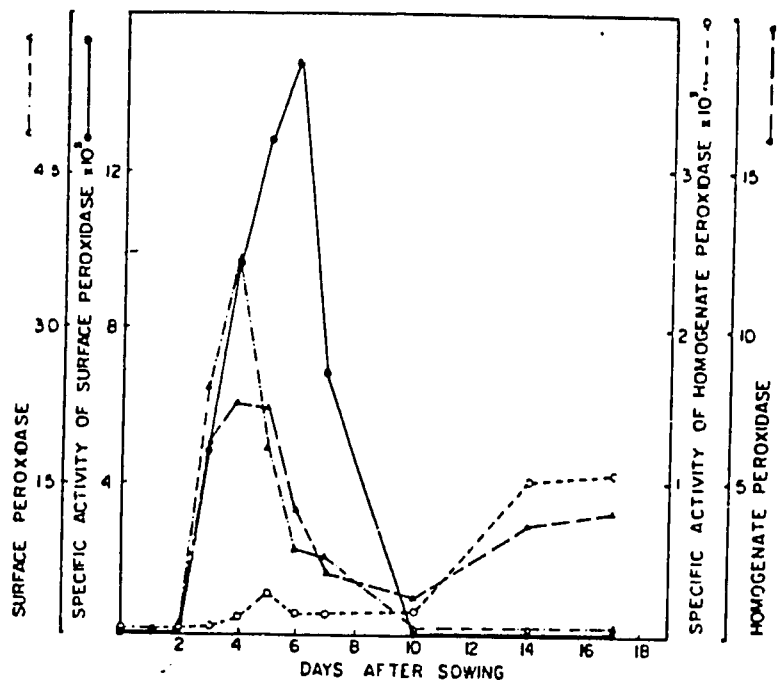

Fig. 3. Time course of peroxidase activity in surface extracts and in homogenates of light-grown cotyledons. Units of peroxidase were calculated in different ways, where W was: , cm^2 of surface area; 0 0 mg protein in homogenates; g fresh weight. 0 0, mg protein in homogenates. Each point is an average of 3 experiments.

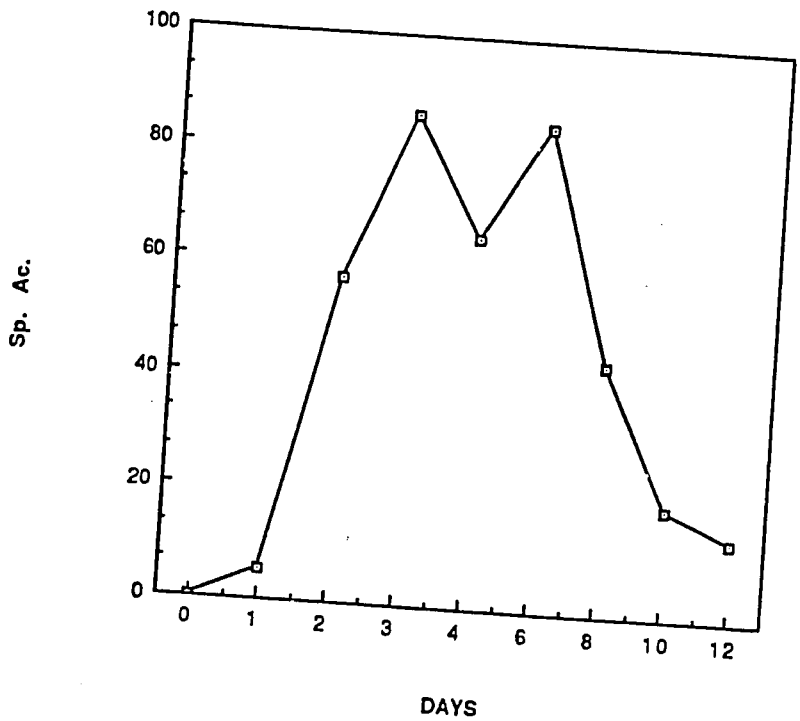

Fig. 4. SOD- like activity of ESPA in developing cucumber cotyledons.

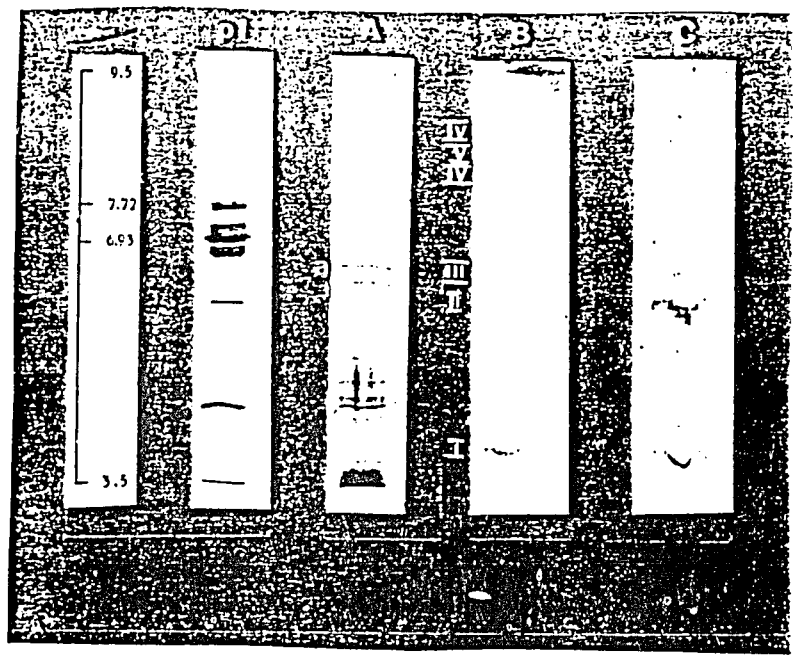

Fig. 5. Isoelectric focusing of surface located proteins. Samples of about 20 mg protein from 4-day-old light-grown cotyledons and hypocotyls were applied to the gels. A. Coomassie blue staining; B. pyrogallol staining; C. guaiacol staining. The gels were electrofocused as indicated under Materials and Methods. The numbers to the left of the pI gel indicate the pI of the focused pI marker proteins. Band a exhibited lectin activity.

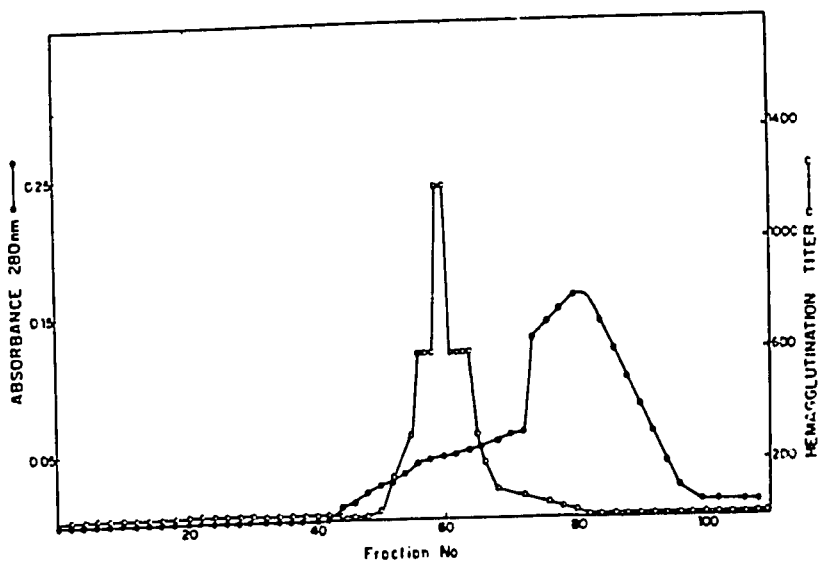

Fig. 6. Gel filtration chromatography of CSA extract on Sephadex G-150

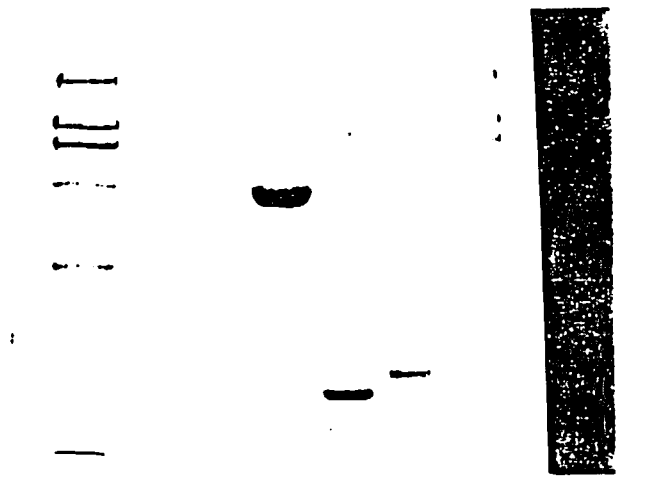

Fig. 7. POLYACRYLAMIDE - SDS ELECTROPHORESIS OF EPSA

The material eluted from the Sephadex G-150 column showed one broad major band with lectin activity on PAGE (Fig. 6). SDS-PAGE showed one major band of approximately 18,000 M.W. (Fig. 7). Isoelectric focusing of the active eluate showed two major bands in which only one (band a) exhibited CSA activity.

The major EPSA peak obtained from Sephadex-150 gel chromatography was taken or further polyacrylamide-SDS gel electrophoresis (Fig. 7). Lanes 2 and 5 show one distinct band each. The band from lane 2 was isolated after PAGE-SDS preparative electrophoresis. Its total amino acid composition is represented in Table 6 which indicated a particularly high percentage of glycine.

Recently, we determined the sequence of the 30 terminal amino acids (Table 7) on the basis of which a number of synthetic 60-mer deoxyoligonucleotides were synthesized (data will be presented at a later stage). The latter will hopefully serve as probes for the production of sufficient amounts of EPSA which will enable the performance of larger experiments in the field of plant protection.

Table 6. Amino acid composition of EPSA

Amino acid	%
Aspartic	4.19
Threonine	7.53
Serine	8.58
Proline	3.06
Glutamic	4.28
Glutamin	1.05
Asparagin	1.45
Alanine	3.59
Valine	5.77
Methionine	1.48
Isoleucine	3.52
Leucine	5.28
Tyrosine	3.77
Glycine	27.49
Arginine	5.20
Phenyl-Alanine	3.25
Histidine	2.05
Lysine	6.86

Table 7. Terminal amino acid sequence of cucumber EPSA

asn-thr-asp-iso-thr-ser-asp-gly-glu-arg-met-pro-glut-ser-thr-lys-ala-ser-gly-pro-phe-lys-gly-val-lys-cys-met-gln

C. Immunoblotting of EPSA

EPSA and tissue proteins of cucumber cotyledons and first leaves were immunoblotted (Fig. 8). EPSA can be detected at the plant surface and in the homogenate. Quantitatively its level seems to be higher in young cotyledons (Fig. 8, lanes 1 and 2) as in the first leaves (Fig. **8**, lanes 3 and 4).

D. Comparison of SOD-like and peroxidase activities of EPSAs of different sources

In Table 8 we represent comparative data of SOD-like and peroxidase activities from some plants which exhibited surface agglutinin activities. Purifications of these EPSAs, their chemical properties and fungicidal activities are presently in progress.

Fig. 8 : Immunoblotting of EPSA from Cotelyden Surface and Homogenate

1 2 3 4

Lane 1 - Cucumber cotelyden surface (3 days old)

Lane 2 - Cucumber cotelyden homogenate (3 days old)

Lane 3 - Cucumber 1st leaf surface (10 days old)

Lane 4 - Cucumber 1st leaf homogenate (10 days old)

Table 8. SOD-like and peroxidase activities of EPSAs from different young plant seedlings. Enzyme activity of SOD is represented as specific activity ($\mu l\ 0_2 \times min^{-1}$ per mg protein).

Plant	Specific Activity SOD	Peroxidase (PU)
Cucumber	61	14
Garden pea	89	12
Eggplant	25	8
Mung bean	9	9
Tobacco	33	3
Papaya	105	22
Tomato	32	12
Chili pepper	48	18

SCREENING OF PLANTS RELEASING EPSA

Cucurbitaceae

Agglutinins were extracted from seeds of *Benincasa cerifera, Cucumis sativus, Luffa cylindrica* Roem and *Luffa acutangula* by using 4 different solvents: 0.02 M phosphate buffer saline (PBS) pH 6.8; 70% ethanol; 0.01 N acetic acid and 0.075 N NaOH. The agglutination activity tested by trypsinized rabbit reticulocytes was highest in 70% ethanol extract of *C. sativus* and *B. cerifera*, whereas *L. acutangula* showed the highest activity in PBS (Table 9). *L. acutangula* or angle loofah was selected for further purification by ammonium sulfate precipitation because of its highest agglutinating activity. The highest hemagglutination titer and SOD activity were found in the pericarp

extract comparing to pulp and seed extracts (Table 10). The sugar inhibition test indicated that the pericarp LSA was specific to N-acetylglucosamine and N-acetylgalactosamine. In loofah seedlings 5-11 days, the PBS extracts of root contained the highest agglutinating activity with respect to hypocotyl and cotyledon (Fig. 9). In addition, LSA crude extract, and ammonium sulfate-precipitated fractions from either pericarp or seedling showed positive SOD activity staining on gel electrophoresis and inhibition of cytochrome C reduction (Fig. 10, 11). LSA therefore resembles CSA with respect to sugar specificity and SOD-like activity, but differs by showing maximum EPSA activity in the root fraction, not cotyledon.

Table 9. Screening of agglutinins from seeds of Cucurbitaceaes. Seed samples were homogenized in 0.02 M PBS and lipid was removed by acetone extraction. The hemagglutinating activity (HA) was tested with trypsinized rabbit red blood cells.

Cucurbitaceae	**Specific HA (HA unit/mg protein)**
White guard (*Benincasa ceriera*)	-
Cucumber (*Cucumus sativus*)	-
Smooth loofah (*Luffa cyclindrica* Roem)	13.9
Angled loofah (*Luffa acutangula*)	34.6

Table 10. Agglutinating activity and SOD activity in different parts of *Luffa acutangula*. Fractions of fruit were extracted with PBS, lipid was removed, and precipitated with 60% saturated ammonium sulfate. The specific HA and specific SOD activities were assayed as described in Methods (SOD 1 unit = 50% inhibition of cytochrome reduction).

Agglutinin type (*L. acutangula*)	**Specific HA** (HA unit/mg protein)	**Specific SOD activity** (SOD unit/mg protein)
Pericarp	919.6	80.2
Cortex	300.1	0.3
Seed	34.6	36.7

Leguminosae and Solanaceae

Screening of agglutinins from seedlings of pea, *Pisum sativum*, mung bean, *Vigna radiata*, tomato, *Lycopersicon esculentum*, eggplant, and *Solanum melongena* by using human red blood cells showed that all plants tested contain agglutinins, except *V. radiata* that trypsinized rabbit reticulocytes has to be used to detect agglutinating activity (Table 11).

Table 11. Agglutinating activity and sugar specificity of aqueous extracts from surface of plant seedlings.

Plant age of seedlings (days)	Surface extract of Root	Hypocotyl	Cotyledon	Epicotyl	Leaf
Tomato (9-day-old)					
Tissue fresh weight (g)	9	6	-	-	4
Total protein (mg)	2.2	1.7	-	-	2.4
Protein conc. (mg/g fr.wt.)	0.24	0.28	-	-	0.6
Total HU (units)	2560	320	-	-	160
Sp. act. (HU/mg prot.)	1163	188	-	-	67
Sugar specificity (mM)	D-mannosamine (150)				
Eggplant (10-day-old)					
Tissue fresh weight (g)	5.7	4.6	-	-	1.5
Total protein (mg)	4.6	3.6	-	-	2.8
Protein conc. (mg/g fr.wt.)	0.81	0.78	-	-	1.9
Total HU (units)	1920	960	-	-	1600
Sp. act. (HU/mg prot.)	417	266	-	-	571
Sugar specificity (mM)	NI	NI	-	-	NI
Pea (7-day-old)					
Tissue fresh weight (g)	3.4	-	116	28	14
Total protein (mg)	9.9	-	11.5	4.6	7.2
Protein conc. (mg/g fr.wt.)	0.29	-	0.10	0.18	0.5
Total HU (units)	960	-	5120	640	1280
Sp. act. (HU/mg prot.)	97	-	441	139	178
Sugar specificity (mM)	NI	-	GalNAc (12.5)	NI	NI
Mung bean (5-day-old)					
Tissue fresh weight (g)	72	110	22	8	11
Total protein (mg)	16.4	15.8	4.6	4.6	11.5
Protein conc. (mg/g fr.wt.)	0.28	0.13	0.21	0.58	1.1
Total HU (units)	5120	1280	1280	1280	5120
Sp. act. (HU/mg prot.)	312	81	278	278	445
Sugar specificity (mM)	NI	NI	NI	NI	NI

Agglutinating activity and sugar specificity were measured by using human RbC; B and O.
NI = not inhibited by any sugar listed in Table 12.

Partial purification of agglutinins from seed extracts by increasing the concentration of ammonium sulfate indicated that 60% saturation was suitable for harvesting agglutinins, among which *P. sativum* showed the highest activity, but these agglutinins showed various sugar specificity when tested with 16 types of sugars (Table 12), for example: *P. sativum*, *V. radiata* and *L. esculentum* were specific for D-mannose, D-galactose and N,N-diacetylchitobiose, respectively, whereas agglutinin from *S. melongena* was not inhibited by any sugar tested.

Table 12. Sugars used for hemagglutination inhibition test at 200 mM.

Monosacharide	Disaccharide	Aminosugar
L-Rhamnose	D-Mellibiose	D-Glucosamine
D-Arabinose	D-Cellobiose	D-Mannosamine
L-Arabinose		N-Acetyl-galactosamine
D-Trehalose		N-Acetyl-glucosamine
D-Mannose		D-galactosamine
D-Xylose		N,N-diacetylchitobiose
D-Fucose		
D-Galactose		

Surface washing from various parts of seedlings of these plants showed different distribution patterns of the maximum agglutinating activity. Besides, surface agglutinins of these seedlings have different sugar specificity from those agglutinins extracted from dry seeds. Since there were problems in obtaining foundation seeds with high germination rate of these plants, soybean (*Glycine max*) was chosen for further study with seedlings' agglutinin. The release of soybean surface agglutinins (SSA) was high in young cotyledon (3-day-old) and rapidly decreased, whereas SSA in root increased with

age (Fig.12). The SSA activity, whether from cotyledon or root, was specifically inhibited by D-galactose and N-acetyl galactosamine. The SOD activity was also detected in the cotyledon SSA fraction (Fig. 13). Since the maximum peak of EPSA was observed in 3-day-old cotyledon fraction, it should differ from SOD which showed maximum activity in 6-8-day-old cotyledon.

Rice (Oryza sativa L.)

Agglutinins from seeds and seedlings of most cereal crops have been extensively characterized (19), especially wheat germ agglutinin (WGA). In this research, EPSA from root and root exudate of rice, subspecies indica cv. RD7 was found to be similar to agglutinin purified from embryo and bran with respect to molecular weight, pI, and amino acids composition (8). They are glycoproteins and differ from each other by carbohydrate moieties. Antiserum against purified embryo agglutinin of rice cv. RD7 cross-reacted with agglutinin in embryo extracts from 30 cultivars (*Oryza sativa* L.) 2 wild rice species and WGA (18). The antibody against agglutinin RD7 was used in combination with FITC-goat antirabbit immunoglobulin to localize surface agglutinins on root and leaf of rice, cv. RD7 seedlings, 4-7-day-old. Rice surface agglutinins (RSA) were located on the root tips, root hair tips, and generally on the root epidermal cells. On the leaf, RSA was specifically observed on hydathodes and opening stoma.

Screening of RSA in 8 cultivars of local rice was performed by indirect ELISA, using goat antirabbit immunoglobulin conjugated with alkaline phosphatase as the second antibody, the highest RSA/plant was found in cv. NMS4, and the lowest in cv. KDML 105, both in the leaf and root fractions (Table 13).

Table 13. Screening of RSA in 8 cultivars of indica rice. Rice seeds were surface-sterilized and germinated in the dark for 4 days, then cut into leaf and root portions, kept frozen at -15°C for 1 hr, then homogenized in PBS. Lectin was assayed by using indirect ELISA.

Rice cultivars	Lectin concentration (ng/mg protein)		Total lectin content (ng/100 plants)	
	Leaf	Root	Leaf	Root
NMS4	228±61	31±7	4230±1135	92±20
RD7	210±42	15±2	2628± 522	58± 6
RD23	307±29	29±2	2405± 224	34± 2
SPBR60	151±19	13±1	1776± 228	33± 3
KTH17	32± 2	8±1	859± 150	31± 2
BMT470	136±14	19±1	773± 80	24± 1
RD25	115±17	10±1	711± 104	17± 0
KDML105	59±15	6±0	598± 151	5± 0
Mean ± SD	155±85	16±9	1785±1167	36±25

Values presented are means of 6 measurements.

Agglutinin in seed extracts

The seeds were screened for agglutinating agents, using red cells of human blood type A, B and O. Among eight kinds of plant extracts tested, seven showed agglutinating activity (Table 14) against all three types of human red blood cells, namely A, B and O. Seed extracts from *Vigna radiata* did not cause agglutination of human red blood cells but caused agglutination of trypsinized rabbit red blood cells.

Table 14. Agglutinin in seed extract

Seed extract from plant	Protein (mg/ml)	Human RBC			Trypsinized rabbit RBC
		A	B	O	
1. Cassia fistula	0.4	/	/	/	ND*
2. Cassia alata	2.28	/	/	/	ND
3. Phaseolus vulgalis (Red kidney bean)	1.53	/	/	/	ND
4. Pisum sativum (Garden pea)	29.5	/	/	/	ND
5. Vigna radiata (Mung bean)		0	0	0	/
6. Solanum melongena (Egg plant)	5.25	/	/	/	0
7. Capsicum minimum (Chilli pepper)	4.15	/	/	/	0
8. Lycopersicon esculentum (Tomato)	5.9	/	/	/	ND

* ND = not determined

Partial purification of agglutinin in seed extract

Partial purification of agglutinin was attained with ammonium sulfate fractionation. To determine the appropriate ammonium sulfate concentration, precipitation of agglutinating activity from seed extracts by ammonium sulfate was performed by using percent saturation ranges from 0-30%, 30-60% and 60-100%. The fraction that showed highest activity (Table 15) was collected and tested for hemagglutination inhibition test using sugars listed in Table 16.

Table 15. Partial purification of agglutinin from seed extracts

Step	Volume (ml)	Activity (U/ml)	Total act. (Units)	Protein conc. (mg/ml)	Spec. Act. (U/mg)	Yield (%)	Purification factor (fold)
1. Cassia fistula							
Crude extract	45	8	304	0.40	20.0	100	1.0
0-30% fraction	12	4	48	0.21	19.0	15.8	0.5
30-60% fraction	6	0	0	0.4	0	0	0
60-100% fraction	9	0	0	0.4	0	0	0
2. Cassia alata							
Crude extract	1.3	32	41.6	2.28	14.0	100	1.0
0-30% fraction	2.3	8	18.4	1.13	7.0	44.2	0.28
30-60% fraction	40	0	0	0.21	0	0	0
60-100% fraction	26	0	0	0.20	0	0	0
3. Phaseolus vulgalis (Red kidney bean)							
Crude extract	142	512	72704	1.53	334	100	1.0
0-30% fraction	21	32	672	0.78	41	0.9	0.1
30-60% fraction	37	512	18944	0.51	1003	26.0	3.0
60-100% fraction	7	64	448	0.78	82	0.6	0.2
4. Solanum melongena (Egg plant)							
Crude extract	155	8	1240	5.25	1.5	100	1.0
0-30% fraction	3.3	4	13	2.34	1.7	0.9	1.1
30-60% fraction	11.5	16	184	5.22	3.0	7.4	2.0
60-100% fraction	6.2	0	0	2.41	0	1.8	0
5. Capsicum minimum (Chili pepper)							
Crude extract	70.0	64	4480	4.5	14.2	100	1.0
0-30% fraction	6.3	16	100	3.7	4.4	7.3	0.3
30-60% fraction	14.0	128	1792	5.6	22.7	25.0	1.6
60-100% fraction	7.5	16	120	3.9	4.1	9.2	0.3
6. Lycopersicon esculentum (Tomato)							
Crude extract	235	1280	330800	15.9	80.5		
30-60% fraction	19.5	5120	99840	59.4	86.2		
7. Pisum sativum (Garden pea)							
Crude extract	183	1280	234240	29.5	43.4		
30-60% fraction	5	10240	41200	37.5	273.1		
8. Vigna radiata (Mung bean)							

Seed extracts did not cause agglutination of human RBC of any blood type but can cause agglutination of trypsinized rabbit RBC and this hemagglutnating activity is inhibited by D-galactose at 1.6 mM concentration.

Table 16. Sugars used for hemagglutination inhibition test

Monosaccharide	Disaccharide
D-Arabinosel [1,2]	Sucrose [2]
D-Xylose [1,2]	Maltose
D-Galactose[2]	Lactose [2]
D-Mannose [2]	
D-Fructose [2]	
D-Fucose [1,2]	
Amino sugars	**Sugar alcohol**
D-Glucosamine [1,2]	Manitol [2]
D-Mannosamine [1,2]	
D-Galactosamine [1,2]	
N-Acetyl Galactosamine [1]	
N-Acetyl Glucosamine [1]	
N,N-Diacetylchitobiose	
N,N,N-triacetylchitotriose	

[1] Sugars used for HA inhibition with seed extracts from Cassia sppes and Phaseolus vulgaiis. There was no inhibition by any sugar at 800 mM conc

[2] Sugars used for HA inhibition with seed extracts from Solanum melongena and Capsicum minimum. There was no inhibition by any sugar tested.

Agglutinating activity of tomato was inhibited by N,N-diacetylchitobiose at 2.3 mM concentration, whereas that of pea was inhibited by D-mannose at 3.1 mM concentration.

Agglutinin in surface extracts of various vegetative tissues of seedling

Table 17 showed the mg of protein per g of plant tissue contained in the aqueous extracts of the various organs of seedling as well as its agglutinating activity and sugar specificity. In 9-day old tomato seedling and 10-day old egg plant seedlings the agglutinating activity was found in the surface extracts of root, hypocotyl and leaf with relatively large amount in the root and leaf surface washes, respectively. In 7-day old pea seedlings the agglutinating activity was found in all surface extracts with relatively large amounts in the cotyledon surface extract. And in 5-day old mung bean seedlings the agglutinating activity was found in all surface extracts examined with relatively large amount in the leaf surface extract.

Table 17. Hemagglutinating activity* and sugar specificity in water-soluble surface extracts from tissues of seedlings

Age of Seedling	Surface extract of				
	Root	Hypocotyl	Cotyledon	Epicotyl	Leaf
9-Day old tomato seedling					
Tissue fresh weight (gm)	9	6	-	-	4
Total protein (mg)	2.2	1.7	-	-	2.4
Protein conc. (mg/gm fr.wg.)	0.24	0.28	-	-	0.6
Total HU (Units)	2560	320	-	-	160
Specific act. (Units/mg prot.)	1163	188	-	-	67
Sugar specificity (mM)	D-Mannosamine (150 mM)				
10-Day old eggplant seedling					
Tissue fresh weight (gm)	5.7	4.6	-	-	1.5
Total protein (mg)	4.6	3.6	-	-	2.8
Protein conc. (mg/gm fr. wg.)	0.81	0.78	-	-	1.9
Total HU (Units)	1920	960	-	-	1600
Specific act. (Units/mg prot.)	417	266	-	-	571
Sugar specificity (mM)	NI	NI	-	-	NI
7-Day old pea seedling					
Tissue fresh weight (gm)	3.4	-	116	28	14
Total protein (mg)	9.9	-	11.5	4.6	7.2
Protein conc. (mg/gm fr. wg.)	0.29	-	0.10	0.18	0.51
Total HU (Units)	960	-	5120	640	1280
Specific act. (Units/mg prot.)	97	-	441	139	178
Sugar specificity (mM)	NI	-	GalNAc (12.5 mM)	NI	NI

5-Day old mung bean seedling

Tissue fresh weight (gm)	72	110	22	8	11
Total protein (mg)	16.4	15.8	4.6	4.6	11.5
Protein conc. (mg/gm fr. wg.)	0.28	0.13	0.21	0.58	1.05
Total HU (Units)	5120	1280	1280	1280	5120
Specific act. (Units/mg prot.)	312	81	278	278	445
Sugar specificity (mM)	NI	NI	NI	NI	NI

*Hemagglutinating activity was measured by using all three types of human red blood cells, namely A, B, and O. Sugar specificity was measured by using human red blood cells types B and O.

NI = not inhibited by sugars listed in Table 2.

Summary of Findings

Agglutinins from Pisum sativum, Vigna radiata, Lycopersicon esculentum and Solanum melongena were isolated. Agglutinins isolated from dry seeds of P. sativum, L. esculentum and S. melongena were able to agglutinate red blood cells of human blood groups A, B, and O, whereas agglutinin from V. radiata could agglutinate only trypsinized rabbit red blood cells.

Agglutinins from seeds of P. sativum V. radiata and L. esculentum were specific for D-mannose, D-galactose and N,N-diacetylchitobiose respectively whereas agglutinin from seed of S. melongena was not inhibited by any sugar tested.

The agglutinating activity in the surface extracts of various parts of seedlings of these plants was studied with the help of the two-fold serial dilution method of agglutinin assay. In 7-day P. sativum seedlings the agglutinating activity was found in the surface extracts of the root, cotyledon, epicotyl and leaf with relatively large amount of agglutinating activity in the cotyledon surface extract. In 5-day V. radiata seedlings the agglutinating

leaf with relatively large amounts of agglutinating activity in the leaf surface extract. In 9-day L. esculentum seedlings and 10-day S. melongena seedlings agglutinating activities were found in the surface extracts of the root, hypocotyl and leaf, with relatively large amounts of agglutinating activity in the root surface extract, and the leaf surface extract respectively.

Agglutinins isolated from the surface of seedlings had a sugar specificity which was different from that of agglutinins isolated from dry seeds. This implies that agglutinins from surface extracts of seedlings were different from seed agglutinins.

According to the proposal we initiated a survey of plants for leaf surface agglutinius which included both Israeli and Thai plants: *Cassia fistula, Cassia alata*, some rice varieties, *Pisum sativum* (garden pea), *Vigna radiata* (mung bean), *Solanum meongena* (egg plant), *Capsicum minimum* (Chili pepper), some *Nicotiana* species and *Lycopersicon esculentum* (tomato).

Seed Extracts

With the exception of mung bean, all seed extracts of the tested plants exhibited agglutinating activity against red blood cells. Mung bean extracts showed agglutinating activity against red blood cells and against trypsinized rabbit red blood cells.

Particularly high agglutinating activities were observed in red kidney beans, tomato, chili pepper and rice. In preliminary experiments we tried to purify partially the agglutimins by differential precipitation with ammonium sulfate. The active fractions are presently tested for superoxide dismutase and peroxidase activities as well as for their fungicidal properties.

Seedling extract.

Tomato: The agglutinating activities were distributed among the different seedling organs according to roots > hypocotyls > leaves. Cotyledon and epicotyl extracts showed no activity. Agglutination was inhibited by D-mannosamine.

Mung beans: Seedling organ activity distribution followed roots = leaves > hypocotyl = cotyledon > epicotyl.

In **peas** the activity pattern between the seedling organs was cotyledons > leaves > roots > epicotyl and in the **egg plants** the pattern roots > leaves > hypocotyls was obtained. In the latter cotyledon and epicotyl extracts were inactive.

Tobacco: Agglutination activity from tobacco seedlings could be observed after the surface extracts were basidified to pH 8.4.

EPSA: The seedling extracts of mung beans, peas and eggplants exhibited EPSA activities.

In tobacco EPSA activity could be detected only after basification.

FUNGISTATIC EFFECTS OF EPSA

We studied the effects of EPSA on the germination and sporulation of a variety of fungi and fungi-plant interactions. Three parasitic fungi are studied in more detail, namely, Sphaerotheca fuliginea which develops powdery mildew in cucurbits, Perenospora tabacina the blue mold fungal disease in tobacco and Erysiphe cichoracearum which leads to powdery mildew in tobacco.

Powdery mildew of tobacco

This disease is of widespread occurrence and causes heavy damage to agricultural plants. The responsible fungus is an ascomycete obligatory exoparasite which is located on the leaf surfaces and in epidermal cells. Its germ tubes penetrate directly into the epidermal cells by excreting "penetrating enzymes" (e.g. cutinase, pectinase, pectine esterase and cellulase) which dissolve the cuticle and epidermal cells walls. In most pathogenic fungi the first germ tube of the germinating spore is responsible for the penetration which occurs 10-15 hrs from infection. After penetration it develops a haustorium. After the latters' formation the two other germ tubes are formed which then ramificate and develop more haustoria. In immune species only the first germ tube develops. One of the mechanisms of resistant varieties and/or resistant developmental stages may be the presence of preformed chemical inhibitors.

In the following experiments we tested age-affected changes in the resistance of tobacco plants to E. cichoracearum and the efficiency of EPSA from cucumber hypocotyls on the germination and penetration of the fungus into the plant.

Table 18. Sporulation of E. cichoracearum and development of powdery mildew on tobacco

Age of Plant (days)	% Germinating Spores 1 tube	3 tubes	No. colonies/cm²	No spores/cm²
13	30.5 [a]	26.8 [a]	-	-
60	22.1 [b]	51.3 [d]	10.7	8.9

Different letters indicate significant differences ($p<0.05$)

Table 19. Effect of EPSA on the germination of E. cichoracearum *in vitro*. EPSA was sprayed on agar blocks. Germination was examined after 16 hrs.

Concentration (μg/ml)	Germination Inhibition %
0	0 [a*]
1	48 [b]
5	63 [c]
10	66 [c]
20	81 [cd]

* Different letters indicate significant differences ($p<0.05$)

Table 18 represents the development of powdery mildew on tobacco plants indicating that the development of the fungus declines as the plant grows older. EPSA inhibited the germination of the spores *in vitro* (Table 19) and *in vivo* (Table 20).

Table 20. Effects of EPSA on the development of powdery mildew *in vivo*.

Conc. (μg/ml)	(No. of germ tubes) 1	2	3
0	28.7 ± 5.2	1.8 ± 1.2	55.7 ± 6.9
1	31.6 ± 4.8	1.6 ± 1.2	16.1 ± 5.3
5	35.3 ± 6.6	2.0 ± 1.6	4.5 ± 3.0
10	33.5 ± 7.4	1.4 ± 1.6	1.4 ± 1.1

Table 20 indicates that EPSA inhibited principally the development of the 3rd germ tube but had practically no effect on the initial stages of spore germination. EPSA also inhibited the development of the fungus (table 21).

Table 21. Effects of EPSA on the development of powdery mildew

Conc. (μg/ml)	No. of Colonies per cm^2 leaf tissue	No. of Conidia per cm^2 (x 10^3)
0	17.8 a	19.7 a
1	12.4 b	13.8 b
5	10.4 b	12.5 b
10	8.1 c	6.2 e
15	0.5 d	0.8 c
20	1.0 e	0.3 d

Table 22 represents data of further experiments on the *in vitro* effect of EPSA on spore germination. Similarly, additional *in vivo* experiments demonstrated the effects of EPSA

on the germination of the powdery mildew fungus spores, its inhibitory effect on the formation of the 3-germ tube structure (Table 23) and of the fungal development (Tables 24-27). It is seen that (1) infection is inversely dependent on the age of the leaves and (2) the effect of EPSA increases with leaf age.

Table 22. Effects of EPSA on germination of the powdery mildew fungus *in vitro*.

Conc. (μg/ml)	% Inhibition	
0	0	a
1	53	b
2	87	c
5	81	d
10	100	cd
0	0	a
1	58	b
2	71	cd
5	66	bc
10	81	bc
0	0	a
1	43	b
2	65	c
5	56	b
10	81	bc

Table 23. Effects of EPSA *in vivo* on the germination of spores and no germination of spores developing 3 germ tubes of powdery mildew fungus.

Conc. (μg/ml)	No germination (%)	Number of 3-germ tubes (%)
0	25.7 ± 5.4	50.7 ± 6.9
1	52.4 ± 9.4	23.7 ± 4.6
2	65.7 ± 17.9	14.5 ± 4.7
5	55.1 ± 6.4	10.6 ± 3.8
10	66.4 ± 16.2	12.2 ± 1.3
0	30.1 ± 7.3	41.5 ± 11.0
1	49.9 ± 13.6	19.5 ± 3.9
2	45.7 ± 12.0	18.9 ± 4.1
5	49.2 ± 10.0	11.7 ± 6.8
10	44.7 ± 9.7	11.0 ± 6.6
0	36.5 ± 10.5	52.4 ± 6.4
1	48.4 ± 2.9	29.7 ± 7.7
2	65.9 ± 6.1	12.8 ± 4.6
5	45.9 ± 6.1	15.9 ± 5.1
10	62.1 ± 10.2	9.2 ± 0.8
0	-	45.7 ± 0.6
1	-	21.7 ± 0.1
2	-	24.3 ± 0.5
5	-	10.9 ± 0.9

Table 24. *In vivo* effects of EPSA on the development of powdery mildew fungus.

Conc. (μg/ml) (%)	Number of Conidia per cm² (x10)	Number of Colonies per cm²
0	25 a	10.9 a
1	10 b	8.5 c
2	6 c	6.0 b
5	4.8 d	
10	4.0 d	3.7 d
0	-	11.2 a
1	-	10.2 a
2	-	10.3 a
5	-	5.9 b
10	-	5.0 b

Table 25. Plant-age-dependent effects of EPSA (10 μg/ml) on germination and development of powdery mildew (in % of control).

Plant Age		% Spore Germination		
No. of leaves	Length of Stem (cm)	1	2	3
4	7	68.5 c	1.0 d	0.5 d
6	10	62.5 c	0.5 d	0.5 d
54	30	59.0 c	0 d	1.0 d

Table 26. Effects of EPSA on spore germination as dependent on leaf age. The plants were 10 weeks old, measured 24 hrs after infection. Data are represented in % germination.

Leaf Age *	% Germination	Number of germ tubes 1	2	3
7	27 ± 1.4	32 ± 7.1	6.0 ± 1.4	27.1 ± 4.3
6	23 ± 3.5	30 ± 7.4	3.3 ± 2.0	29.2 ± 0.8
5	25 ± 1.2	22 ± 2.6	5.1 ± 1.1	26.4 ± 7.3
4	26 ± 4.6	16 ± 4.3	3.0 ± 1.9	26.5 ± 4.2
3	21 ± 3.5	16 ± 0.9	2.0 ± 1.9	18.1 ± 3.9
2	24 ± 2.6	18 ± 2.3	1.5 ± 1.2	11.6 ± 1.5
1	24 ± 9.2	16 ± 6.4	0.5 ± 0.7	5.2 ± 2.2

* Leaf number 1 is the oldest

Table 27. Effect of EPSA on spore germination as function of leaf age. The plants were 10 days old. Counts were taken 6 days after infection.

No. of Leaf	No. of Colonies		No. of Spores per cm²		No. of Spores per Colony	
	-EPSA (total no)	+EPSA (% inhibition)	-EPSA (total no)	+EPSA (% inhibition)	-EPSA (total no)	+EPSA (% inhibition)
4	10.5	35	9.[illegible]	42	8.6	45
3	12.8	48	6.1	45	5.0	41
2	11.8	47	6.2	68	4.9	68
1*	12.4	61	4.2	77	3.5	73

* Leaf no. 1 is the oldest

Table 28. The effect of leaf surface application of EPSA upon the development of S. fuliginea on the first leaf of cucumbers at various time intervals after infection.

A. Sporulation (no. of conidia per cm^2);

B. Colonies (no. per leaf).

Days after infection		Control	EPSA
	7	21,400 ± 1,160	4,900 ± 2,300
A.	12	29,100 ± 2,400	8,500 ± 2,950
	18	32,300 ± 5,922	18,250 ± 8,120
	5	162 ± 75	92 ± 88

Soybean surface agglutinin (SSA)

The 60% ammonium sulfate precipitated fraction of cotyledon SSA showed fungistatic effect on *Curvularia* sp. by inhibiting spore germination (Fig. 14). The per cent inhibition was dependent on the SSA concentration (Table 29).

Table 29. The effect of SSA on spore germination of *Curvularia* sp.

SSA Concentration (ug protein/ml)	Spore germination (%)
0	98.25 ± 7.74
3	95.25 ± 3.10
6	55.00 ± 4.16
12	28.25 ± 4.65
24	1.50 ± 1.29

Rice agglutinin

Binding of purified rice agglutinin to the spores and emerging germ tubes of *Trichoderma viride* was evident by immunofluorescent staining (Fig. 15). In the absence of rice agglutinin, Fig. 16 shows that spores of *T. viride* germinated after 12 hr of incubation and formed adventitious branches of germ tubes about 22 hr after incubation, but in the presence of 1 $mg.ml^{-1}$ lectin, germination was delayed to about 22 hr and a significantly lower number of branching can be observed. When the concentration of lectin was reduced to 0.5 $mg.ml^{-1}$, and the minimal medium was used instead of yeast extract 0.2%, the inhibitory effect of rice lectin on spore germination was still significant (Fig. 18). Since RSA was localized on the root surface, root tips and root hair tips, opening stoma and hydathodes of seedlings' leaves, which are the potential infection sites of many pathogens, their locations correspond well with plant protective function. However, our findings that lectin content in embryo and seedling of rice varies with cultivars, developmental stage, and exogenous nitrogen (18) should encounter for variation in the degree of protection mediated by natural agglutinins. Purified EPSA from cucumber which resembles rice lectin in terms of sugar specificity, pH stability and thermostability has been reported to inhibit formation of conidiospores in powdery mildew, and inhibit spore germination in *T. viride*, *T. lignorum*, *Pseudomonas lacrimans*, *Uromyces phaseoli*, *Fusarium solanii*, *Fusarium oxysporum*, *Sclerotium rolfsii* and *Rhizocotonia solanii*. CSA also reduced the per cent formation of lesions in tobacco plant infected by tobacco necrosis virus (TNV). It is hoped that RSA could also inhibit several types of pathogens. As for the mechanism of action, it is possible to study RSA at the molecular level because cDNA clones encoding the embryo lectin of rice were already isolated, and used to characterize the expression of RSA in developing embryo (20).

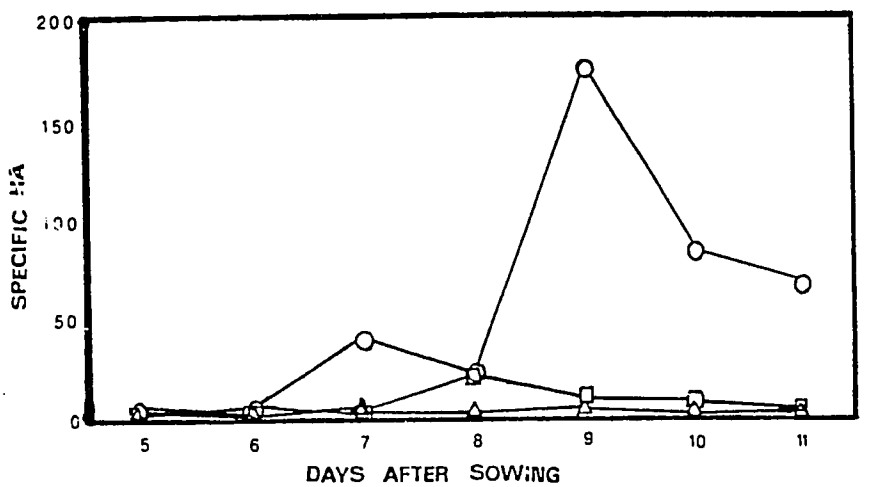

Fig. 9 Specific activity of agglutinins from different parts of loofah seedling; cotyledon (Δ), hypocotyl (□), and root (O) at different developmental stages.

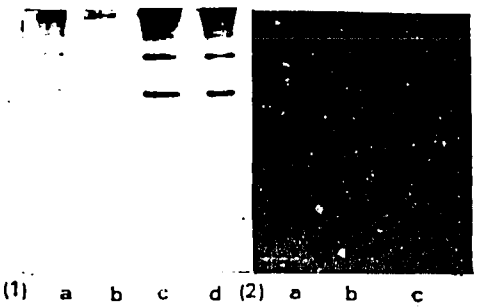

Fig. 10 Protein and SOD activity stains applied to polyacrylamide gel electrophoregram of crude extract and 70% $(NH_4)_2SO_4$ fractions of loofah fruit pericarp. Lane 1a and 1b are 50 and 25 ug crude extract, whereas 1c and 1d are 130 and 65 ug of partially purified fractions stained for protein. Lane 2a, 2b are 20 and 10 ug crude extract, and 2c is 65 ug partially purified fraction stained for SOD activity.

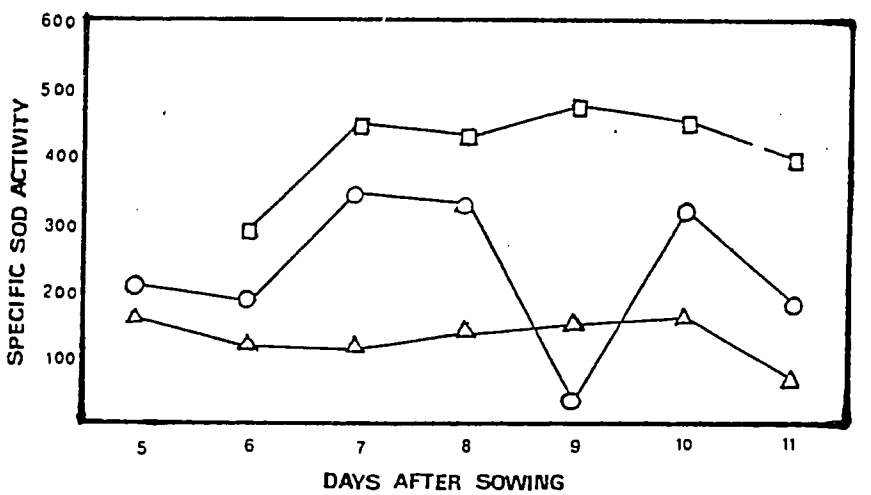

Fig. 11 Specific activity of SOD in different parts of loofah seedling; cotyledon (△), hypocotyl (□), and root (○) during development.

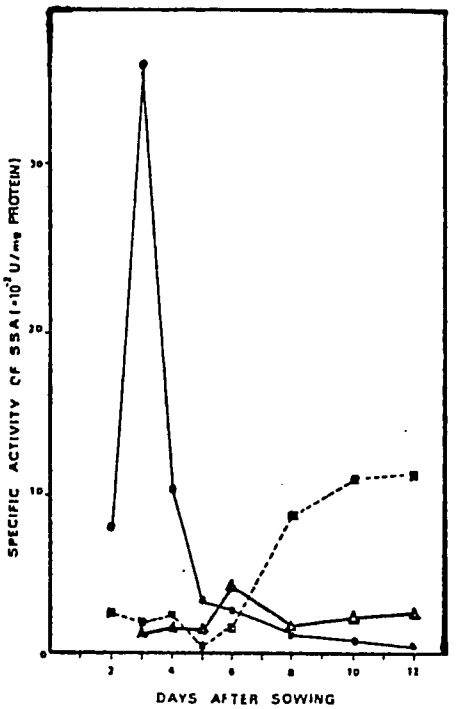

Fig. 12 Specific agglutinating activity of SSA (90 minutes washing) at different developmental stages from cotyledon (●), hypcotyl (△), and root (■).

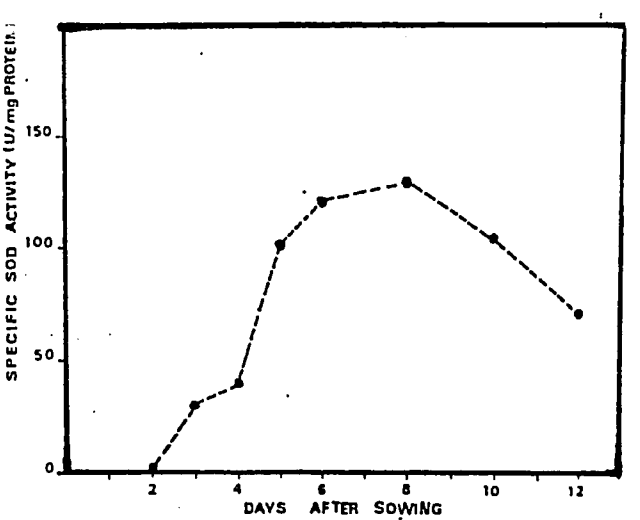

Fig. 13 Specific activity of SOD in cotyledon surface washing of soybean at different ages of seedlings.

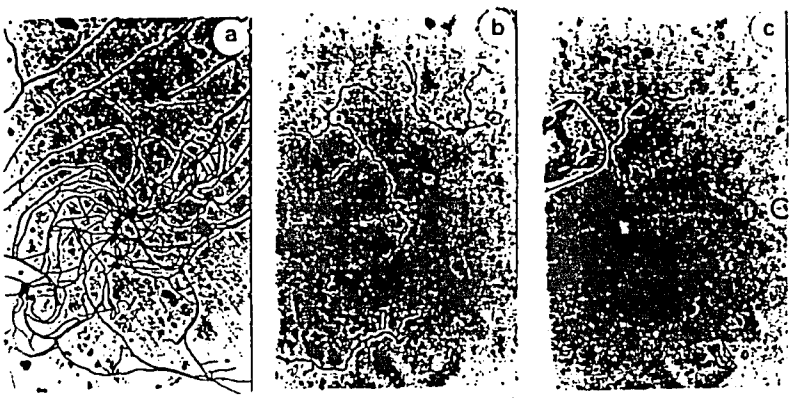

Fig. 14 Germination of *Curvularia* sp. spores in the absence of SSA (a), in the presence of SSA (b and c), the arrow (↓) showes ungerminating spores.

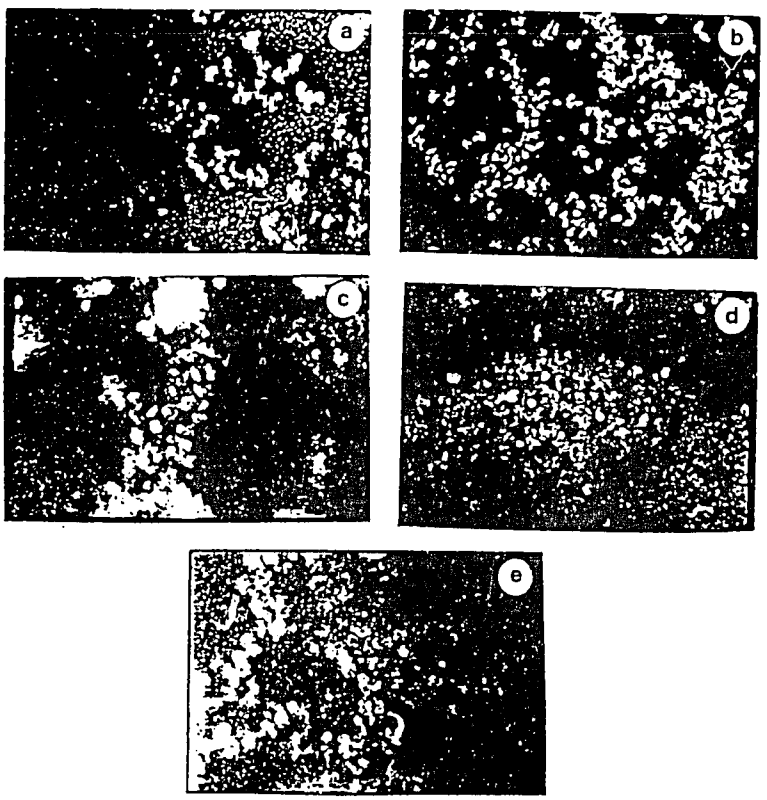

Fig. 15 Binding of rice lectin to spores of *Trichoderma viride*. The spores were incubated with rice lectin (1 mg.ml^{-1}), antilectin (dilution 1:10), followed by goat antirabbit IgG-FITC conjugate (dilution 1:500). Photos were taken at; a = 0h; b = 2h; c = 4h; d = 12h; and e = 18h after addition of lectin (a,b,c,d, and e x 80).

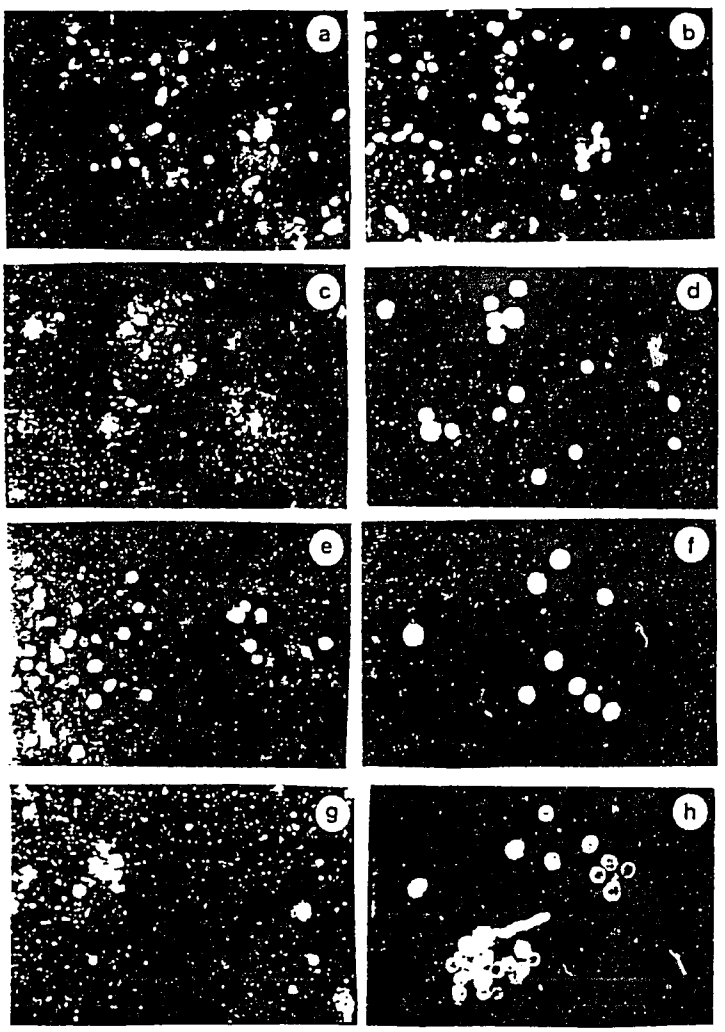

Fig. 16

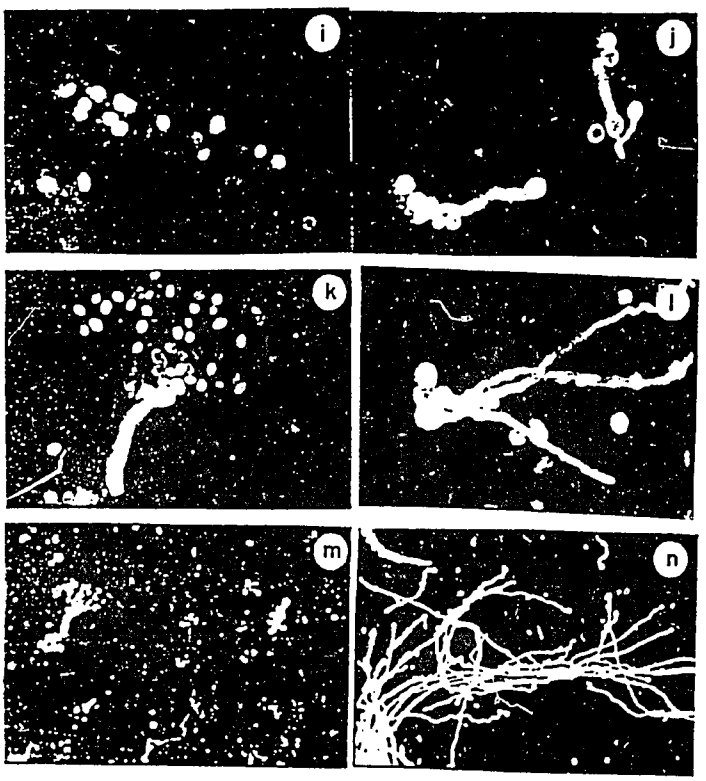

Fig. 17 Spores germination in 0.2% yeast extract in the presence and absence of 1 $mg.ml^{-1}$ rice agglutinin. The spores were stained with 0.01% calcofluor (a,b,c,d,e,f,g,h,i,j,k,l x 200; m,n x 50). Photos were taken at; a,b = 0h; c,d = 2h; e,f = 4h; g,h = 12h; i,j = 18h; k,l,m and n = 22h after addition of lectin in a,c,e,g,i,k and m.

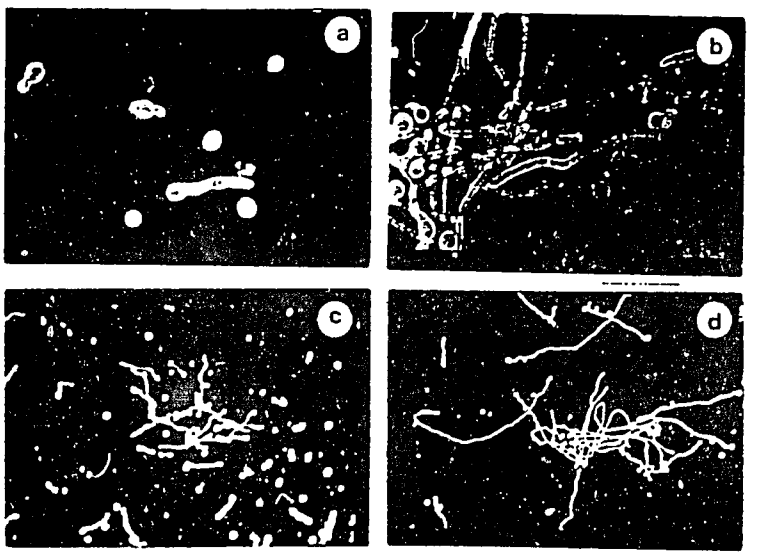

Fig. 18 Spores germination in minimal medium in the presence of 500 $\mu g.ml^{-1}$ rice lectin. The spores were stained with 0.01% calcofluor (a,b X 200; c,d x 50). Photos were taken at 24h after addition of lectin in a and c.

DISCUSSION

Most of the available published information on plant surfaces is concerned with the lipid constituents of the cuticle. In this work we devoted our main attention to water-soluble materials of plant surfaces, which, as found in this work, also contained proteins.

Lectins, as well as peroxidase are found in membranes and cell walls. It is possible that EPSA, peroxidases and other proteins might be released from epidermis as a result of changes in membranes or cell walls as in imbibed seeds. The release of proteins might also result from a secretion process such as the selective release of peroxidase to intercellular spaces (24). Like many lectins (25), EPSA apparently exists at physiological pH in an aggregate form of a Mr of about 140,000. It apparently consists of subunits of about Mr 18,000 molecular weight. Like few other lectins, CSA probably does not require metal ions for its activity. EPSA is extremely stable to high temperatures, chemical denaturants such as urea, guanidine, organic solvents and high salt concentrations. It is also resistant to acid and alkaline pH as to proteolytic enzymes.

The stability of EPSA appears to be an intrinsic property of the molecular structure, since no stabilizing components could be detected during the stages of purification. Absence of the substrate or metal ions had no influence on its stability. Hydrophobic bonds are thought to contribute to the heat stability of proteins (26) and possible also for EPSA.

Biological significance of EPSA

Although the amount of EPSA is very low, it may nevertheless play an ecological role. Its localization, high sugar specificity and stability possibly enables EPSA to react with pathogens on the plant surfaces as has indeed been demonstrated in this report. Its resistance to high temperatures and proteolytic enzymes probably prevents its degradation by pathogens and environmental conditions. EPSA thus could remain in an active form on the surface area during the development of the seedlings.

The inhibition of agglutination activity by $(GlcNAc)_3$, chitin and chitosan also hints at its possible role in the recognition of microorganisms. Chitin is absent in higher plants but is a common wall constituent of fungi and algae (27). Chitosan, which is a deacetylated derivate of chitin, is a component of the cell wall of many fungi (28). Therefore, EPSA may be involved in the protection of plants against pathogens. The accumulation of chitosan in pea - *Fusarium solani* interaction (29) and in the infection of wheat by *Puccinia striiformis* (30) supports this hypothesis.

Table 30 summarizes some properties of EPSA demonstrated in the present account.

Table 30. Summary of some properties of EPSA

1. Inhibited by N, N', N'' - triacetylchitotriose (> 35 μg/ml), chitosan (> 20 μg/ml) and chitin
2. Thermostabile up to 85° at pH 4-10
3. Stable to pH 3-10 at 25°. Inactivated at 85° at pH 3 and pH 10
4. Inhibited by horseradish peroxidase but not by other proteins tested
5. Resistant to pronase and trypsin (20-30% inactivation), but not to papain
6. Resistant to urea and guanidine
7. Agglutinating activity
8. SUPEROXIDE DISMUTASE - LIKE ACTIVITY
9. Contains a markedly high content of glycine
10. Inhibits germination and development of some fungi

In this report we demonstrated the existence of agglutinating proteins on plant surfaces, some properties of which are summarized in Table 30. Further investigation of surfaces of plants are needed in order to understand how and why plants release proteins to the surface during growth. Studies of the composition, structure and arrangement of the surface-located proteins seem to be of importance for an understanding of recognition phenomena. If EPSA is involved in the protection of plants against pathogens, it could possibly be used for blocking receptor or target surfaces for protection from pathogens as in the case of the Chlorella lectin, where administration of the Chlorella lectin protected mice against the bacteria (31).

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