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Ethylene Inducing Protein - Biochemistry and Mode of Action in Plants

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ABSTRACT

The ethylene-inducing protein that was purified from Cellulysin was also shown to be endoxylanase (EIX). On SDS-PAGE it was resolved into 3 polypeptides of 22, 14 and 8 kD. Antiserum to the native EIX immunoprecipitated both the enzymatic and biological activities. Synthesis of a single 22 kD extracellular polypeptide detectable on immunoblots with the 3 antisera against the EIX polypeptides was inducible in Trichoderma viride during growth on D-xylose, xylan or crude cell-wall preparations. Other fungal species also synthesised a 22 and a 14 kD polypeptides similar to that found in Cellulysin.

When EIX was applied to petioles of isolated tobacco leaves, it was taken up in the vascular system and as a result an ACC synthase activity, a drop in pH, an increase in electrolyte leakage, necrosis and wilting of the leaves were observed. When EIX was injected into the vascular system of intact plants through a petiole, it was translocated and induced ethylene as well as necrosis in leaves above and below the leaf of injection.

Endoxylanase activity was found in extracts of avocado fruit, however it did not cross react with antibodies to EIX. We did not reach yet a complete purity of this enzyme. Based on Superose 12 column data with FPLC, the apparent molecular weight of the avocado endoxylanase is 67 kD. Avocado fruit extract were found to contain also xylosidase (1,4-exoxylanase) activity. Both enzymic activities were determined during avocado fruit ripening.

OBJECTIVES OF THE ORIGINAL RESEARCH PROPOSAL

- a. To study the chemical and physical structure as well as biological characteristics of the EIP which is found in the cellulolytic enzyme preparation, Cellulysin.
- b. To explore different systems (i.e. higher plants, fungi and bacterial) for the presence of EIP (identical or similar) using antibodies raised against EIP.
- c. To study in more detail the mechanism by which EIP induces ethylene biosynthesis by stimulating ACC-synthase activity. Attempts will be made to determine if EIP binds to specific receptors, does EIP actually get into the cell or does it act as an enzyme to produce an oligosaccharide-type component which gets into the cell to increase ACC-synthase activity. Using radiolabelling studies and monoclonal antibodies to ACC-synthase, determine if the increase in ACC-synthase is due to activation or de novo synthesis.

For the most part we did follow these objectives. As for part (a) there were a few changes in procedures when new information became available. Our initial objective to purify the ethylene biosynthesis-inducing protein was accomplished as well as making antibodies to the native and denatured form of the protein. Once we discovered that the ethylene biosynthesis-inducing protein also contained an endoxylanase activity, we started to culture the fungus to see if we could induce the production of this protein. We found that we could induce the synthesis of this protein when T. viride was cultured on xylan and were able to feed radioactive amino acids and get radioactive EIX. However, the specific activity of the protein was low. Attempts to cleave the polypeptide with proteases in urea were not that successful. However, we did find we could chemically cleave EIX with N-chlorosuccinamide. This cleaves at tryptophan residues. The protein contains no methione and therefore we were not able to cleave with cyanogen bromide. We did run Cleveland gels using the chemically cleaved polypeptides. Several attempts

were made to microsequence the polypeptide, but these were unsuccessful because it is N-terminally blocked. However, we were able to isolate some natural degradation products which were partially sequenced.

We did not deviate from part (b) of the objectives. Here we looked for similar type of proteins in several fungi and higher plants. The antibodies did cross react with several of the fungi xylanases but not with the partially purified avocado xylanase. For part (c) we made some progress. We found that EIX is capable of moving in the vascular system, presumably in the xylem, of detached leaves and intact plants. EIX movement was monitored with tissue blotting techniques as well as western blotting. Progress on understanding how ACC synthase is induced is slow. However, we have been able to get good enzyme activity out of green tobacco leaves that were induced with EIX. Attempts to purify this enzyme and compare it to other enzymes are in progress. Besides inducing ACC synthase activity we were able to show that EIX induces a hypersensitive-type response. This was not foreseen in our proposal.

BODY OF THE REPORT

1. Purification and Biological Characteristics of The Ethylene-Inducing Protein.

1.1. THE NATURE OF AN ETHYLENE BIOSYNTHESIS-INDUCING FACTOR FOUND IN CELLULYSIN

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ABSTRACT

The proteinaceous ethylene biosynthesis-inducin factor (EIF) that was isolated from Cellulysin was shown to contain a xylanase activity. In all non-denaturing protein separation methods employed (Sephacryl S-200 chromatography, preparative isoelectric focusing and agarose electrophoresis), xylanase activity co-purified with the ethylene biosynthesis-inducing activity. Treatment with heat (60°C) or proteases in 8 M urea inhibited both ethylene biosynthesis-inducing and xylanase activities. Antibodies raised against EIF immunoprecipitated both ethylene biosynthesis-inducing and xylanase activities. The purified EIF contained no detectable cellulase, polygalacturonase, protease or other hydrolytic activities as estimated by using p-nitrophenyl derivatives of several sugars as substrates. Based on these data, we suggest that EIF contains a specific xylanase activity which is involved in inducing ethylene biosynthesis.

INTRODUCTION

It is well established that some isolated protoplasts produce ethylene (Anderson et. al., 1979; Cassells et. al., 1980; Facciotti and Pilet, 1981; Guy and Kende, 1984) and that this ethylene biosynthesis was induced by a component(s) of the cell wall digesting enzymes prior to the release of protoplasts (Anderson et. al., 1982; Chalutz et. al., 1984). In the case of the enzyme mixture, Cellulysin, proteins that induce ethylene biosynthesis have been purified and partially characterized (Fuchs and Anderson, 1987). The purified ethylene biosynthesis-inducing factor (EIF) contains 3 polypeptides of 18, 14, and 10 kD. In this paper, we summarize our studies on the purification and characterization of this factor, the enzymatic properties of EIF and the preparation of antibodies.

MATERIALS AND METHODS

The purification of EIF was according to Fuchs and Anderson (1987). After initial absorption to Sephacryl S-200, desorption with salt, followed by ammonium sulfate

precipitation (90% saturation), preparative isoelectric focusing, agarose mini-gels electrophoresis, preparative agarose gel column electrophoresis (BRL), freeze drying and Superose 12 sizing column HPLC. The EIF is quite stable at room temperature and can be frozen and thawed many times without significant loss of activity.

Assays: Tobacco (*Nicotiana tabacum* L., cv., Xanthi) leaves from plants grown in the greenhouse were pretreated for 16 h with 100 μ l/l ethylene prior to harvesting leaf discs (1 cm in diameter) for the induction of ethylene biosynthesis. Six leaf discs (approximately 50 mg) were incubated at 23°C for 4 h in 25 ml Erlenmeyer flasks containing 1 ml of basal medium [700 mM sorbitol, 10 mM Mes-NaOH (pH 6.0), 10 mM CaCl_2 , 50 μ g/ml streptomycin sulfate and 50 units/ml penicillin G] together with sample to be bioassayed. Ethylene was quantified by gas chromatography (Chalutz et al., 1984).

The EIF used for enzyme assays was the most highly purified fraction described previously (Fuchs and Anderson, 1987). Xylan substrates, prepared from oat spelts (Sigma) or birch wood (Roth) were used for enzyme assays according to Wood and McCrae (1986). Reducing sugars were determined by the method of Sumner (1924). The assay using Remazol brilliant blue xylan (Sigma) was done according to Biely et al. (1985). p-Nitrophenyl derivatives of: β -D-xylopyranoside, β -D-glucopyranoside, α -D-galactopyranoside, α -L-arabinofuranoside, and α -D-glucopyranoside, as well as CMC, polygalacturonic acid and BSA were purchased from Sigma. The p-nitrophenyl sugar derivatives were used as substrates according to Boller and Kende (1979). Cellulase and polygalacturonase activities were determined by measuring reducing groups released from CMC and polygalacturonic acid, respectively, and proteolytic activity by measuring an increase in A_{280} of acid soluble material from BSA. Reaction products from xylanase activity using oat spelts or birch wood xylan were separated on silica gel G TLC plates with ethylacetate/acetic acid/water (2:1:1). Reducing sugars were detected with p-anisidine-phthalic acid reagent (Randerath, 1963).

Inhibition studies: Proteolytic digestions of EIF [20 μ g protein separated on a Sephacryl S-200 column (Fuchs and Anderson, 1987)] with 10 μ g trypsin or V_0 in a total of 30 μ l were carried out at 30°C in the presence of 8 M urea. EIF (20 μ g in 40 μ l water) was also heat inactivated at 60°C.

Antibodies production: New Zealand female rabbits were immunized with the major fraction of purified EIF from agarose electrophoresis (Fuchs and Anderson, 1987). Two doses of 200 μ g of antigen in complete Freund's adjuvant (Sigma) were given subcutaneously at two-week intervals. One week following the second subcutaneous immunization, 100 μ g of antigen was given intravenously. Animals were bled weekly for several weeks thereafter. The Ig fraction was partially

purified from the serum by precipitation with 40% saturated ammonium sulphate.

Immunoprecipitation: Immunoprecipitation reactions were carried out in ELISA plates or in 1.5 ml eppendorf centrifuge tubes at 4 C for 16 h. Supernatants were collected following centrifugation and were assayed for xylanase and ethylene biosynthesis-inducing activities.

RESULTS

Enzymatic activity: We have made many attempts to associate an enzymatic activity with the purified protein from Cellulysin that induces ethylene biosynthesis in tobacco leaf discs (Fuchs and Anderson, 1987). The activity of several exo-hydrolases using p-nitrophenyl-derivatives of xylose, galactose, glucose and arabinose were not detected in purified EIF. Yet these are known to be present in Cellulysin (Boller and Kende, 1979). Other enzyme activities such as cellulase, polygalacturonase, and proteinase were also not detected in purified EIF. A xylanase activity using xylan from oat spelts, birch wood or Remazol brilliant blue xylan was found. Analysis, by TLC, of products liberated by EIF from oat spelts and birch wood xylan after prolonged (20 h) digestion gave no indication that xylose (as monomer) was liberated.

Fig. 1. Electrophoresis and subsequent bioassay of EIF in 1% agarose (pH 6.4); BPB, bromophenol blue (negatively charged); O, origin; MG, methylgreen (positively charged). Data from Fuchs and Anderson (1987).

Agarose electrophoresis: Electrophoresis, in 1% agarose at pH 6.4 and subsequent bioassay of the protein showed that the ethylene biosynthesis-inducing activity migrated towards the cathode (Fig.1). Preparative agarose gel electrophoresis separated 3 main peaks of A_{280} material, ethylene biosynthesis-inducing and xylanase activities (Fig. 2). Inhibition studies: Heat (60°C) inactivated both ethylene biosynthesis-inducing and xylanase activities in a similar pattern. Likewise, digestion by trypsin or V_8 protease in 8 M urea (Fig. 3). The EIF was more resistant to trypsin than to V_8 protease.

Immuno-cross reactivity: Anti-EIF Ig's precipitated both xylanase and ethylene biosynthesis-inducing activities in an almost parallel manner (Fig. 4). Ig's from non-immunized rabbits did not show any inhibition of xylanase or ethylene biosynthesis-inducing activities.

Fig. 2. Protein, ethylene biosynthesis-induction and xylanase activity of fractions eluted from agarose column electrophoresis of partially purified EIF. Data from Fuchs et. al. (in press).

Fig. 3. Rates of loss of ethylene biosynthesis- induction (—○—) and xylanase activities (—■—) as affected by high temperature (60°C) (●) and by incubation with trypsin (▲) and protease from *Staphylococcus aureus* (V₈) (■) in the presence of 8 M urea. Data from Fuchs et. al.(in press).

DISCUSSION

In previous studies it has been shown (Anderson et. al., 1984; Fuchs and Anderson, 1987) that Cellulysin contains a protein fraction that is capable of inducing ethylene biosynthesis in tobacco leaf discs, but it had no known enzymatic activity. In our attempt to place an enzyme activity to this protein we assayed for various hydrolases. Our choice of substrates was based on what activities were known to occur in Cellulysin and substrate availability (Biely et. al., 1985; Boller and Kende, 1979; Labavitch and Greve, 1983). We found xylanase activity but were unable to detect cellulase, polygalacturonase, or protease. Neither

were we able to detect other hydrolase activity with any of the p-nitrophenyl sugar derivatives used. Based on our inability to detect free xylose after prolonged incubation of EIF with xylan, our inability to detect hydrolysis of p-nitrophenyl-xylopyranoside, and the ability of the protein to hydrolyze specific endoxylanase substrate, Remazol brilliant blue xylan (Biely et.al., 1985), we conclude that this activity is of an endoxylanase.

Fig. 4. Inhibition of ethylene biosynthesis-inducing and xylanase activities by EIF antibodies. Concentration dependent immunoprecipitation of xylanase (A) and ethylene biosynthesis-inducing activity (B). Data from Fuchs et. al. (in press).

Because we were unable to physically separate xylanase activity from EIF we tried to differentiate between them by various inhibition studies. If the two activities represent two separate proteins, we would expect them to be inhibited differently. However, we found that xylanase and ethylene biosynthesis-inducing activities were inhibited similarly by heat, proteases with urea (Fig. 3) and by Ig's (Fig. 4). The temperature sensitivity and the size (about 18 kD) of the EIF are very similar to the 18 kD "xylanase 2" which was purified from *T. koningii* (Wood and McCrae, 1986).

Cell wall fragments are becoming recognized as possible regulators of growth and development as well as elicitors (Fry, 1986; Tran Than Van et. al., 1985). The role this xylanase plays in producing biologically active compounds, if any, waits to be discovered. However, an endoxylanase activity been shown to hydrolyze pear fruit structural polysaccharides (Labavitch and Greve, 1983) and was found in protein elicitor from Phytophthora parasitica (Farmer and Helgeson, 1987).

The data which are presented in this paper show inseperable association between the Cellulysin ethylene biosynthesis-inducing protein and a xylanase activity under a range of non-denaturing coditions. Based on all these data we conclude that the ethylene biosynthesis-inducing protein we have isolated and purified from Cellulysin also has an endoxylanase activity.

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DESCRIPTION OF COOPERATION

This has been a true cooperative study between the two groups. Dr. Fuchs spent a sabbatical year in the laboratory of Dr. Anderson where it was discovered that the ethylene biosynthesis-inducing protein contained an endoxylanase activity and lead to the production of antibodies to the native protein. Dr. Fuchs was able to bring purified protein and antibodies back to Israel for use in his Laboratory. Dr. Anderson spent a year sabbatical at the Weizmann Institute of Science, Rehovot, Israel and was able to do some collaboration with Drs. Fuchs and Chalutz. The Laboratory of Dr. Anderson concentrated on characterizing the protein while the Laboratory of Dr. Fuchs and Chalutz concentrated on finding xylanase in ripening fruits and correlating activity to ripening and ethylene production.

EVALUATION OF THE RESEARCH ACHIEVEMENTS

The main achievements of the project concern the identification of a fungal protein with an endoxylanase activity that seems to have some properties that regulate the biosynthesis of the ripening hormone ethylene (2,6,7,9). This lead to an approach that was not foreseen when the project was conceived. Here, we were able to culture the fungus, T. viride, and to show that the synthesis of EIX was induced when the fungus was cultured on xylan or its monomer (1,4,5). This also prompted us to develop a new, modified, purification scheme to accommodate liters of culture filtrate (4). With this scheme, it is possible to isolate mg quantities of pure EIX from several liters of culture filtrate in a couple of days.

It also seems to induce a hypersensitive-type response in tobacco leaf tissue (3). This protein induces the activity of ACC synthase in tobacco leaf tissue (8). If the protein is

applied to the cut petiole of a leaf, the protein is translocated throughout the leaf and induces ethylene production and necrosis in the leaf (3). If injected into the petiole of an intact leaf, the protein seems to be translocated both up and down the stem through the xylem and into the leaves (2,3). The protein does not seem to spread horizontally. Work by another group (Dr. Robert Fluhr, personal communication) has shown that EIX induces the synthesis of the pathogen related protein, chitinase. These studies have implications concerning how plants respond to fungi and fungal metabolites and may shed more light on defense mechanisms in higher plants.

To the best of our knowledge, it is the first time that xylanase and xylosidase activities are described in avocado fruit (9). These may be related to regulation of ethylene biosynthesis-induction, cell-wall degradation and softening in fruits.

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(Resulting from the research project)

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