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Ethylene Biosynthesis-Inducing Protein from Cellulysin Is an Endoxylanase[1]

Yoram Fuchs[2], Abha Saxena[3], H. Ray Gamble, and James D. Anderson*

U.S. Department of Agriculture, Agricultural Research Service, Plant Hormone Laboratory (Y.F., A.S., J.D.A.), *Helminthic Disease Laboratory* (H.R.G.), *BARC, Beltsville, Maryland 20705, and Department of Horticulture* (A.S.), *University of Maryland, College Park, Maryland 20742*

ABSTRACT

The proteinaceous ethylene biosynthesis-inducing factor (EIF) that was purified from Cellulysin was also shown to contain a xylanase activity. In all nondenaturing protein separation methods employed (Sephacryl S-200 chromatography, and preparative isoelectric focusing and agarose electrophoresis), xylanase activity copurified with the ethylene biosynthesis-inducing activity. Treatment with heat (60°C) or proteases in 8 molar urea inhibited both ethylene-inducing and xylanase activities. Antibodies raised against purified EIF, which contains three polypeptides of 18, 14, and 10 kilodaltons, immunoprecipitated both ethylene biosynthesis-inducing and xylanase activities. The purified EIF contained no detectable cellulase, polygalacturonase, or protease activity. Other hydrolytic activities as estimated by using *p*-nitrophenyl derivatives of several sugars as substrates also were not detected. Different commercially available hydrolytic enzyme preparations were tested for both ethylene biosynthesis-inducing and xylanase activities. All enzymes tested contained xylanase activity, but only a few induced ethylene biosynthesis. Western blots of proteins separated by SDS-PAGE, using antibodies prepared against the non-denatured purified EIF, revealed two major bands of about 18 and 14 kilodaltons in EIF. These antibodies seem to be specific for these proteins from *Trichoderma viride*, because there was little cross-reactivity with the other proteins in Cellulysin and other commercial enzyme preparations. Based on these data, we suggest that EIF contains a specific xylanase activity which is involved in inducing ethylene biosynthesis.

It is well established that some isolated protoplasts produce ethylene (3, 8, 10, 15) and that this ethylene biosynthesis is induced by a component(s) of the cell wall digesting enzymes prior to the release of protoplasts (4, 9). In the case of the enzyme mixture, Cellulysin, proteins that induce ethylene biosynthesis have been purified and partially characterized (13). The purified EIF[4] contains 3 polypeptides of 18, 14, and 10 kD. To date, attempts by us to identify some enzymic activity or function other than ethylene biosynthesis induction for these proteins have been unsuccessful. In this paper, we report that the ethylene biosynthesis-inducing proteins from Cellulysin contain an endoxylanase activity. In addition, we have raised antibodies against the ethylene biosynthesis-inducing proteins and have shown that the Ig fraction inhibits both the ethylene biosynthesis-inducing and xylanase activities. A preliminary report has been presented (14).

MATERIALS AND METHODS

Bioassay of Ethylene Production

Tobacco (*Nicotiana tabacum* L. cv Xanthi) leaves from plants grown in the greenhouse were pretreated for 16 h with 100 μL/L ethylene prior to cutting 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 with 1 mL of basal medium (700 mM sorbitol, 10 mM Mes-NaOH at pH 6.0, 10 μM $CaCl_2$, 50 μg/mL streptomycin sulfate, and 50 units/mL penicillin G) together with the sample to be bioassayed. Ethylene was quantified by gas chromatography (9).

Enzyme Activity Assays

The EIF used for enzyme assays was the most highly purified fraction described previously (13). This was obtained from Cellulysin (Calbiochem) which is produced by the fungus *Trichoderma viride*. The xylan substrates, prepared from oat spelts (Sigma[5]) or from birch wood (Roth) were used for enzyme assays according to Wood and McCrae (26). Reducing sugars were determined by the method of Sumner (22). The xylanase assay using Remazol brilliant blue xylan (Sigma) was done according to Biely *et al.* (5). Polygalacturonic acid, CMC, BSA, and the *p*-nitrophenyl derivatives of: −β-D-xylopyranoside, −β-D-glucopyranoside, −α-D-galactopyrano-

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[2] On leave from the Department of Fruit and Vegetable Storage, Institute for Technology and Storage of Agricultural Products, ARO, The Volcani Center, P. O. Box 6, Bet Dagan 50250, Israel.

[3] Currently located at National Institutes of Health, Bethesda, MD.

[4] Abbreviations: EIF, ethylene biosynthesis-inducing factor from Cellulysin; IEF, isoelectric focusing; PBS, 10 mM Na_2HPO_4, 0.9% NaCl (pH 7.2); TTBS, 50 mM Tris, 0.9% NaCl (pH 7.2) in 0.05% Tween-20; CMC, carboxymethyl cellulose; V_8, protease from *Staphylococus aureus*, V_8; Remazol brilliant blue xylan, 4-*o*-methyl-D-glucurono-D-xylanremazol brilliant blue R xylan.

[5] The mention of specific instruments, trade names, or manufacturers is for purpose of identification and does not imply any endorsement by the United States government.

side,−α-L-arabinofuranoside, and −α-D-glucopyranoside, were purchased from Sigma. The *p*-nitrophenyl sugar derivatives were used as substrates according to Boller and Kende (7). Cellulase and polygalacuronase 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 (21).

Electrophoresis

Preparative IEF column electrophoresis (pH 3.0 to 10.0) and preparative agarose electrophoresis were performed as previously reported (13). Different hydrolytic enzymes were separated by SDS-PAGE (18) and are listed in the legend of Figure 5. Polyacrylamide gels composed of 15% (w/v) acrylamide and 0.7% (w/v) bisacrylamide were used with a 3% acrylamide stacking gel. Prestained mol wt markers were from BRL. Electrophoretic separations of samples (10 μg) were carried out at 25 mA per gel. Protein was determined according to Marder *et al.* (20), but without the acetone wash. After electrophoresis, gels were used for Coomassie blue staining as well as for protein blotting (Western) onto nitrocellulose (24).

Ethylene Induction and Xylanase Inhibition Studies

Proteolytic digestions of EIF (20 μg protein separated on a Sephacryl S-200 column [13]) with 10 μg trypsin or V_8 in a total of 30 μL were carried out at 30°C in the presence of 8 M urea. EIF (20 μg in 40 μL water) was also heat inactivated at 60°C. At various time intervals during protease and heat treatments, samples (5 μl) were removed and ethylene biosynthesis-inducing and xylanase activities were determined.

Antibody Production

New Zealand female rabbits (Commando Farms, Landover, MD) (2–3 kg) were immunized with the major fraction of purified EIF from agarose electrophoresis (13). Two doses of 200 μg of antigen in complete Freund's adjuvant (Sigma) were given subcutaneously at 2-week intervals. One week following the second subcutaneous immunization, 100 μg of antigen was given intravenously. Animals were bled after another week, the blood was allowed to clot and serum collected. The Ig fraction was partially purified from the serum by precipitation with 40% saturated ammonium sulfate.

Immunoprecipitation

Immunoprecipitation reactions were carried out in ELISA plates or in 1.5 mL eppendorf centrifuge tubes preblocked with 5% powdered dry milk in PBS. Reaction volumes of 100 μL contained partially purified EIF from Sephacryl S-200 (13), Ig's and PBS. Immunoprecipitation was at 4°C for 16 h. Supernatants were collected following centrifugation and were assayed for xylanase and ethylene biosynthesis-inducing activities.

Western Blotting

Samples of protein were subjected to SDS-PAGE as described above and electroblotted to nitrocellulose (24). Nitrocellulose sheets were incubated for at least 2 h in 5% dry milk powder (Carnation) (19) dissolved in TTBS. Blots were then incubated in partially purified Ig (0.8 μg/mL) in 1% dry milk powder in TTBS for 4 h, rinsed several times with TTBS, then incubated for 1 h in 1:3000 dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Calbiochem) in 1% dry milk powder in TTBS, and again rinsed several times with TTBS. The blot was then allowed to react with 4-chloro-1-naphthol/H_2O_2 developer in order to visualize the cross-reacting bands.

RESULTS

Enzymic Activity of Purified EIF

We have made many attempts to associate an enzymic activity with the purified protein from Cellulysin that induces ethylene biosynthesis in tobacco leaf discs (13). The activities of several exo-hydrolases using *p*-nitrophenyl-derivatives of xylose, glucose, galactose, and arabinose were not detected in the purified EIF; yet these enzyme activities are known to be present in Cellulysin (7). 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 of products liberated by EIF from oat spelts and birch wood xylan by TLC after prolonged (20 h) digestion gave no indication that xylose was liberated.

Agarose Electrophoresis

Preparative agarose gel electrophoresis separated three main peaks of A_{280} material, ethylene biosynthesis-inducing and xylanase activities (Fig. 1). The SDS-PAGE profile of the proteins in the three fractions were similar to those previously published (13).

Inhibition of Ethylene Biosynthesis-Inducing and Xylanase Activities

Heat (60°C) inactivated both ethylene biosynthesis-inducing and xylanase activities in a similar pattern, as did digestion by trypsin or V_8 protease in 8 M urea (Fig. 2). The EIF was more resistant to trypsin than to V_8 protease.

Immunocross-Reactivity of EIF and Xylanase

Anti-EIF Igs precipitated both xylanase and ethylene biosynthesis-inducing activities in an almost parallel manner (Fig. 3). Igs from nonimmunized rabbits did not show any inhibition of xylanase or ethylene biosynthesis-inducing activities (data not presented). Thus, the antibodies are specific to the antigen, which contains three polypeptides of 18, 14, and 10 kD.

Antibodies to Purified EIF React with Ethylene Biosynthesis-Inducing Fractions

Ethylene biosynthesis-inducing activity in the material purified by Sephacryl S-200 focused between pH 6 and 10 in

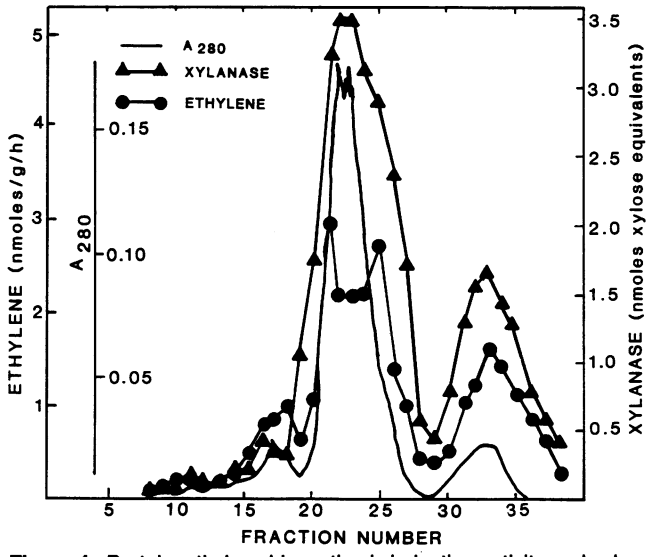

Figure 1. Protein, ethylene biosynthesis induction activity and xylanase activity of fractions eluted from agarose column electrophoresis of partially purified EIF. One mg protein of combined Sephadex G-100 fractions with ethylene inducing activity (13) was subjected to preparative agarose gel (1%) electrophoresis. Constant current of 7.5 mA was applied and 1 mL fractions were eluted and collected continuously from the lower end of the column. Aliquots of these fractions were assayed for ethylene induction (●) and xylanase activity (▲). Elution of protein was continuously monitored at A_{280}.

the preparative IEF column while xylanase activity focused below pH 3 and from pH 4 to 10 (Fig. 4). Xylanase activity was present in all fractions with ethylene biosynthesis-inducing activity; however, there were fractions containing xylanase activity without ethylene biosynthesis-inducing activity. All IEF fractions which induce ethylene biosynthesis were recognized by the antibodies to EIF on Western blots (Fig. 4).

Electrophoretic Comparison of Some Commercial Enzyme Preparations

The protein patterns of commercial enzyme preparations from various organisms (Fig. 5A) are presented. Cellulysin that was passed through Sephacryl S-200 (lane 2) shows fewer bands than crude Cellulysin (lane 1), particularly at 18 and 14 kD. Cellulase RS Onozuka, lane 8, is somewhat different than lane 1 despite the fact it was produced from the same organism. With the exception of lanes 1 and 8, Western blots of the various enzyme preparations showed little cross-reactivity with the antibodies raised against EIF (Fig. 5B). The purest EIF preparation (lane 13) showed only one visible band when stained with Coomassie blue, but when more protein was applied three bands were visible at 18, 14, and 10 kD. Two of these proteins are evident on Western blots at about 18 and 14 kD. A minor band was also recognized in the crude enzyme preparation of lanes 1 and 8 at about 10 kD as well as a band at about 25 kD in lane 1. The antibodies at the concentration used, only recognized specific proteins which originated from *T. viride*, lanes 1, 8, and 13 (Fig. 5B). Preimmune serum did not cross-react with any protein in Cellulysin on Western blots, even when assayed at a 1000-fold greater concentration than immune serum.

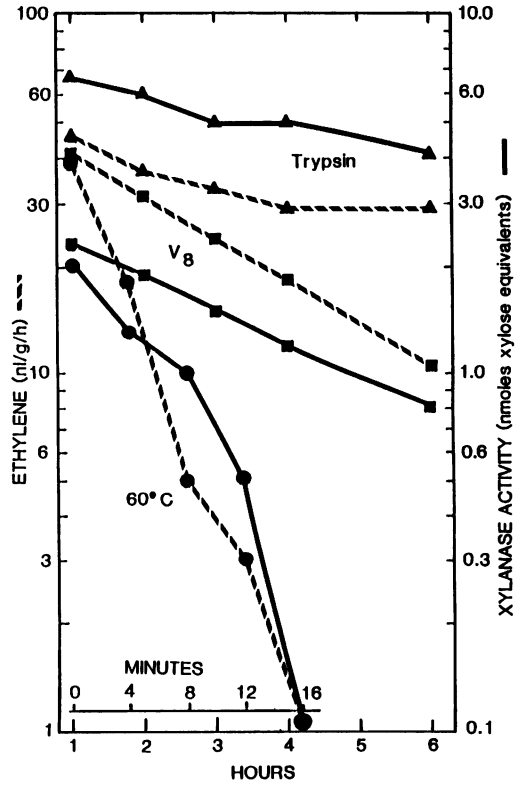

Figure 2. Loss of ethylene biosynthesis induction (– – –) and xylanase activities (——) as affected by high temperature treatment (60°C) (●) and by incubation with trypsin (▲) and protease from *Staphylococcus aureus* (V_8) (■) in the presence of 8 M urea. Aliquots for the protease and heat experiments, contained 1.3 and 2.5 μg EIF per assay, respectively.

Xylanase and Ethylene Biosynthesis-Inducing Activities of Commercial Enzymes

All enzyme preparations tested contained xylanase activity. However, only Pectolyase (lane 6) and Cellulase RS Onozuka (lane 8) induced ethylene biosynthesis as effectively as Cellulysin (Fig. 5C). Cellulase PB (lane 7), for example, contained about the same xylanase activity as Cellulase RS Onozuka (lane 8), but did not induce much biosynthesis of ethylene. The enzyme preparations containing the highest xylanase activity (lane 9, hemicellulase; and lane 10, cellulase from *Aspergillus niger*), had little or no ethylene biosynthesis-inducing activity. The purified EIF (lane 13) had the highest specific activity for ethylene biosynthesis-inducing and xylanase activities.

DISCUSSION

In previous studies it has been shown (1, 13) that Cellulysin contains a protein fraction composed of 3 polypeptides (18, 14, and 10 kD) that is capable of inducing ethylene biosynthesis in tobacco leaf discs. However, it had no known enzymic activity. In our attempt to place an enzyme activity to EIF we assayed for various hydrolases. Our choice of substrates was based on what activities were known to occur in Cellulysin and substrate availability (6, 7, 17). We found xylanase activity but were unable to detect cellulase, polygalacturonase, protease, or other hydrolase activity with any of

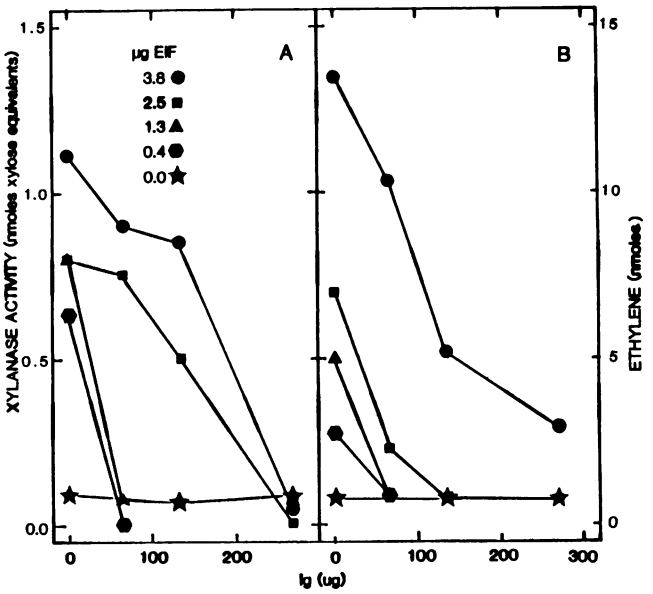

Figure 3. Inhibition of ethylene biosynthesis-inducing and xylanase activities by EIF antibodies. Dependency of immunoprecipitation of xylanase (A) and ethylene biosynthesis-inducing activity (B) in Sephacryl S-200 purified material (13) on the concentration of antibodies prepared against EIF. The various symbols represent different concentrations of EIF. Activity is expressed as nmol ethylene produced by 6 leaf discs in 4 h at 23°C and xylanase activity as nmol of xylose equivalents released in 1 h at 30°C.

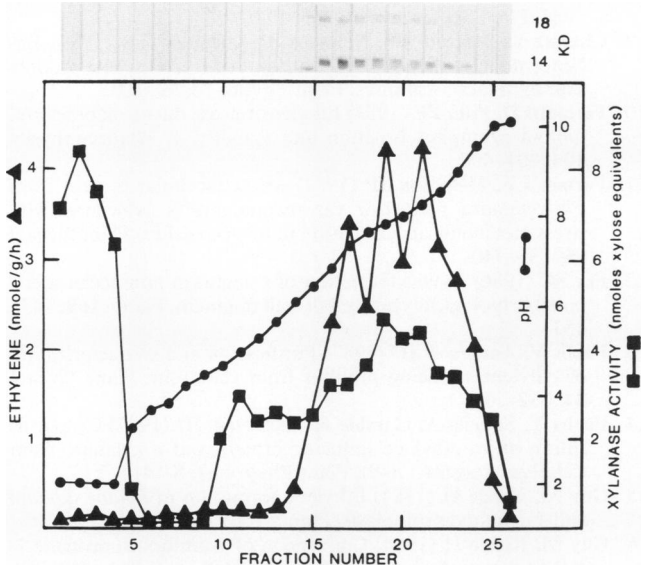

Figure 4. Ethylene biosynthesis induction, xylanase activity, and Western blot of isoelectric focused fractions of partially purified EIF. One mg protein collected from a Sephacryl S-200 column was applied to the isoelectric focusing column (13). After electrophoresis, 1-mL fractions were collected and pH (●) was measured for each fraction and a 10 µL aliquot was used in each assay of ethylene induction (▲) and xylanase activity (■). Top, Western blot of each fraction.

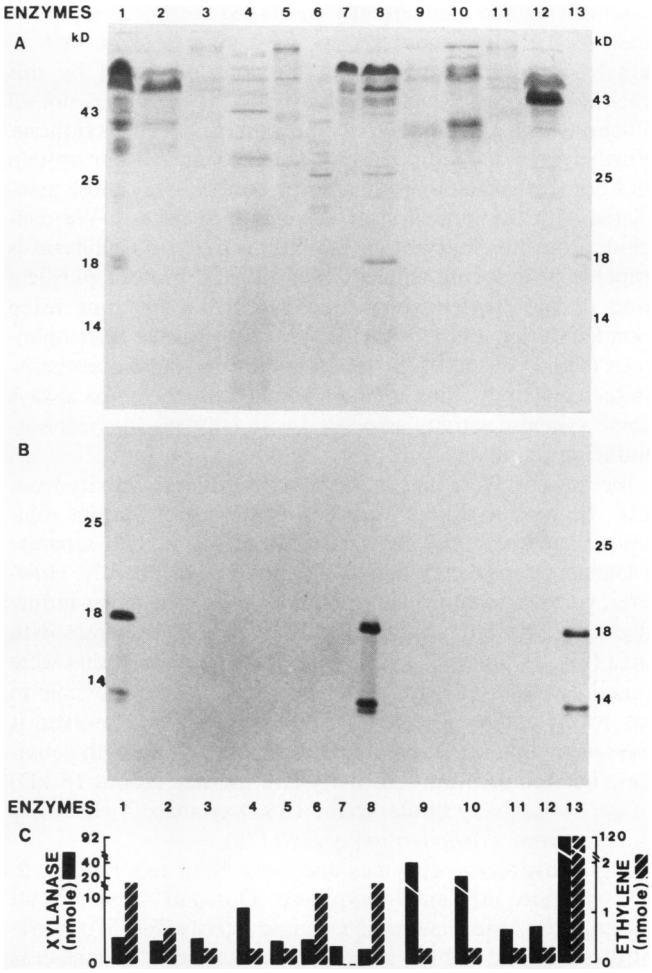

Figure 5. SDS-PAGE gel, Western blot, and ethylene biosynthesis-inducing and xylanase activities of some cell wall hydrolyzing enzymes: A, Coomassie blue stained gel following SDS-PAGE; B, Western blot with anti-EIF antibodies; C, xylanase and ethylene biosynthesis-inducing activities of the different enzymes. Ten µg of protein of each enzyme mixture and 1 µg of EIF were used for electrophoresis, 20 µg of protein of each enzyme mixture were used for xylanase activity and 100 µg of protein were used for ethylene induction. One µg of EIF was used for xylanase and ethylene biosynthesis-inducing assays. Xylanase activity is expressed as nmol xylose equivalents released per 20 µg protein during 20 min at 30°C. Ethylene biosynthesis-inducing activity is expressed as nmol ethylene produced during 4 h by 6 discs, per 100 µg protein. The enzymes used are as follows: 1, Cellulysin (Calbiochem, *Trichoderma viride*); 2, Cellulysin passed through Sephacryl S-200; 3, Rhozyme (Rohm and Hass, *Aspergillus niger*); 4, Macerase (Calbiochem, *Rhizopus sp.*); 5, Pectinase (Sigma, *A. niger*); 6, Pectolyase (Seishin Pharmaceutical, *A, japonicus*); 7, Cellulase PB (Worthington, *T. reesei); 8, Cellulase RS Onozuka (Yakult Pharmaceutical, T. viride*); 9, Hemicellulase (Sigma, *A. niger*); 10, Cellulase (Calbiochem, *A. niger*); 11, Cellulase (Sigma, *A. niger*); 12, Cellulase (Sigma, *Penicillium funiculosum*); and 13, EIF (purified from Cellulysin).

the *p*-nitrophenyl sugar derivatives used. This activity appears to be an endoxylanase. We base this conclusion on our inability to detect free xylose after prolonged incubation of EIF with xylan, inability to detect hydrolysis of *p*-nitrophenyl-xylopyranoside, and the ability to hydrolyze the endoxylanase substrate, Remazol brilliant blue xylan (5).

Once we found that the EIF had xylanase activity, attempts were made to determine if the two activities were separable using the methods we had previously used to isolate the material. Here we wanted to determine if in the pooling of

fractions we had inadvertently combined the activities. In the case of IEF electrophoresis, we were able to show that all ethylene biosynthesis-inducing fractions separated by this method also had xylanase activity (Fig. 4). However, not all fractions with xylanase activity induced ethylene biosynthesis. Furthermore, the antibodies we prepared against our protein did not recognize other apparent xylanases, only those associated with the induction of ethylene biosynthesis. We conclude from this that not all xylanase activity in Cellulysin is capable of inducing ethylene biosynthesis. Further purification of the ethylene biosynthesis-inducing fractions using nondenaturing methods such as 1% (w/v) agarose electrophoresis (Fig. 1) did not fully separate the ethylene biosynthesis-inducing activity from xylanase activity, *i.e.* there was always some xylanase activity associated with ethylene biosynthesis-inducing activity.

Because we were unable to separate xylanase activity from EIF, we tried to differentiate between them by various inhibition studies. If the two activities represent two separate proteins, we expected them to be inhibited differently. However, we found that xylanase and ethylene biosynthesis-inducing activities were inhibited similarly by heat, proteases with urea (Fig. 2), and by Igs (Fig. 3). Because the antibodies were raised against a mixture of polypeptides, it is not possible to say which antibody precipitated which activity. However, it does show that the active material is antigenic to both activities. 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 *Trichoderma koningii* (26).

Other hydrolytic enzymes also have been reported (1, 2, 23) to induce ethylene biosynthesis. Our data show that all the enzymes tested had some xylanase activity (Fig. 5C). Some of these have about the same specific activity of xylanase as Cellulysin (*e.g.* Rhozyme, lane 3; pectinase, lane 5; Pectolyase, lane 6; cellulase, lanes 11 and 12). But only Pectolyase had comparable ethylene biosynthesis-inducing activity. Other enzymes (*e.g.* hemicellulase, lane 9 and a cellulase, lane 10 from *A. niger*) had much higher specific activities for xylanase, but these had little or no ethylene biosynthesis-inducing activity. Thus, there appears to be no direct correlation between total xylanase activity and ethylene biosynthesis-inducing ability. We do know that if xylanase activity from these different enzyme mixtures is involved in ethylene biosynthesis, it is different than what we purified from Cellulysin, because they do not interact with the antibodies raised against our protein.

Cell wall fragments are becoming recognized as possible regulators of growth and development as well as elicitors (12, 25). The role this ethylene biosynthesis-inducing xylanase plays in producing biologically active compounds, if any, remains to be discovered. However, an endoxylanase activity has been shown to hydrolyze pear fruit structural polysaccharides (17) and was found in the protein elicitor from *Phytophtora parasitica* (11).

The data which are presented in this paper show inseparable association between the Cellulysin ethylene biosynthesis-inducing protein and a xylanase activity under a wide range of nondenaturing conditions. Based on all these data we conclude that the ethylene biosynthesis-inducing protein we isolated and purified from Cellulysin also has an endoxylanase activity. It is not clear if the two different activities are associated with the same polypeptide or with different polypeptides in the EIF fraction.

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