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Cosgrove et al.

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(54) **PURIFIED PLANT EXPANSION PROTEINS AND DNA ENCODING SAME**

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(22) Filed: **Jun. 5, 1998**

Related U.S. Application Data

(60) Division of application No. 08/440,517, filed on May 12, 1995, which is a continuation-in-part of application No. 08/242,090, filed on May 12, 1994, now abandoned, which is a continuation-in-part of application No. 08/060,944, filed on May 12, 1993, now abandoned.

(51) **Int. Cl.⁷** **C07H 21/02**; C07H 21/04; C12P 21/06; C12N 1/20; C12N 15/00

(52) **U.S. Cl.** **536/23.1**; 536/23.2; 536/23.5; 536/23.6; 530/350; 435/69.1; 435/252.3; 435/320.1

(58) **Field of Search** 536/23.1, 23.6, 536/23.2, 23.5; 530/350; 435/69.1, 252.3, 320.1

(56) **References Cited**

PUBLICATIONS

Shcherban et. al, PNAS (Sep. 26, 1995), 92 (20) : 9245-9.*

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(57) **ABSTRACT**

The sequence is disclosed both as the nucleic acid sequence SEQ ID NO: 1 and as the corresponding amino acid sequence SEQ ID NO: 7. Five other amino acid sequences are also disclosed, two from rice and three from Arabidopsis, SEQ ID NO: 2 through SEQ ID NO:6, respectively.

3 Claims, 24 Drawing Sheets

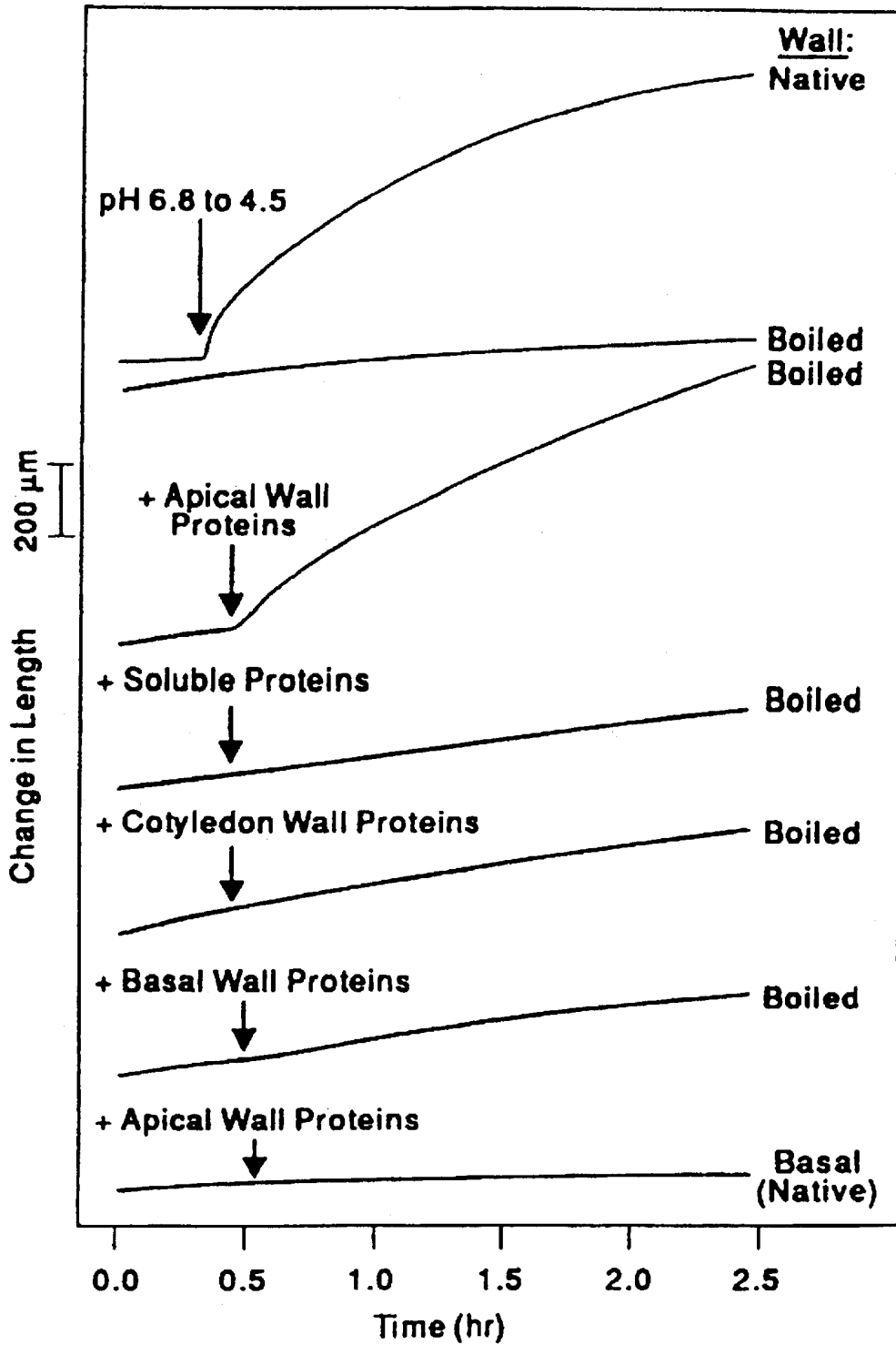


Fig.1A

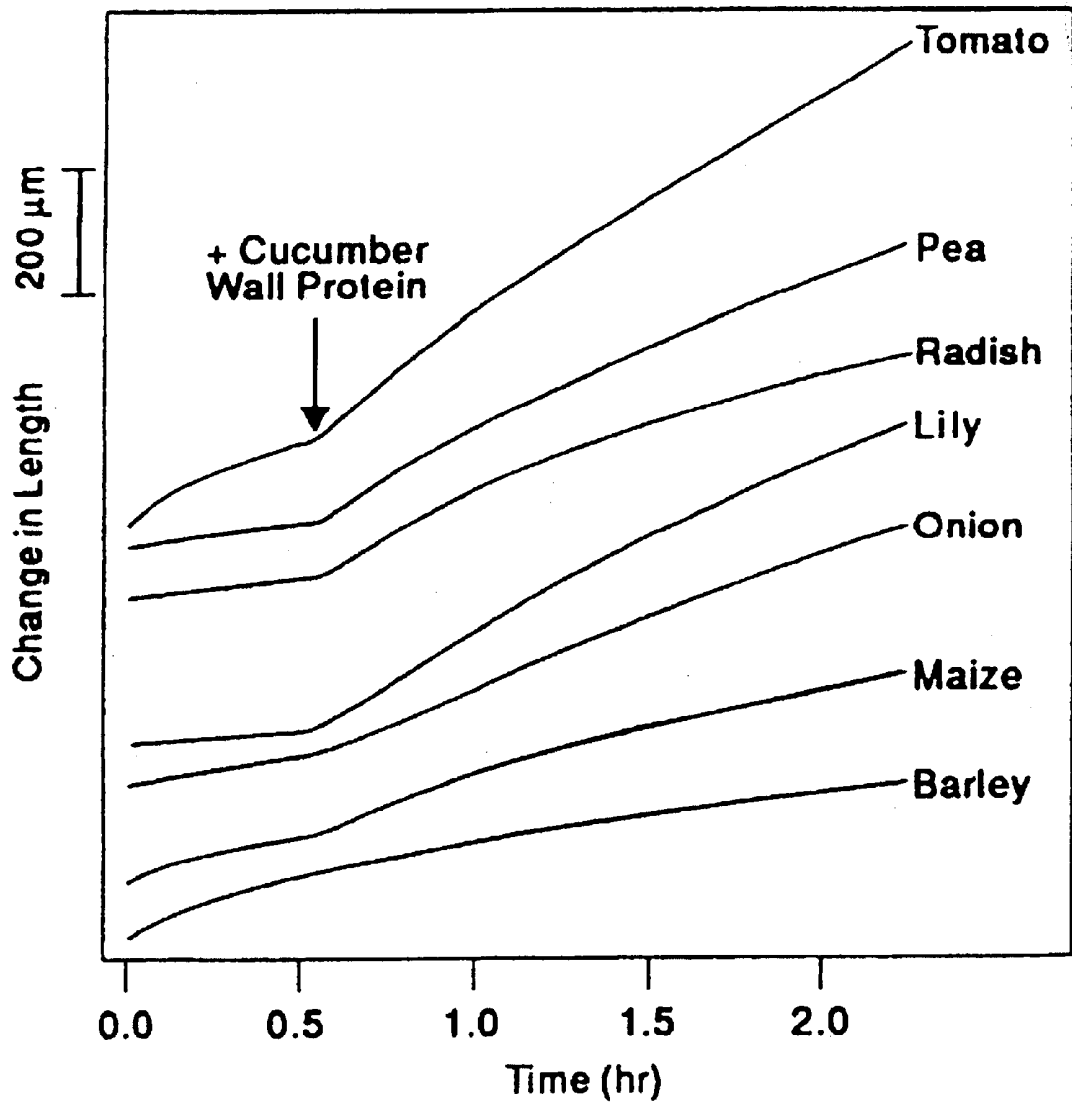


Fig.1B

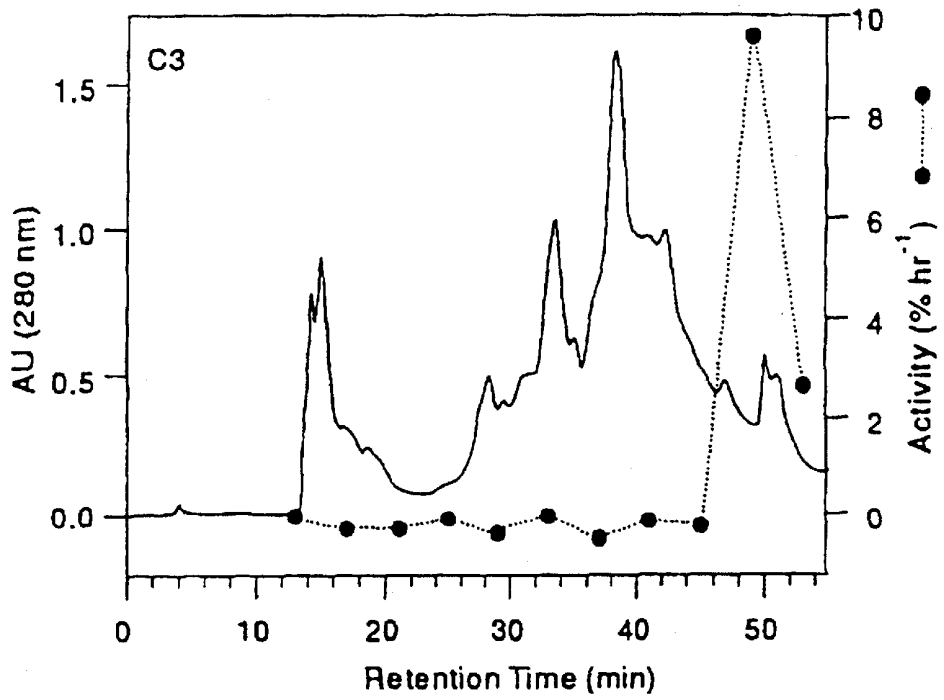


Fig.2A

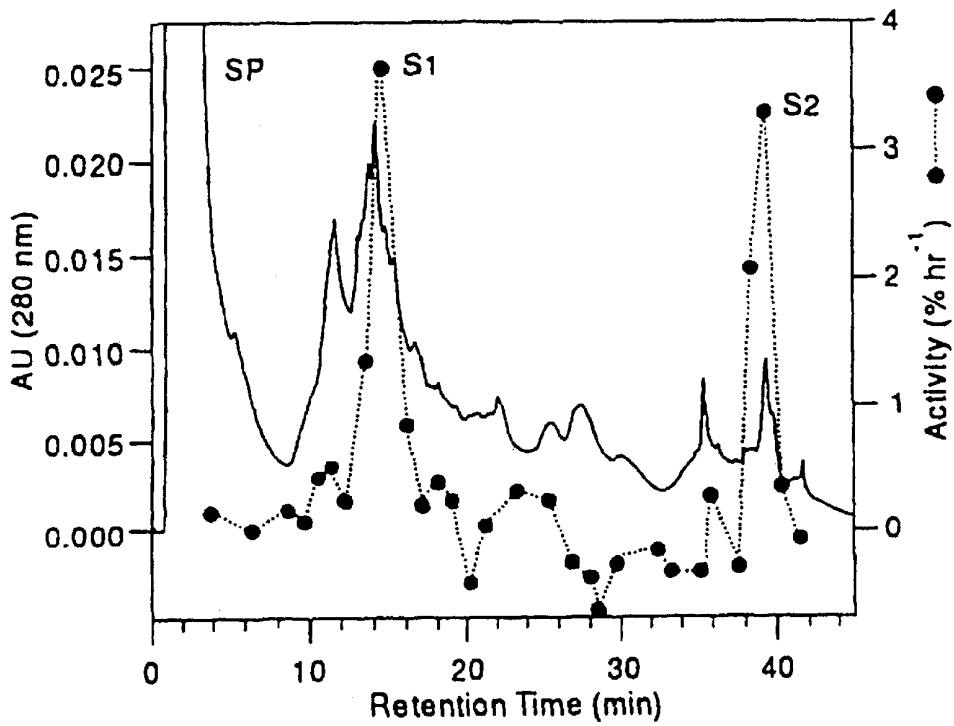


Fig.2B

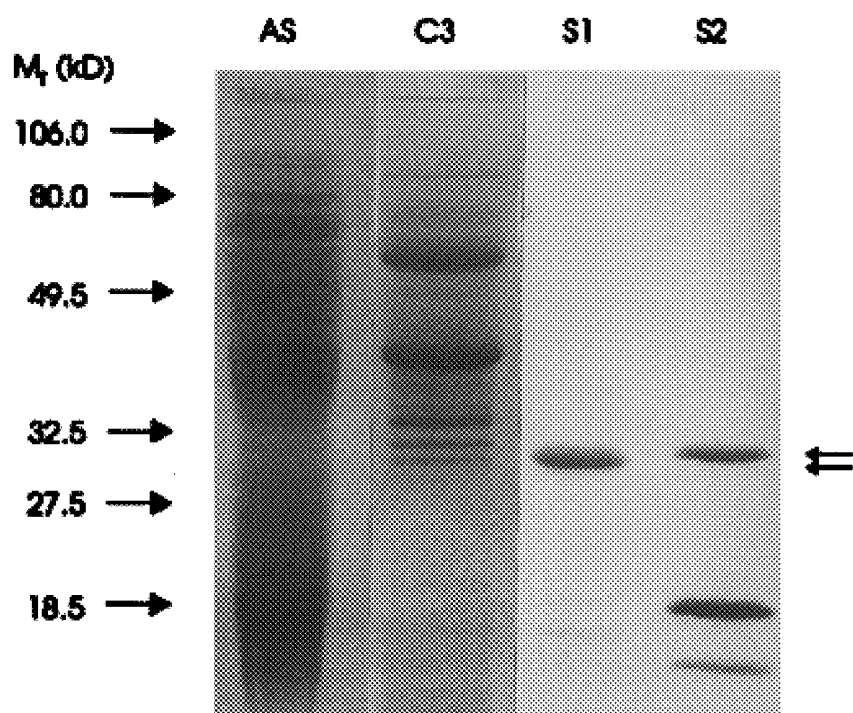


Fig.3

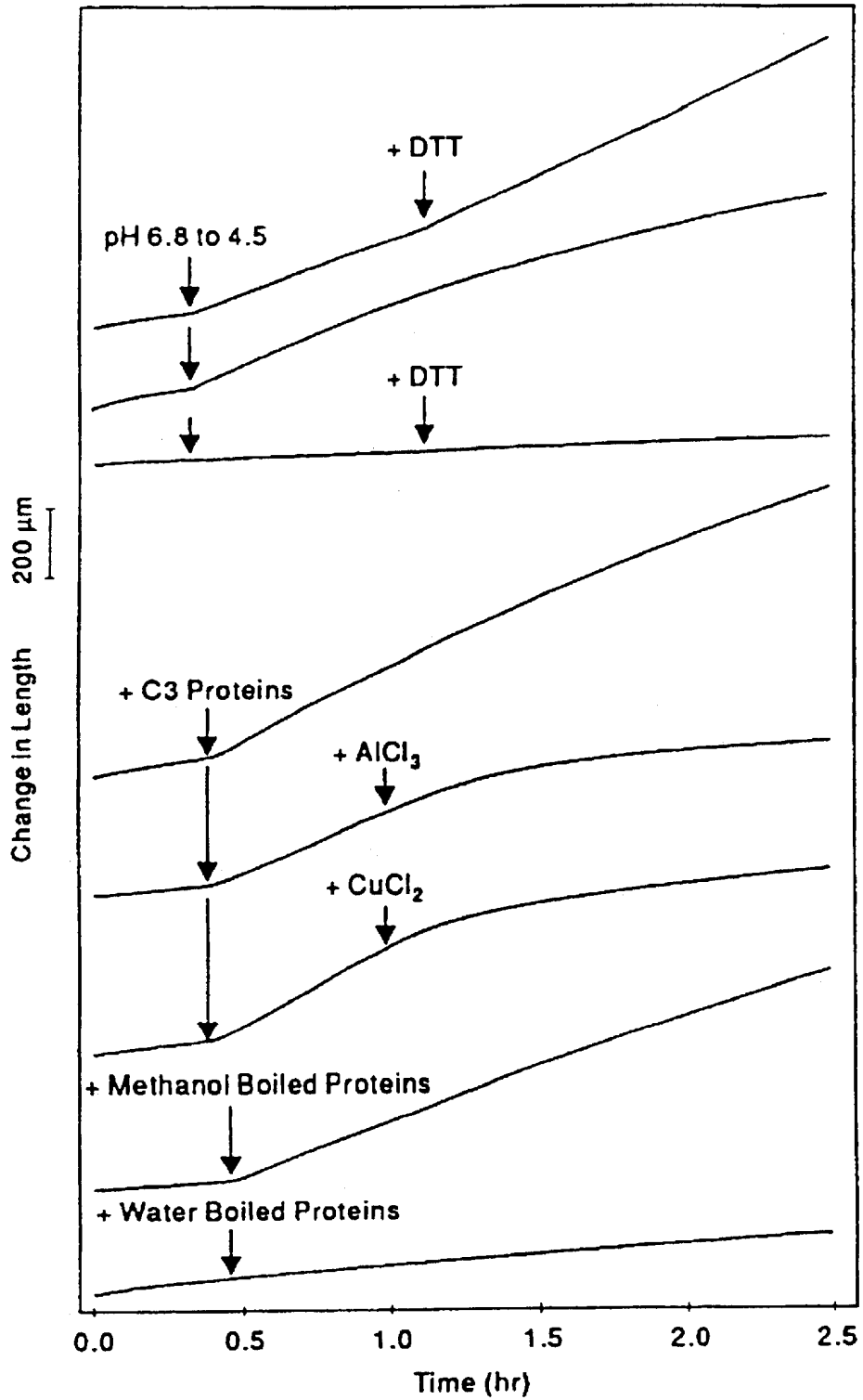


Fig.4A

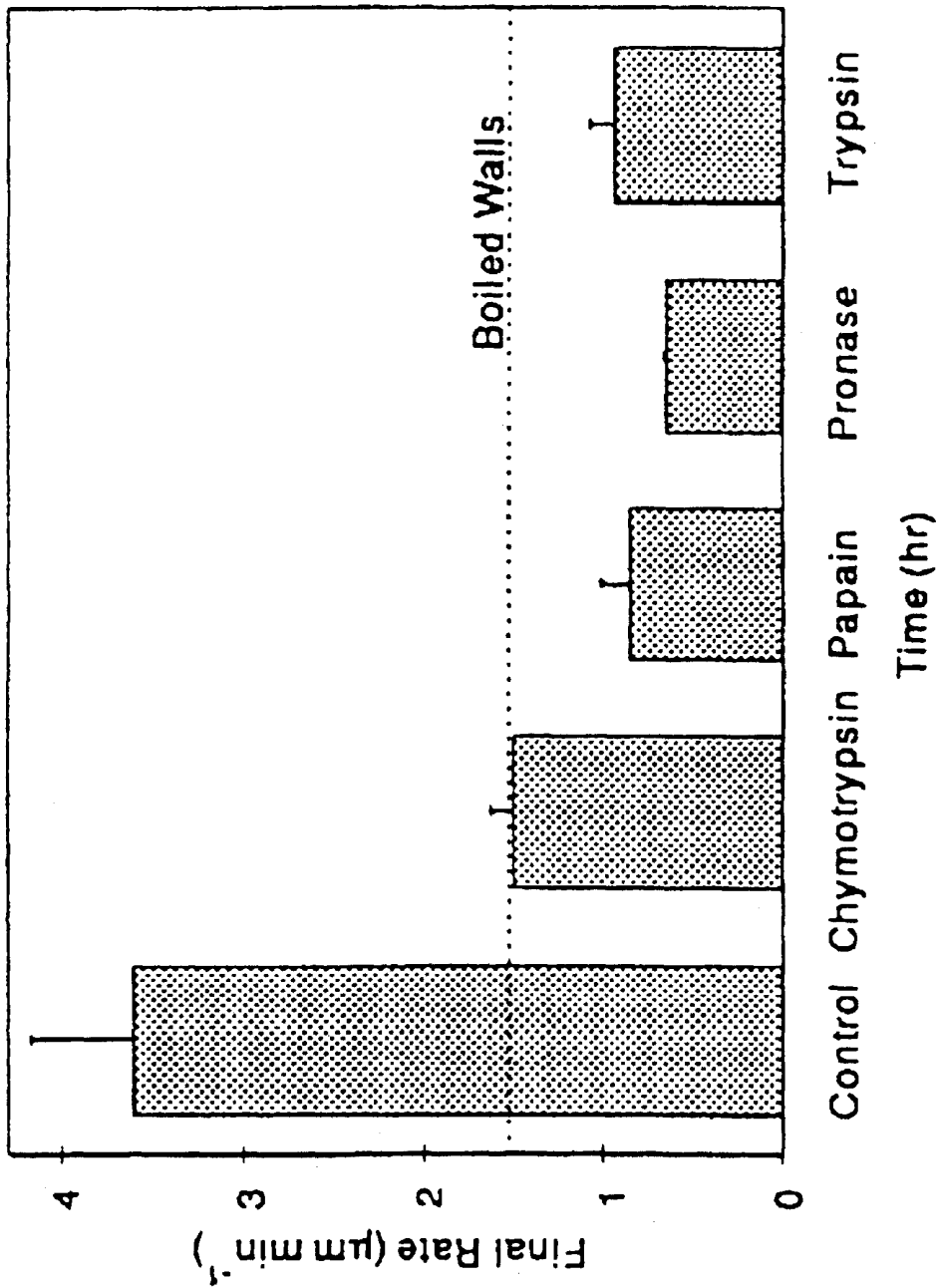


Fig.4B

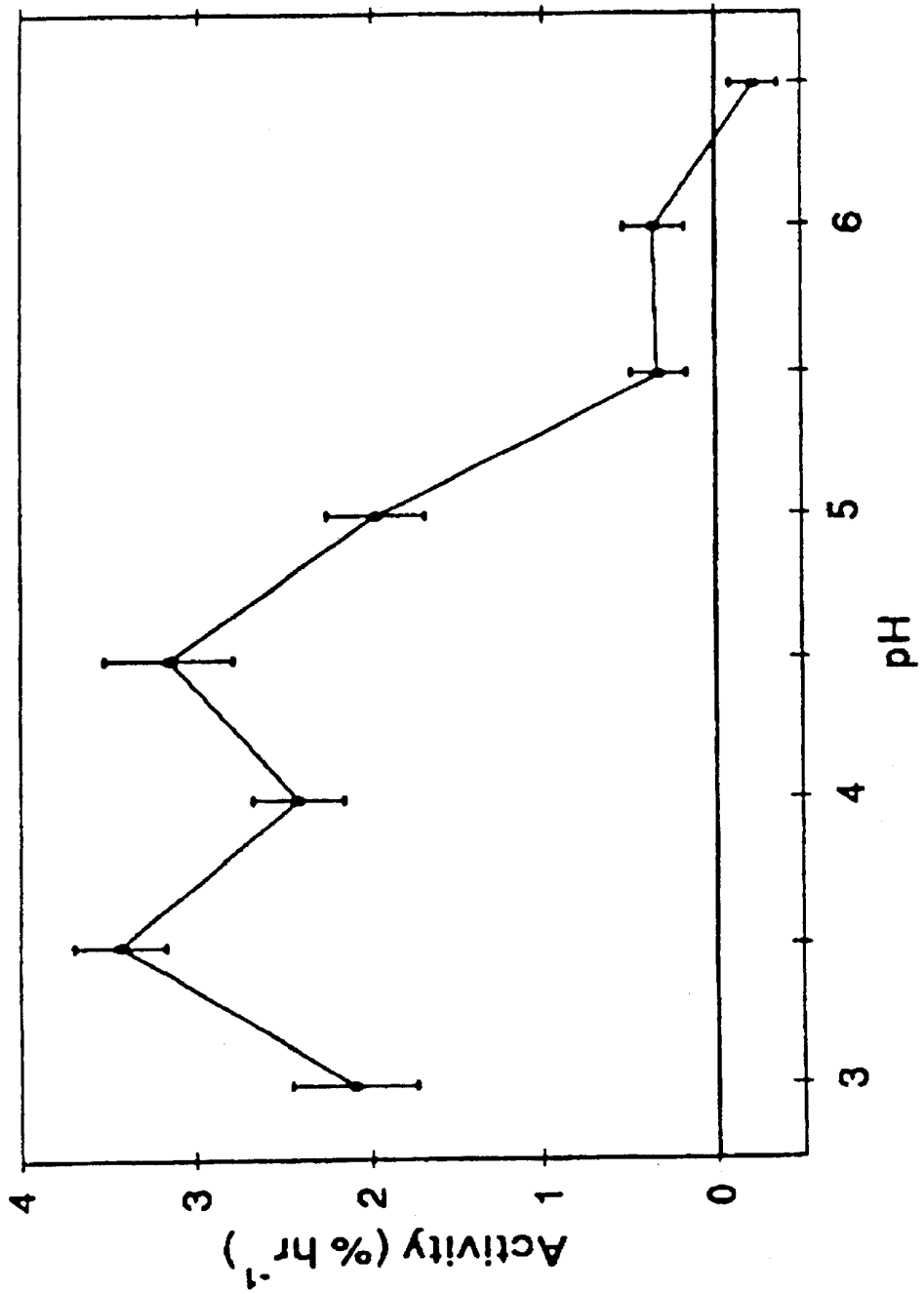


Fig.5

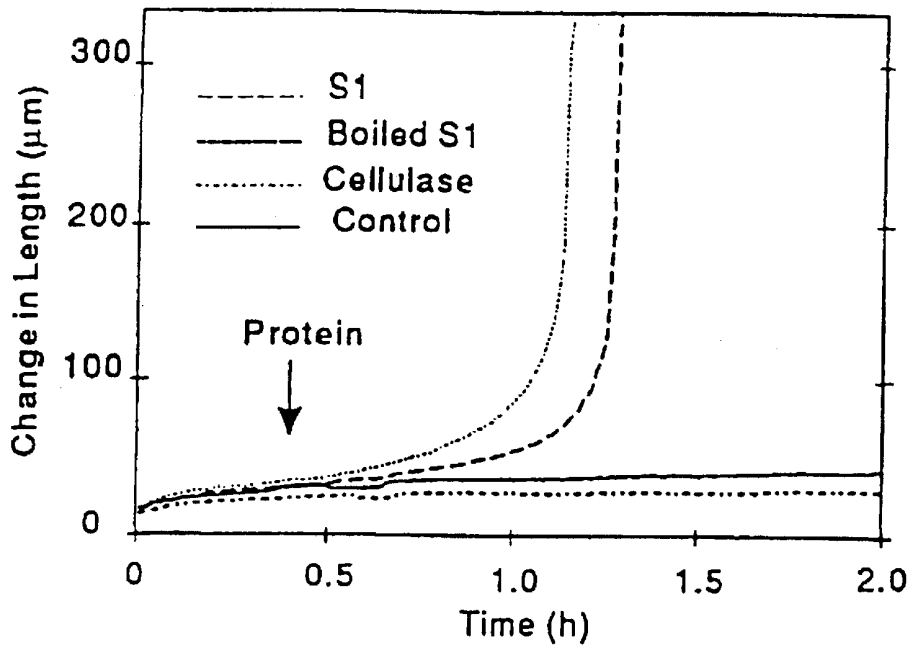


Fig.6A

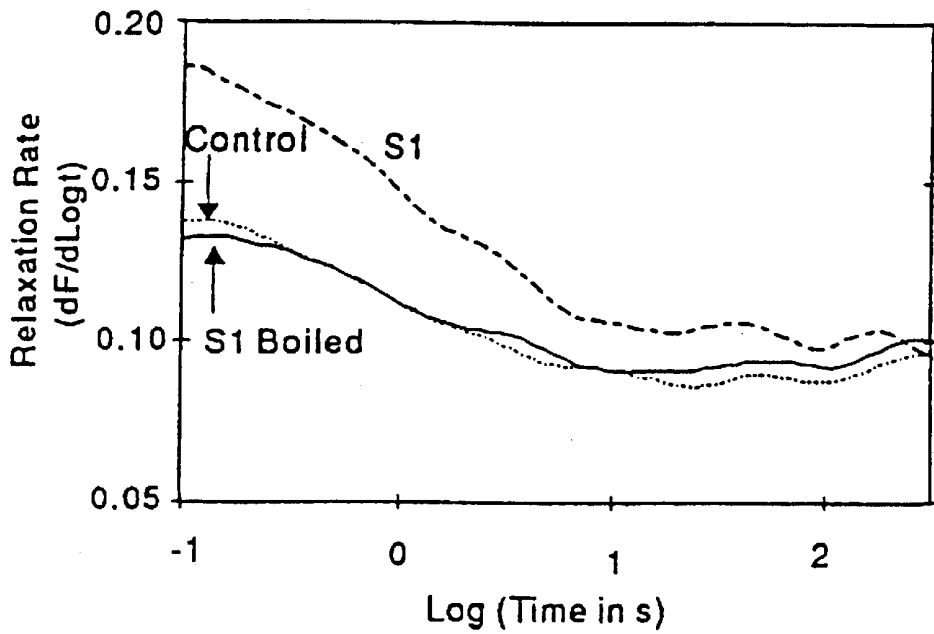


Fig.6B

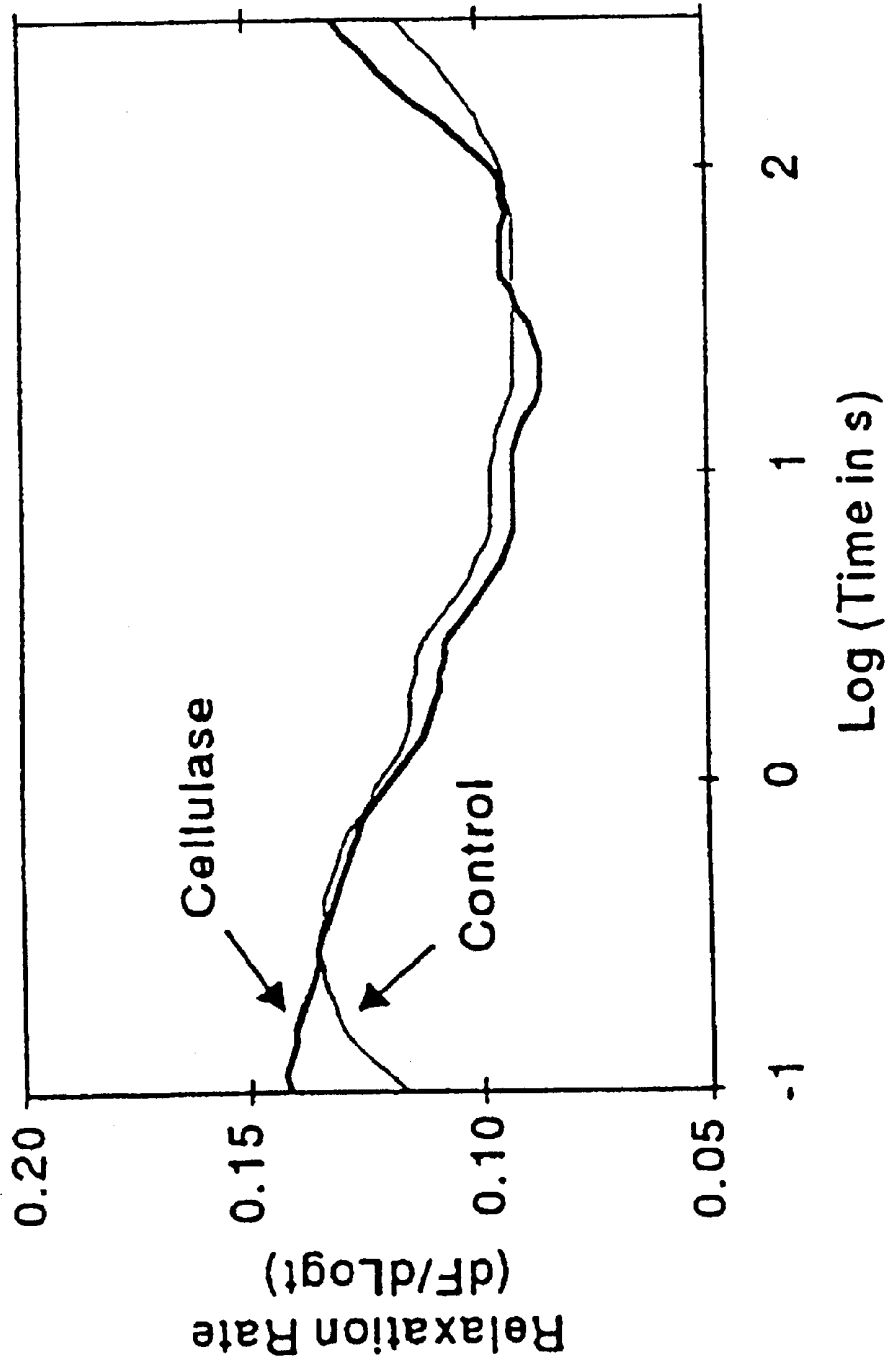


Fig.6C

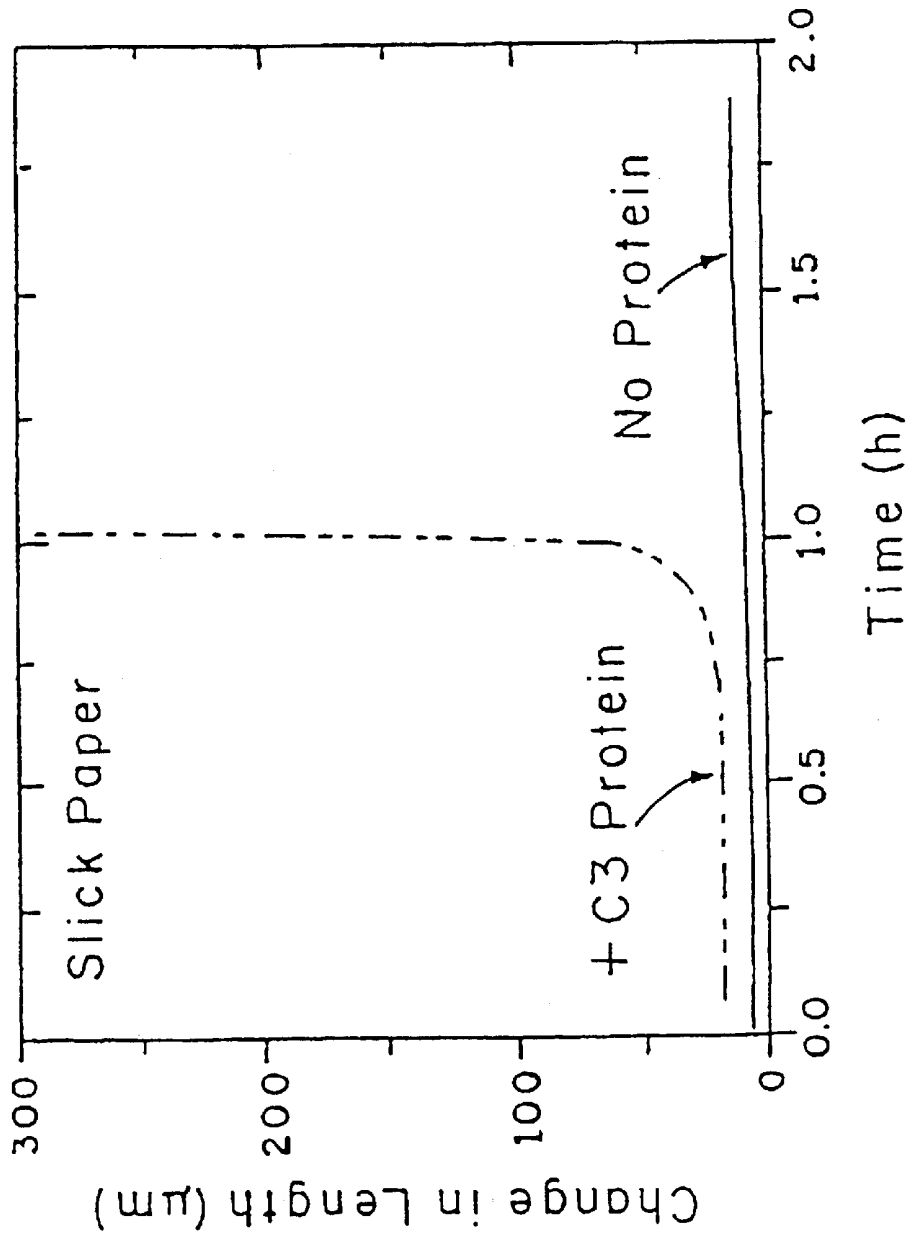


Fig.7

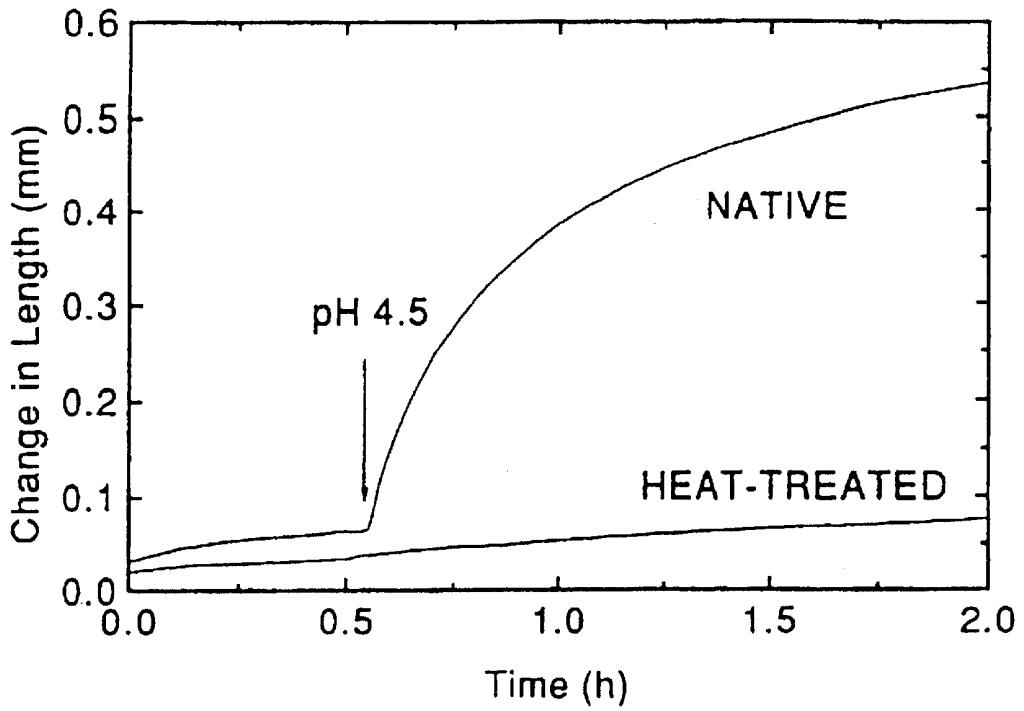


Fig.8A

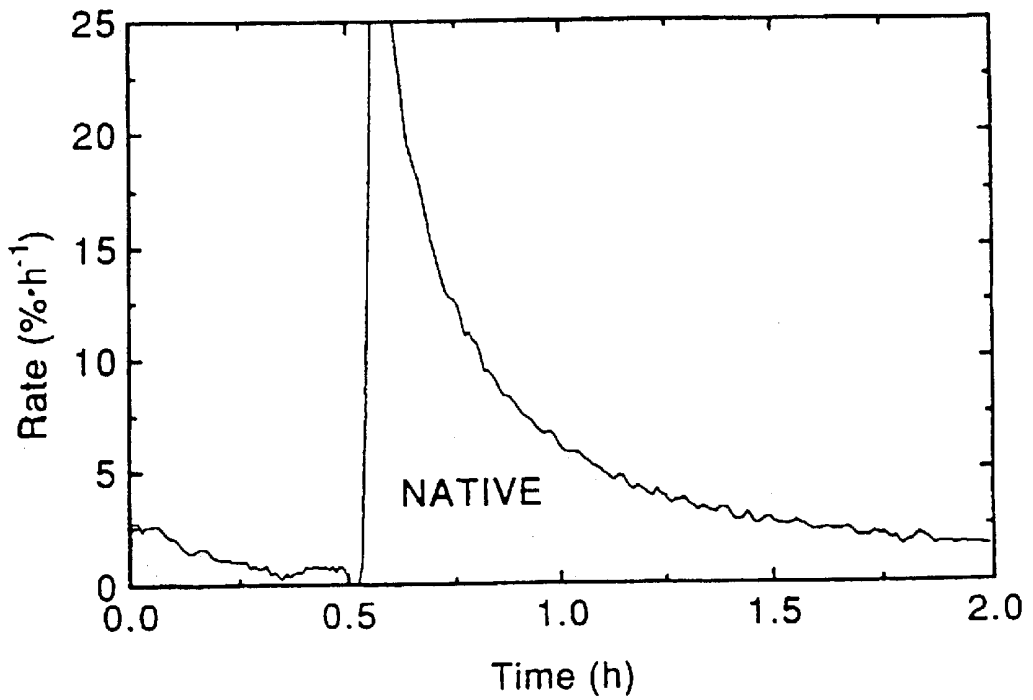


Fig.8B

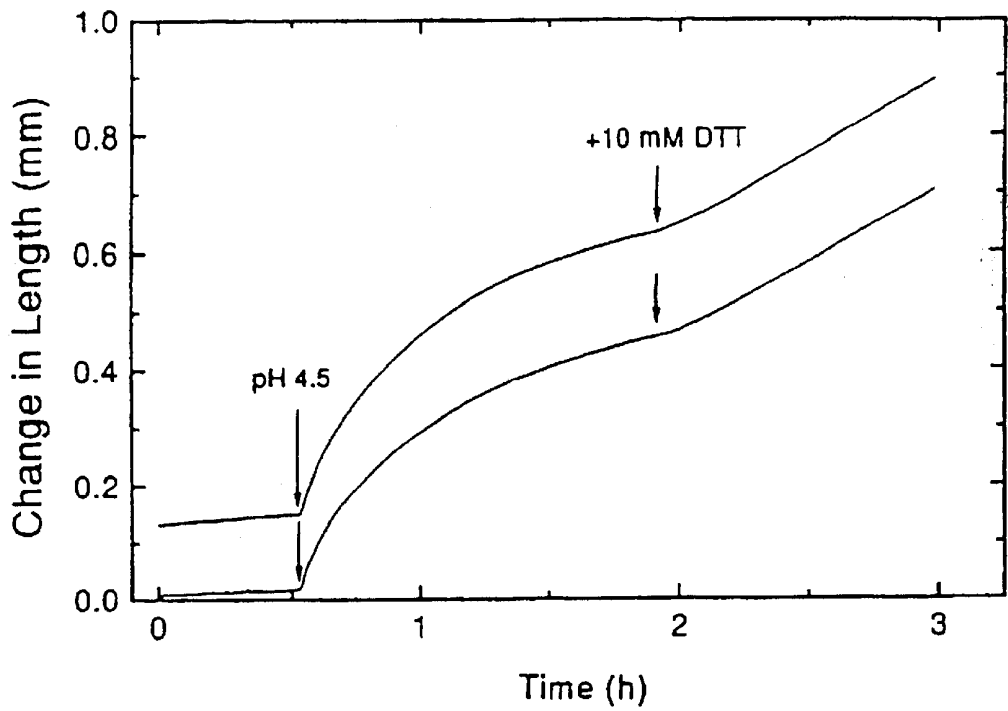


Fig.9A

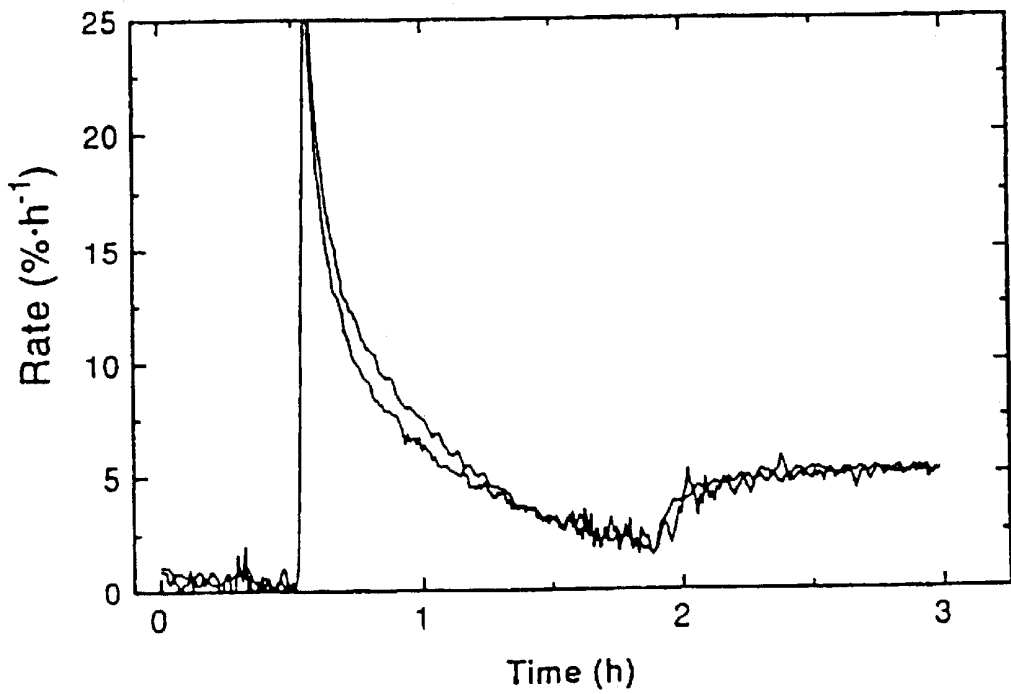


Fig.9B

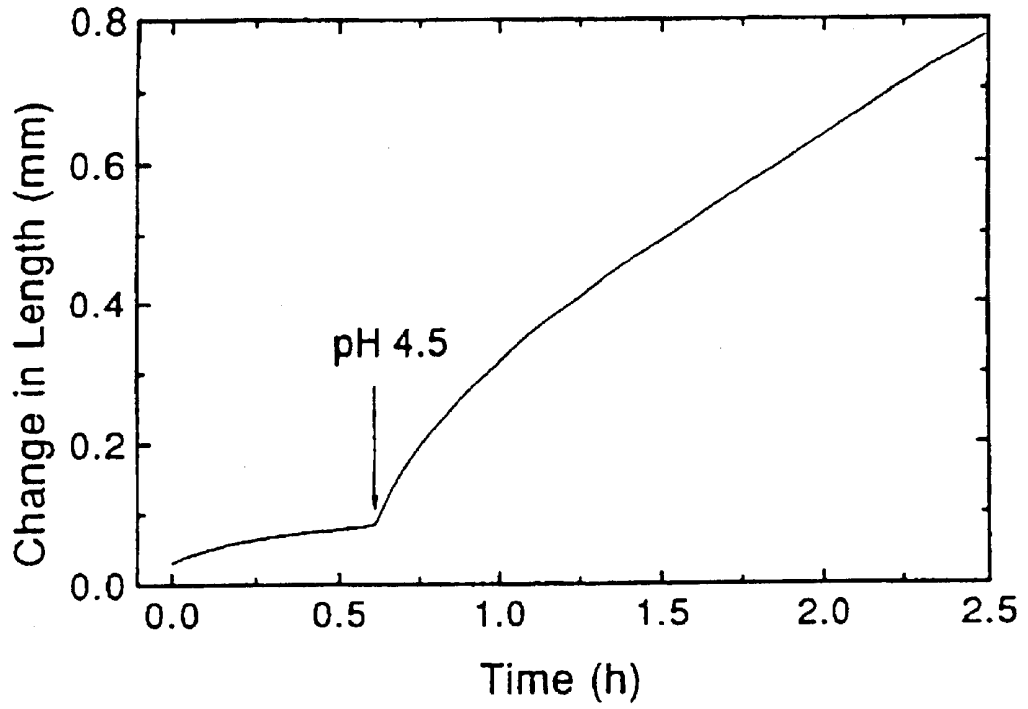


Fig.10A

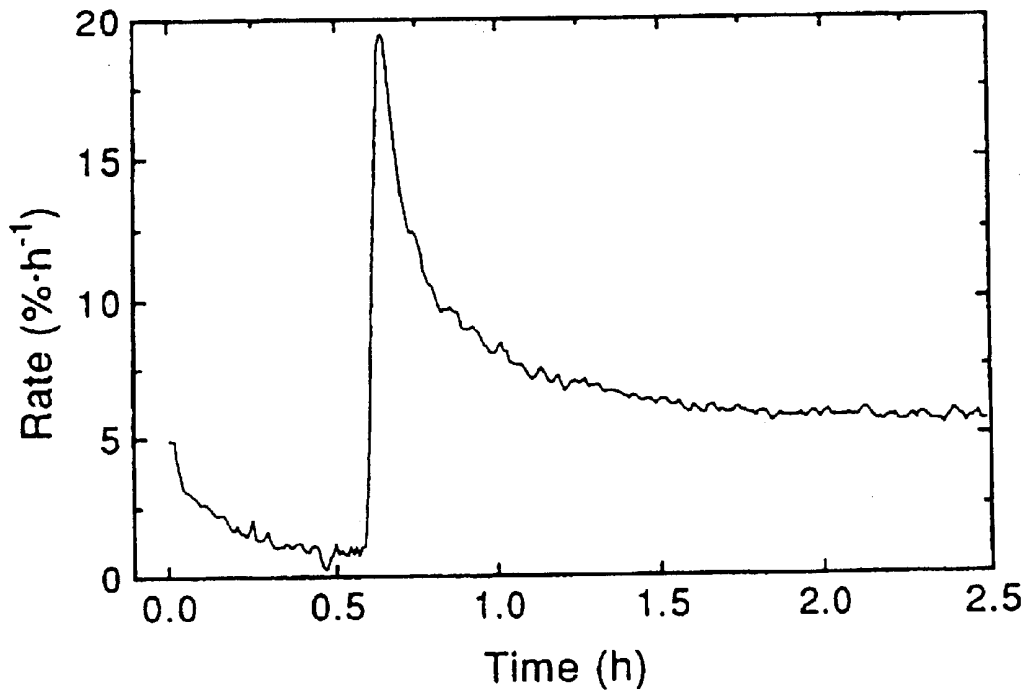


Fig.10B

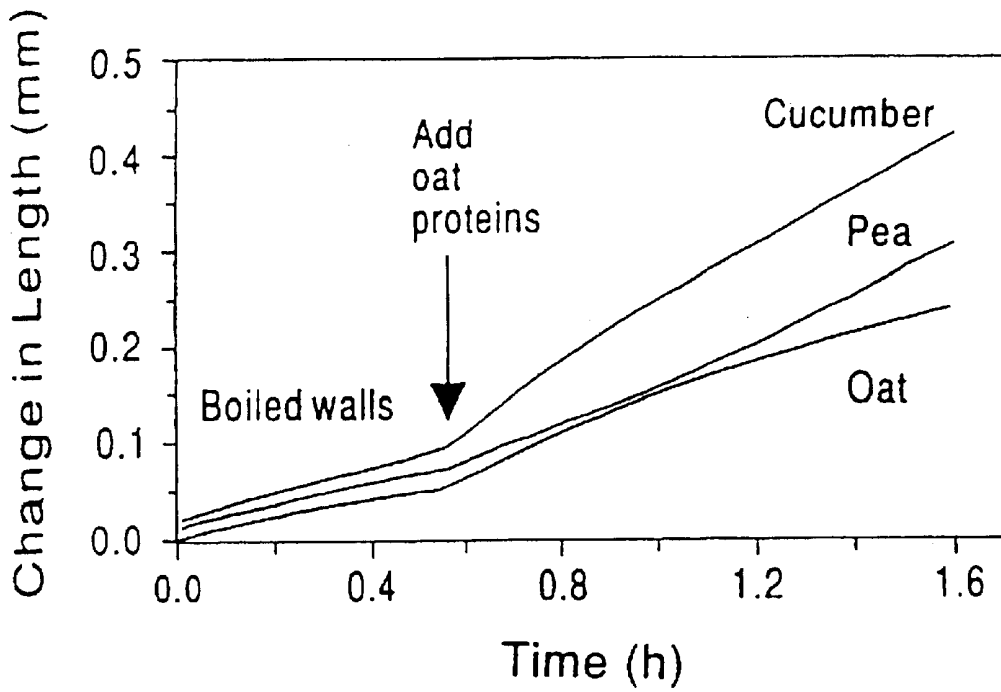


Fig.11A

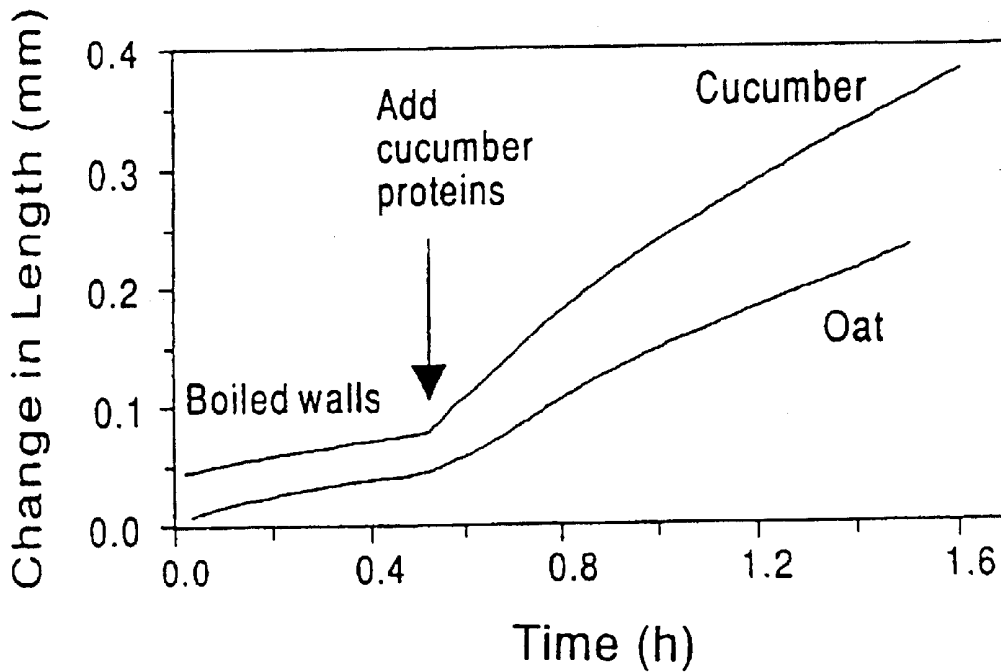


Fig.11B

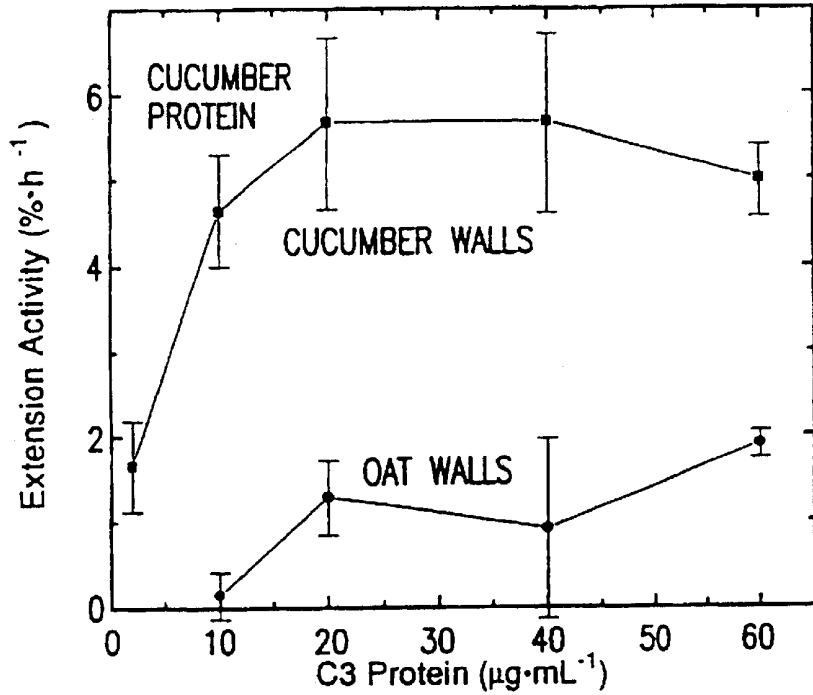


Fig.12A

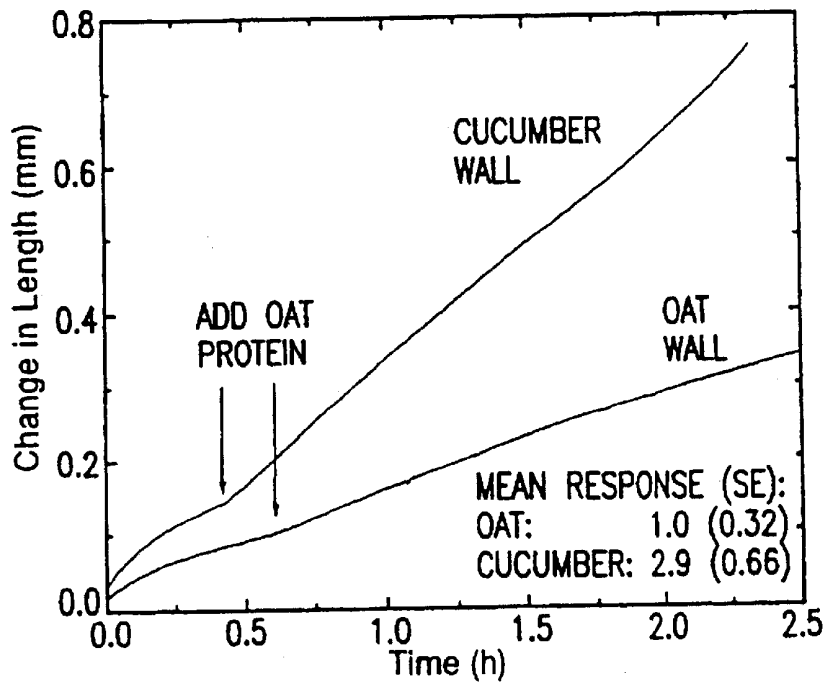


Fig.12B

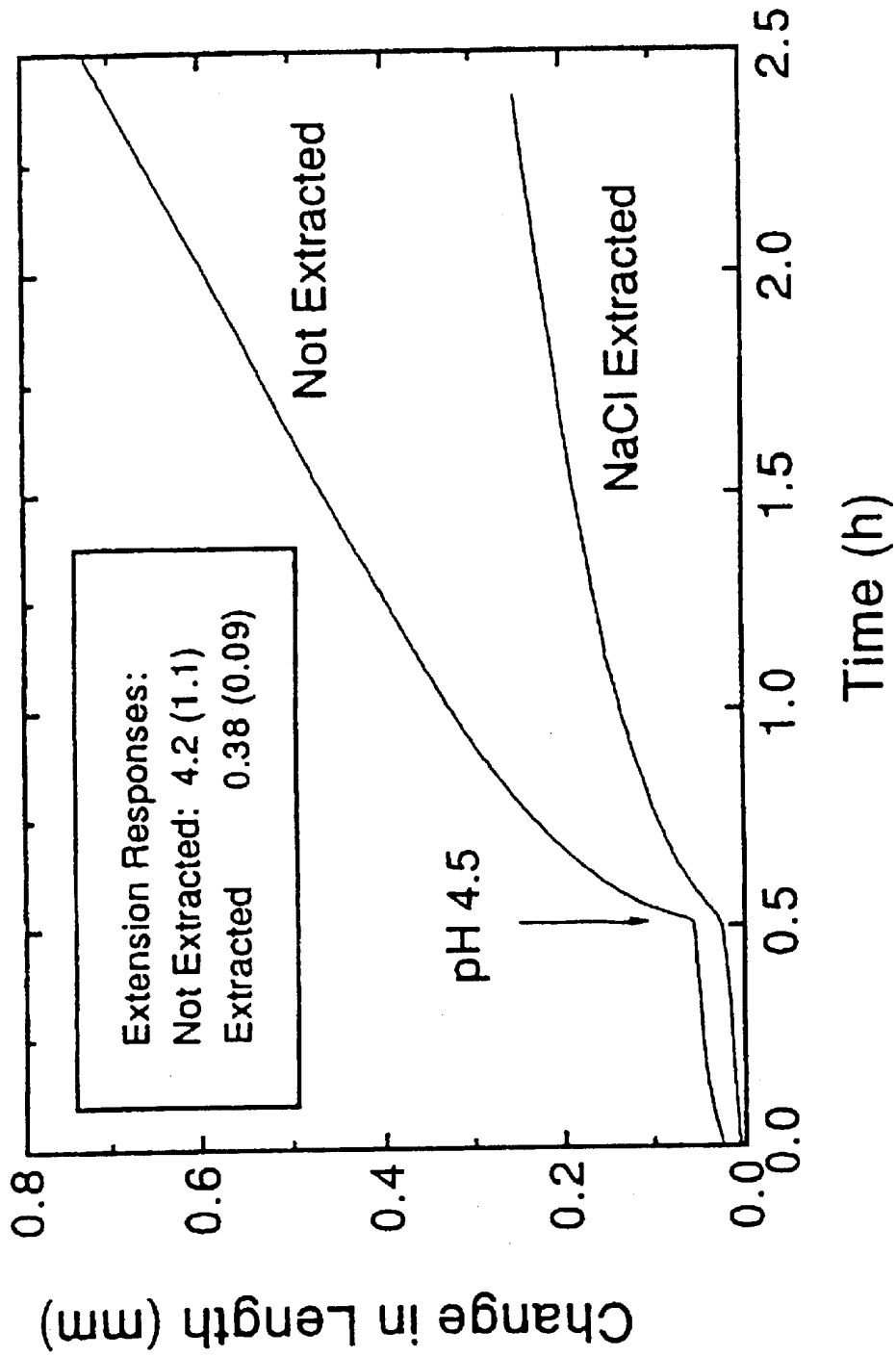


Fig.13

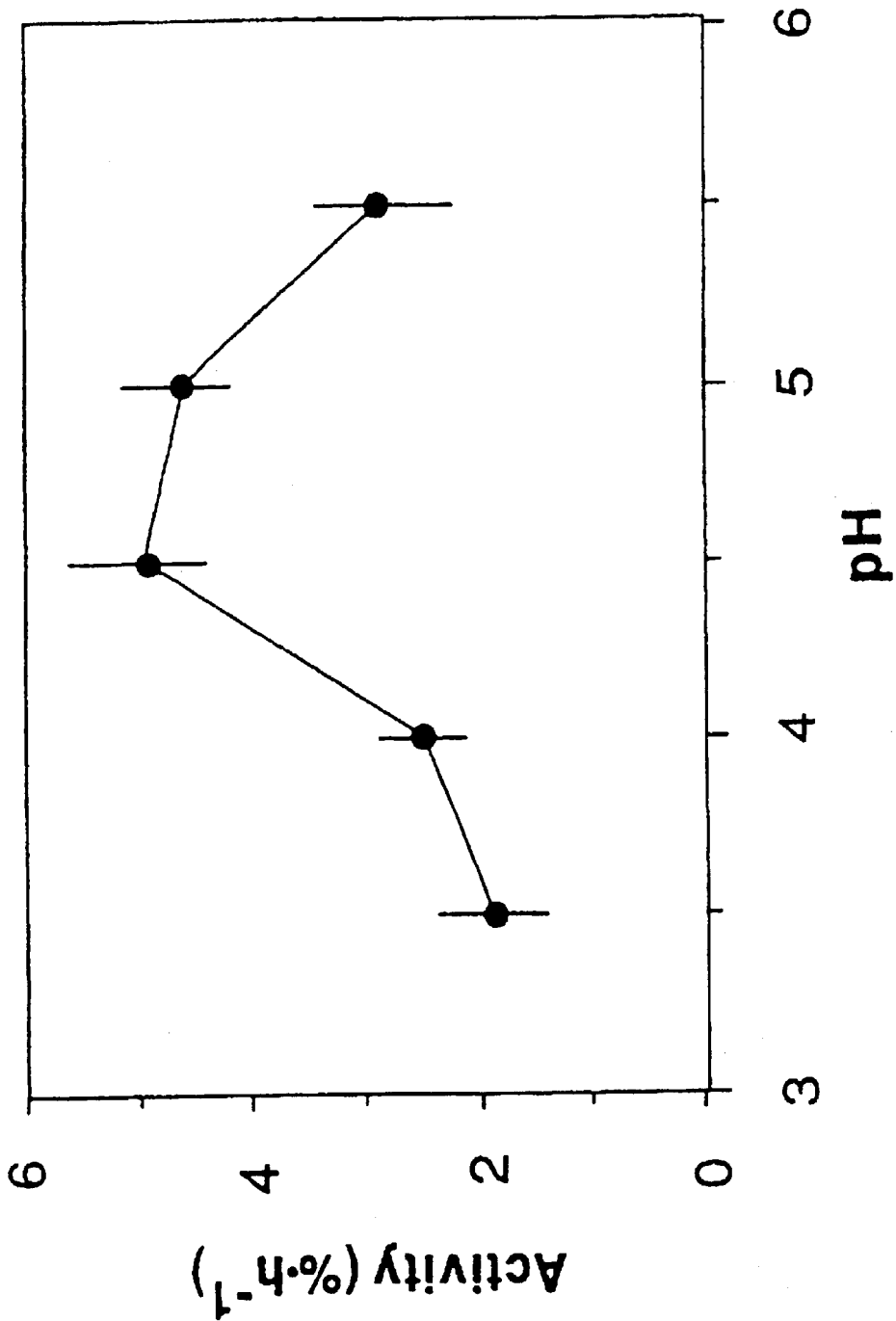


Fig.14

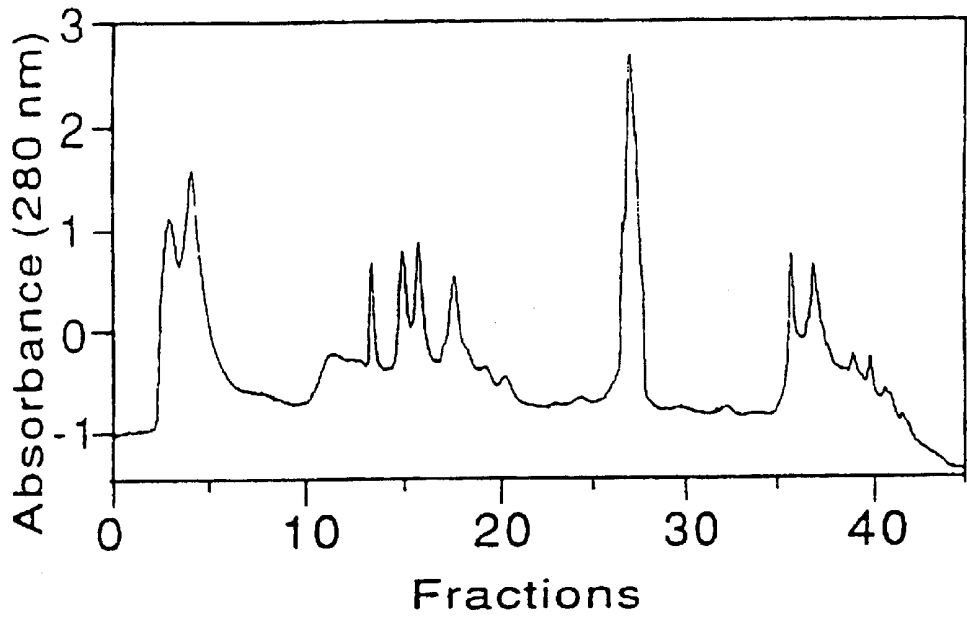


Fig.15A

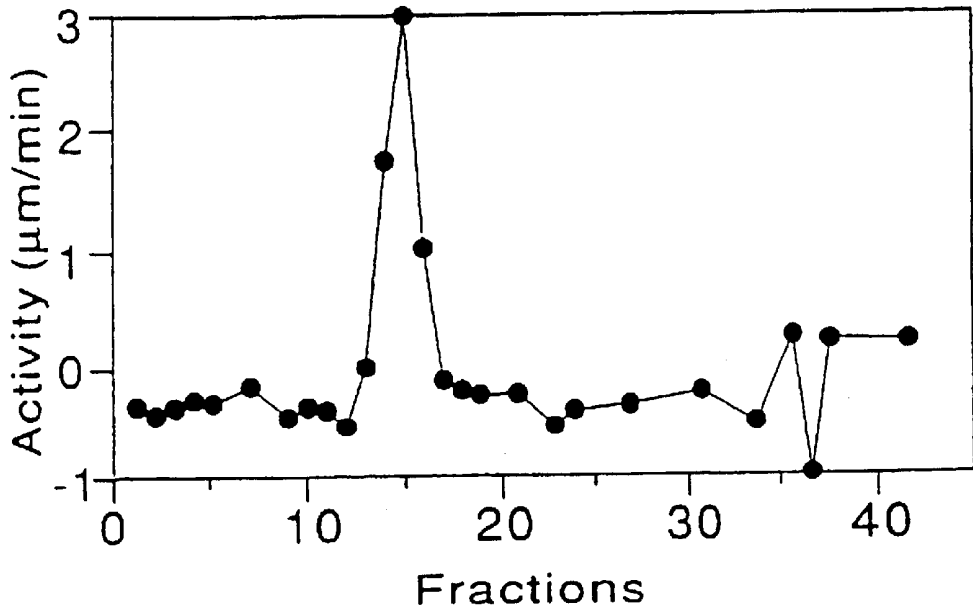


Fig.15B

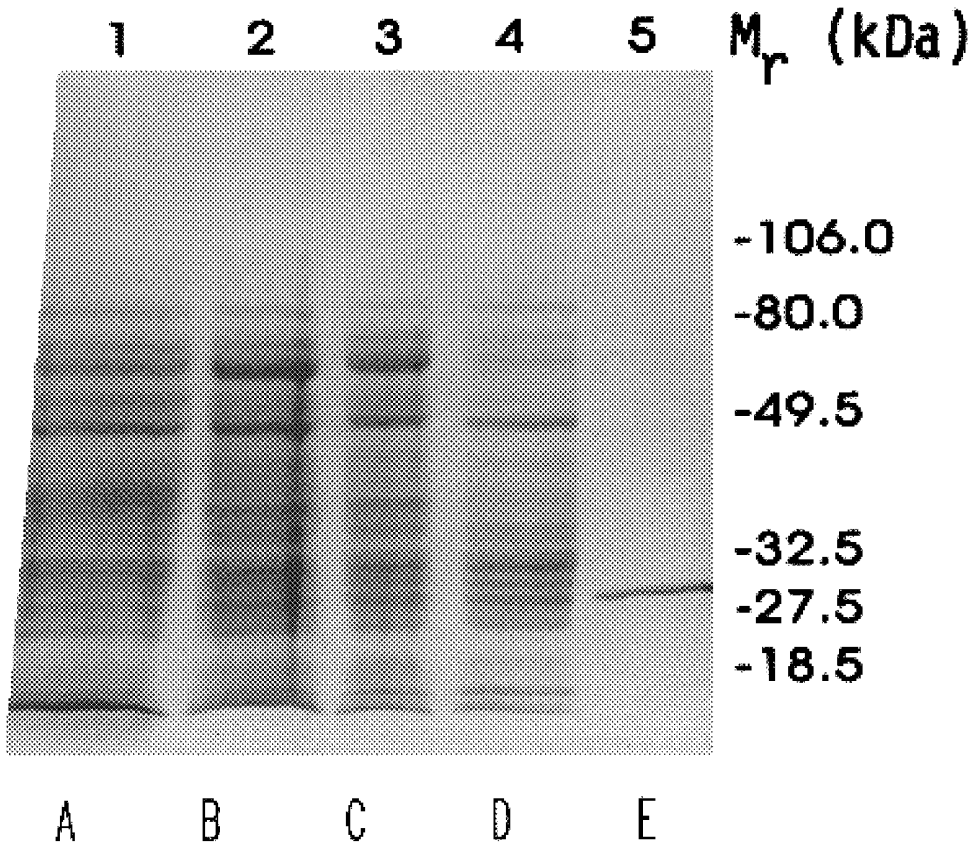


Fig.16

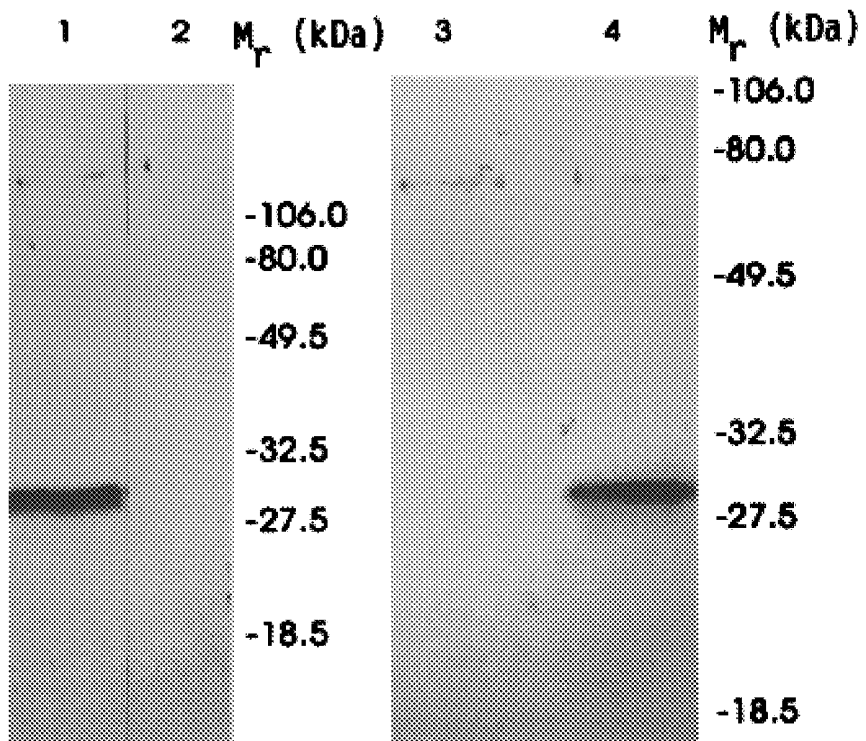


Fig.17

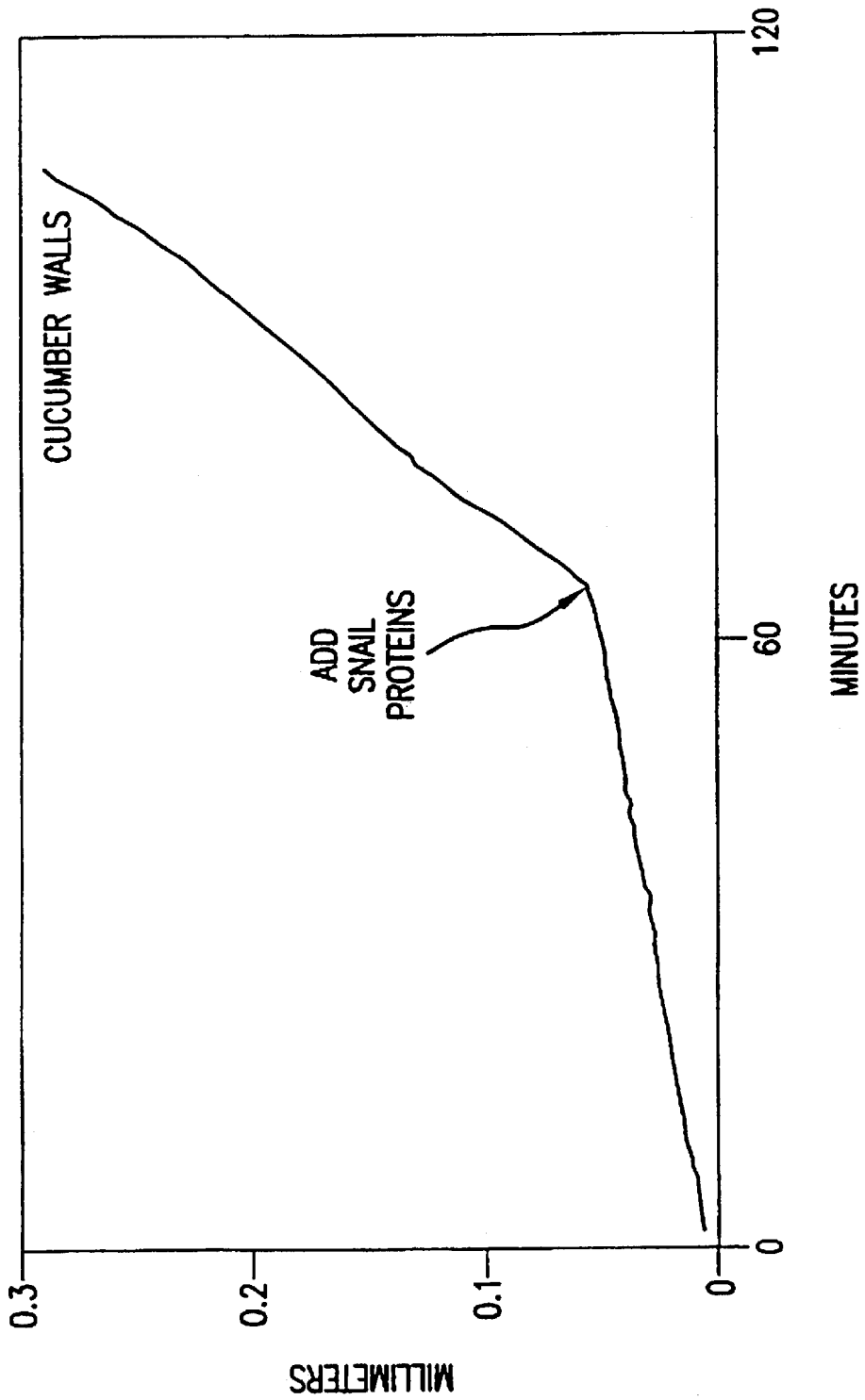


Fig.18

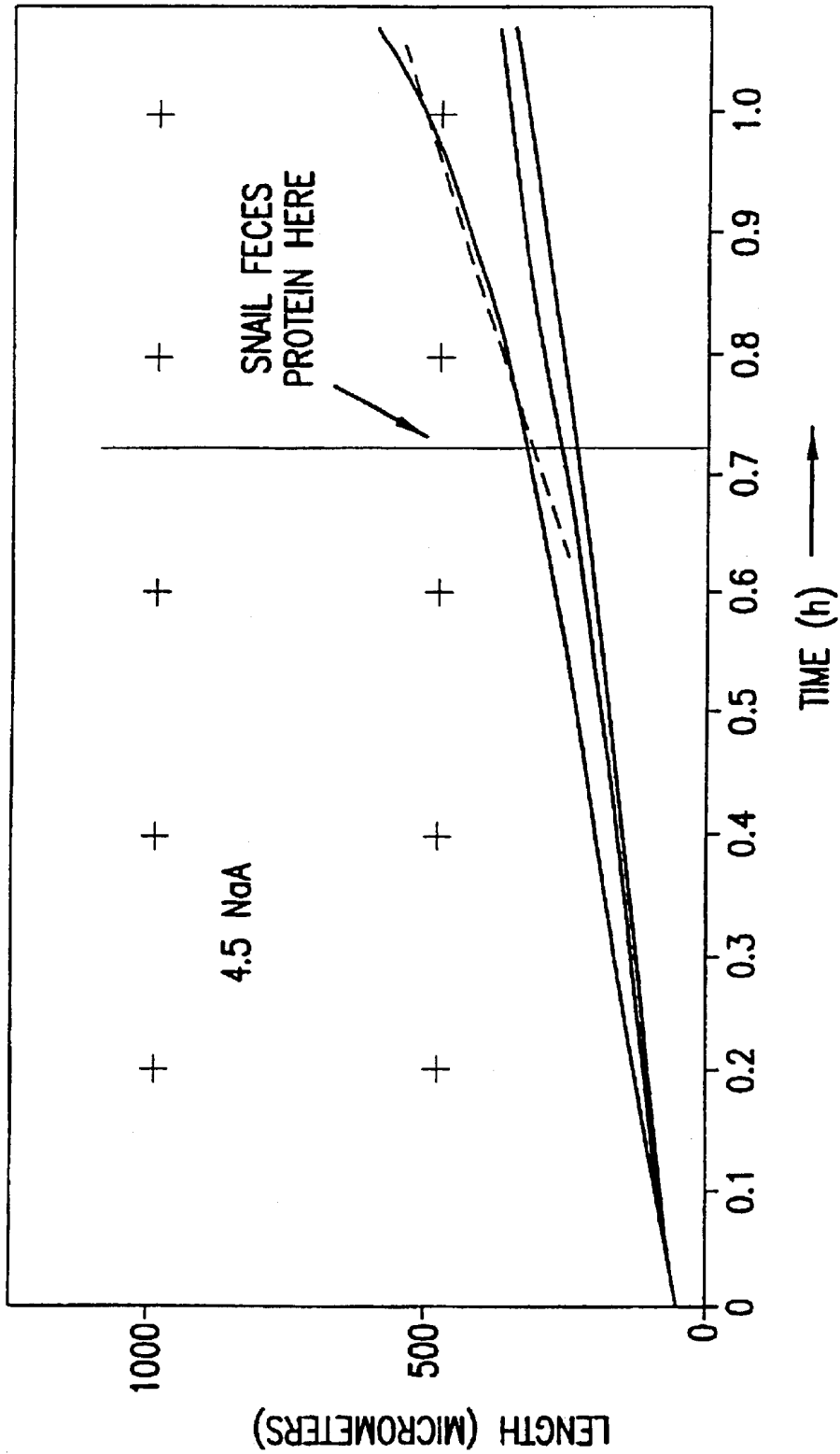


Fig.19

MW STANDARDS

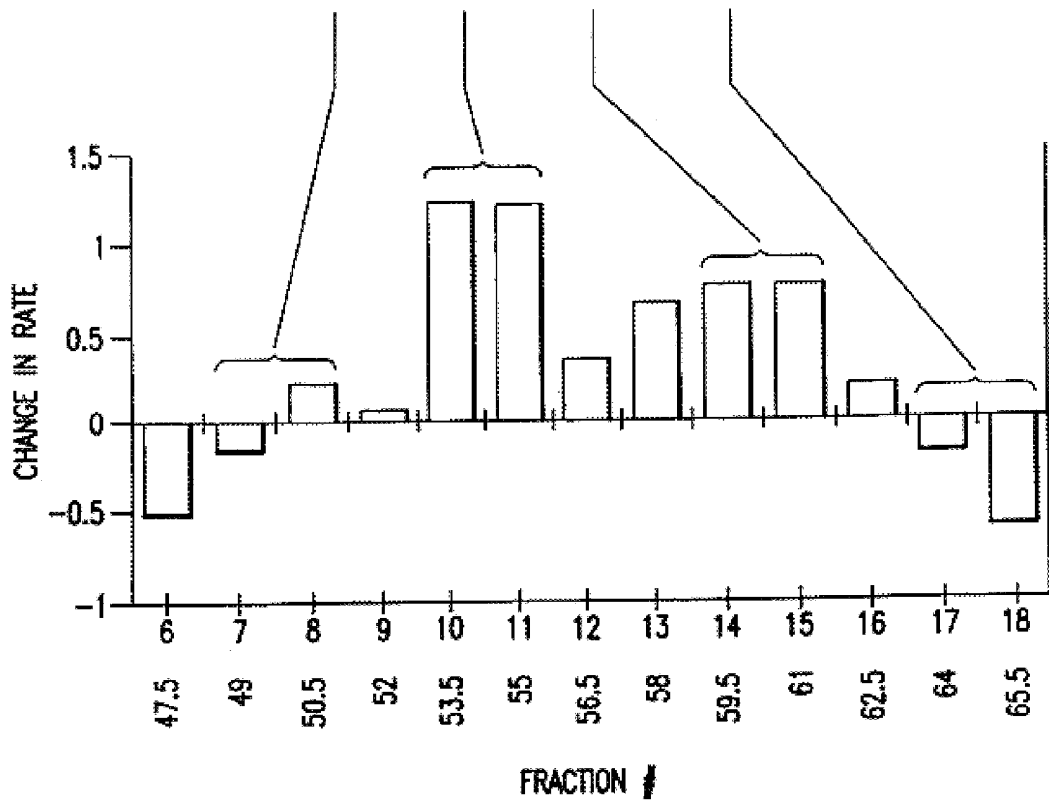
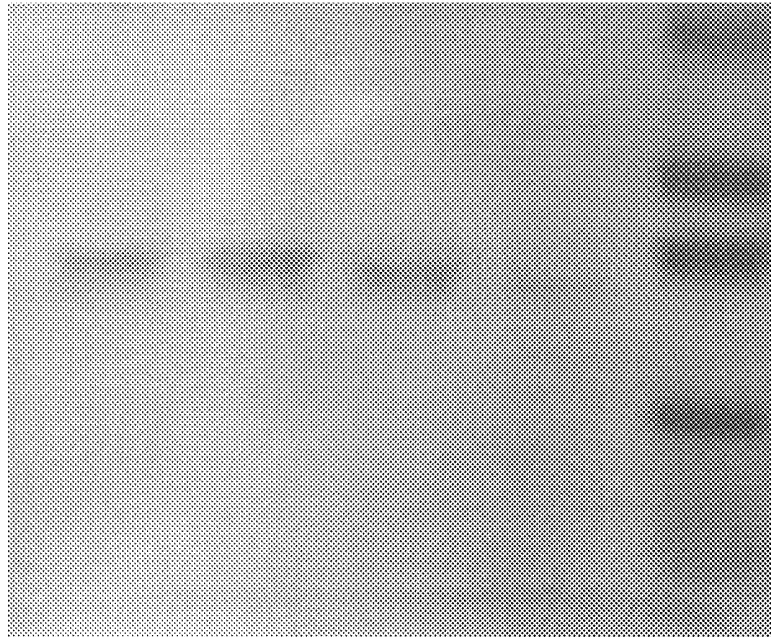


Fig.20

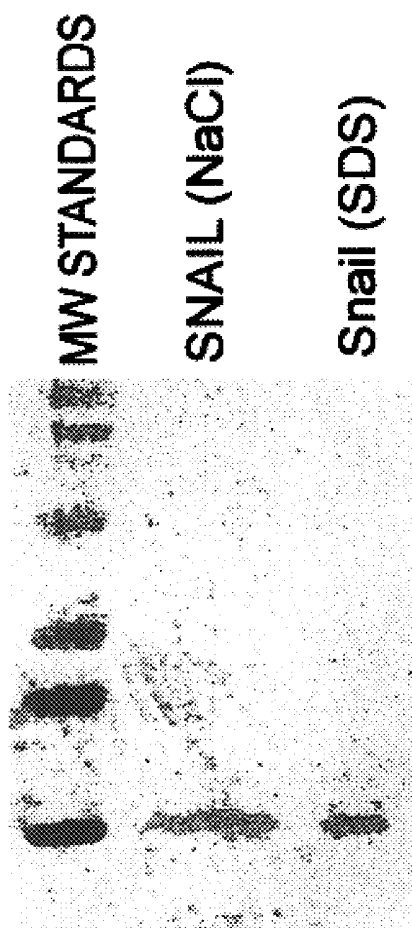


Fig.21

PURIFIED PLANT EXPANSION PROTEINS AND DNA ENCODING SAME

CORRESPONDING U.S. APPLICATIONS

This specification is a divisional of U.S. application Ser. No. 08/440,517, filed May 12, 1995, which is a continuation-in-part of U.S. application Ser. No. 08/242,090, filed May 12, 1994, now abandoned which is a continuation-in-part of U.S. application Ser. No. 08/060,944, filed May 12, 1993, now abandoned.

INTRODUCTION

The present invention relates to a new class of proteins, known as expansins, and their isolation, sequencing, genesis by expression systems, and utilization. These proteins have been identified in a wide variety of plant and other materials and have a variety of applications, including but not limited to agricultural and/or food applications and industrial uses such as their use in the paper industry as a catalyst for weakening the strength of paper products. For example, they can prove especially useful in the recycling of paper.

By the way of background, for many years wall "loosening enzymes" have been implicated in the control of plant cell enlargement (growth), largely on the basis of rapid biophysical and biochemical changes in the wall during auxin-induced growth (reviewed by Cleland and Rayle, *Bot. Mag. Tokyo*, 1:125-139, 1978; Taiz, *Annu. Rev. Plant Physiol.*, 35:585-657, 1984). Plant walls contain numerous hydrolytic enzymes, which have been viewed as catalysts capable of weakening the wall to permit turgor-driven expansion (reviewed by Fry, *Physiol. Plantarum*, 75:532-536, 1989). In support of this hypothesis, Huber and Nevins (*Physiol. Plant.*, 53:533-539, 1981) and Inoue and Nevins (*Plant Physiol.*, 96:426-431, 1991) found that antibodies raised against wall proteins could inhibit both auxin-induced growth and wall autolysis of corn coleoptiles. In addition, isolated walls from many species extend irreversibly when placed under tension in acid conditions in a manner consistent with an enzyme-mediated process (Cosgrove D. J. *Planta*, 177:121-130, 1989). Despite these results and other evidence in favor of "wall-loosening" enzymes, a crucial prediction of this hypothesis has never been demonstrated, namely, that exogenously added enzymes or enzyme mixtures can induce extension of isolated walls. To the contrary, Ruesink (*Planta*, 89:95-107, 1969) reported that exogenous wall hydrolytic enzymes could mechanically weaken the wall without stimulating expansion. Similarly, autolysis of walls during fruit ripening does not lead to cell expansion. Thus a major piece of evidence in favor of wall-loosening enzymes as agents of growth control has been lacking.

Once identified, expansins—proteins capable of inducing cell wall extension—would have utility not only in the engineered extension of cell walls in living plants but foreseeably in commercial applications where their chemical reactivity could prove useful. If expansins can disrupt noncovalent associations of cellulose, as they have been shown to do, then they would have particular utility in the paper recycling industry. Paper recycling is a growing concern and will prove more important as the nation's landfill sites become more scarce and more expensive. Paper derives its mechanical strength from hydrogen bonding between paper fibers, which are composed primarily of cellulose. During paper recycling, the hydrogen bonding between paper fibers is disrupted by chemical and mechanical means prior to re-forming new paper products. Proteins

which cause cell expansion are thus intrinsically well suited to paper recycling, especially when the proteins are nontoxic and otherwise innocuous, and when the proteins can break down paper products which are resistant to other chemical and enzymatic means of degradation. Use of proteins of this type could thus expand the range of recyclable papers.

Other modes of application of expansins, once they are found, include production of virgin paper. Pulp for virgin paper is made by disrupting the bonding between plant fibers. For the reasons identified above, expansins are useful in the production of paper pulp from plant tissues. Use of expansins can substitute for harsher chemicals now in use and thereby reduce the financial and environmental costs associated with disposing of these harsh chemicals. The use of expansins can also result in higher quality plant fibers because they would be less degraded than fibers currently obtained by harsher treatments.

Thus, a need remains for the identification, characterization and purification of expansins—proteins which can be characterized as catalysts of the extension of plant cell walls and the weakening of the hydrogen bonds in the pure cellulose paper—and the incorporation of DNA sequences which give rise to such proteins in appropriate expression systems.

SUMMARY OF THE INVENTION

In accordance with the present invention, a new class of proteins and methods related thereto are presented. The proteins, which can be characterized as catalysts of the extension of plant cell walls and the weakening of the hydrogen bonds in pure cellulose, are referred to as expansins. Two proteins have been isolated by fractionation techniques from washed wall fragments of cucumber hypocotyls, referred to as "cucumber expansin-29" and "cucumber expansin-30" (abbreviated cEx-29 and cEx-30). Moreover, three peptide fragments from the purified cEx-29 protein were sequenced, then oligonucleotide primers were designed to amplify a portion of the expansin cDNA using polymerase chain reaction with a cDNA template derived from cucumber seedlings, and then the PCR fragment was used to screen a cDNA library to identify full length clones. Another expansin protein has been isolated from oat coleoptiles (oat expansin oEx-29), while three additional expansin sequences have been identified in Arabidopsis and an additional two in rice. Expansins appear to be broadly distributed throughout the plant kingdom and can be identified in stem and leaf vegetables (i.e., broccoli, cabbage), fruit and seed vegetables (i.e., tomato), fiber crops and cereals (i.e., corn), and forest and ornamental crops (i.e., cotton). Also, expansin-like protein has been found in proteins obtained from the digestive tract of snail and its feces (sEx). These novel proteins can find use in a variety of applications including the paper industry in production of paper pulp in preparation of virgin paper and in paper recycling as a preferred way of disruption of paper fibers due to their nontoxic and environmentally innocuous nature in contrast with the harsh chemical treatments applied today, which are environmentally noxious and in many cases completely unacceptable.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1. Extension curves of native and reconstituted isolated cell wall specimens obtained using a constant load extensometer.

Ten-mm sections of tissue were frozen and thawed, and lightly abraded prior to suspension in the extensometer.

Tissues were clamped under an applied force of 20 g and extension recorded using a linear voltage displacement transducer. The length of tissue between the clamps was 5 mm.

(A) Extension curves of native and reconstituted cucumber hypocotyl walls. From top to bottom: native walls were suspended under tension in 50 mM Hepes, pH 6.8, for 20 min. after which the bathing solution was replaced by 50 mM sodium acetate, pH 4.5, (arrow). Inactivated walls were treated for 15 sec with boiling water prior to suspension; as shown in the second line, this treatment eliminated acid induced extension. For reconstitution experiments, inactivated walls were suspended in 50 mM sodium acetate, pH 4.5, for 30 min. at which point the bathing solution was replaced by 0.5 ML of fresh solution (arrows) containing 2–3 mg of proteins extracted from growing cell walls (apical wall protein), or with soluble proteins from growing cells (apical soluble proteins), or proteins extracted from cell walls from the nongrowing cotyledon (cotyl. wall protein) or from walls of the basal hypocotyl (basal wall protein).

(B) Reconstitution of extension activity in inactivated (heat treated) walls from growing tissues of different species by the active cucumber wall extract. Walls from the growing region from tomato, pea and radish hypocotyls, lily and onion leaves, and coleoptiles of maize and barley were prepared, heat-treated, and suspended as given above, except that a force of only log was applied to the more fragile tomato and radish walls. Walls were first suspended in 50 mM sodium acetate, pH 4.5, after 30 min. (arrow) the bathing solution was replaced with 0.5 mL of the same buffer containing 2–3 mg of active wall protein from cucumber hypocotyls (apical 3 cm).

FIG. 2. Fractionation of extension-inducing cucumber wall extracts by HPLC.

(A) Ammonium sulfate precipitates of salt extracted cucumber wall extracts were resuspended and loaded onto a C3 hydrophobic interactions column in 50 mM sodium acetate, pH 4.5, 20% (saturation) ammonium sulfate. Proteins were eluted in a descending gradient (20–0%) of ammonium sulfate. Fractions were desalted and checked for extension inducing activity with inactivated cucumber tissues as in FIG. 1. A_{280} nm is shown by a solid line and extension inducing activity by a broken line.

(B) Active fractions from (A) were concentrated and desalted into 15 mM Mes, pH 6.5, and loaded onto a sulfopropyl anion exchange column equilibrated with the same buffer. Proteins were eluted with an ascending gradient of NaCl (0–1M) in this buffer. Extension inducing activity (broken line) of fractions was checked directly after adjusting the pH to 4.5 with 1M acetic acid.

FIG. 3. SDS-PAGE of ammonium sulfate precipitate (AS), and active fractions from C3 and sulfopropyl (S1 and S2) EPLC separations of cucumber proteins.

Protein samples were concentrated, desalted, and run on SDS-polyacrylamide gels according to the method of Laemmli. Gels were stained with Coomassie Brilliant Blue R 250. Arrows indicate the major protein bands at 29 and 30 kD in the S1 and S2 fractions which appear to possess the extension-inducing activity.

FIG. 4. The effects of DTT, metal ions, methanol and water boiling, and protease treatments on reconstituted extension.

(A) The effect of DTT on reconstituted extension. Growing wall specimens of cucumber hypocotyl were inactivated by heat and then reconstituted by shaking in a solution of active C3 proteins (estimated concentration of 50 micro-

grams per mL). Walls were then clamped under constant load (as described in FIG. 1) firstly in a bathing solution of 50 mM Hepes, pH 6.8. After 20 min. the solution was changed for 50 mM sodium acetate, pH 4.5 (first arrow). After a further 40 min. DTT (from a 100 mM stock solution) was added to give a final concentration of 10 mM. The first two lines represent typical traces from 4 experiments with and without DTF addition.

For the effects of metal ions, growing wall specimens from cucumber hypocotyl were inactivated by heat and then clamped under constant load (as described in FIG. 1) in a bathing solution of 50 mM sodium acetate, pH 4.5. After 20 min. the bathing solution was exchanged for a fresh one containing 50 micrograms per mL of active C3 proteins (first arrow). After a further 40 min. $AlCl_3 \cdot 6H_2O$ or $CuCl_2 \cdot 2H_2O$ (from 100 mM stock solutions) was added to bring the bathing solution to a final concentration of 1 mM (second arrow). All experiments were repeated 4 times. Line 3 shows extension without the addition of metal ions, the next two lines show typical data obtained with the addition of $AlCl_3$ and $CuCl_2$ respectively.

For the effects of boiling cell walls in methanol or boiling in water on the recovery of extension inducing activity, growing cucumber hypocotyl tissue was first boiled for 3 min. in methanol or for 30 sec in distilled water, wall fragments were recovered, cleaned and extracted (as described in FIG. 1). Proteins were precipitated with $(NH_4)_2SO_4$ and resuspended in 50 mM sodium acetate, pH 4.5 before being tested in the reconstitution assay described in FIG. 1. Lines 6 and 7 are representative data from 4 experiments.

(B) Growing wall specimens were inactivated by heat and reconstituted with C3 proteins. Reconstituted walls were incubated with 1000 units of trypsin or chymotrypsin for 4 hr at 30° C., in 50 mM Hepes, pH 7.3, or with 2 milligrams per mL of pronase or papain for 4 hr at 30° C., in 50 mM sodium acetate, pH 5.0. Controls were reconstituted and incubated in the same manner without the addition of proteases. At the end of the incubations tissues were clamped under constant load (as described in FIG. 1), first in 50 mM Hepes, pH 6.8. After 30 min. the bathing solution was replaced by 50 mM sodium acetate, pH 4.5. The difference in the two rates of extension was calculated. Data presented are the means of four experiments in each case.

FIG. 5. Effect of pH on reconstituted extension.

Cucumber wall specimens, in activated by heat, were clamped under constant load (as described in FIG. 1). Initial bathing solutions were 50 mM citric acid, titrated to pH 3.0, 3.5, 4.0, 4.5, 5.0, 5.5, 6.0 or 6.5 with 1M K_2HPO_4 . After 30 min., the bathing solution was changed for a 1:1 dilution of active C3 proteins with the relevant buffer (final estimated protein concentration was 50 micrograms per mL), where necessary the final pH was adjusted using either 1M citric acid or 1M K_2HPO_4 . Extension was recorded for a further 2 hr and change in rate of extension calculated as initial-final rates.

FIG. 6. The effects of cucumber expansins on cellulose paper.

(A) Strips of Whatman No. 3 filter paper were clamped in a constant load extensometer with 5 mm of paper between the clamps (described in FIG. 1). Paper strips were initially bathed in 50 mM sodium acetate, pH 4.5. After about 20 min. of extension, the bathing solution was replaced with either: 5 mg of S1 protein in 15 mM Mes, pH adjusted to 4.5 with 1M acetic acid (SI); 5 mg of S1 protein, in the same buffer, which had been heated to 100° C. for two minutes

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(Boiled S1); 100 mg of cellulase (from *Trichoderma viride* Boehringer Mannheim) in 50 mM sodium acetate (Cellulase), pH 4.5; or 50 mM sodium acetate pH 4.5 (Control). Extensions were recorded for a further 100 min. The figure shows representative traces from at least six independent experiments, all of which showed similar results.

(B) Strips of Whatman No. 3 paper were soaked in a solution containing 5 mg/mL S1 protein in 15 mM Mes, pH adjusted to 4.5 with 1M acetic acid, (SI); in the same protein solution which had been boiled for two minutes (Boiled S 1); or in 50 mM sodium acetate, pH 4.5 (Control). Stress relaxation tests were performed by standard methods. Data represent the average of ten measurements in each case. Experiments were repeated three times with similar results.

(C) Conditions were the same as in (B) except that the paper strips were soaked in a solution containing 100 mg/mL of *Trichoderma cellulase* in 50 mM sodium acetate, pH 4.5 (Cellulase), or 50 mM sodium acetate, pH 4.5 (Control). Data are the averages of ten measurements in both cases. Experiments were repeated three times with similar results.

FIG. 7 The effect of cucumber expansins on slick paper.

Paper strips of 2–3 mm width were cut and hung in our extensometer, such that a 5-mm long strip was put under 20-g force. The buffer contained 50 mM sodium acetate, pH 4.5 with or without added C3 protein (about 20 pg per paper strip was used). The length of the paper strips was recorded for up to 5 h. Four samples of each paper were used with each treatment (+/- protein). The untreated (control) paper showed very little change in length, and maintained its mechanical strength throughout the test period. In contrast, the treated papers began to extend and quickly broke once extension began. The time for breakage after addition of protein varied from 45 min. to 2.5 h, but all samples broke.

FIG. 8. Acid-induced extension in native and heat-treated oat coleoptile walls.

(A) When switched from a neutral buffer (50 mM Hepes, pH 6.8) to an acidic buffer (50 mM sodium acetate, pH 4.5), native walls extended at a high rate which fell continuously with time, whereas heat-treated walls lacked a significant response. The native and heat-treated (15 sec in boiling water) walls were prepared and mounted on an extensometer (5 mm between two clamps) with 20 g of tension as described by Cosgrove (1989) and McQueen-Mason et al. (1992).

(B) A plot of extension rate for the native walls shown in A demonstrates that the rate decreases continuously. Similar results were obtained in 10 repetitions.

FIG. 9. Effect of dithiothreitol (DTT) addition to acid-induced extension of oat coleoptile walls.

Native walls were prepared as in FIG. 8 and the buffer was exchanged for the same buffer containing 10 mM DTT at approximately 80 min after start of acid-induced extension

(A). The plot of extension rate (B) shows that the rate is stable at about 5% per h after addition of DTT. Similar results were obtained in 6 independent trials.

FIG. 10. Acid-induced extension in native oat coleoptile walls in the continuous presence of DTT.

(A) Walls were prepared as in FIG. 8 and incubated in 50 mM Hepes, pH 6.8, with 2 mM DTT; after about 35 min the buffer was switched to 50 mM sodium acetate, pH 4.5, with 2 mM DTT.

(B) The plot of extension rate shows that the decay in extension rate after 1 h is greatly diminished (compare with FIG. 8). This experiment was repeated four times with similar results.

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FIG. 11. Extension induced in oat, cucumber and pea walls by proteins extracted from oat coleoptile walls (A) and cucumber walls (13).

Coleoptile wall proteins and cucumber wall proteins were extracted with 1M NaCl, precipitated with ammonium sulfate, desalted and partially purified on a DEAE Sephadex column. Heat-inactivated cell walls from oat coleoptiles, cucumber hypocotyls or pea epicotyls were clamped at 20-g tension in 50 mM sodium acetate, pH 4.5. After about 0.5 h, the solution was replaced with 0.4 mL of the same buffer containing about 10 mg proteins partially purified from oat or cucumber cell walls by DEAE-anion exchange chromatography. The data represent a typical experiment from at least four replicates.

FIG. 12. Comparative responsiveness of oat and cucumber walls to exogenously added oat or cucumber proteins.

(A) Concentration dependence of cucumber and oat walls to exogenous cucumber protein. Cucumber walls were frozen, thawed, abraded, heat-inactivated and loaded in the extensometer, as in FIG. 8. Oat coleoptiles were treated similarly, except that the epidermis was stripped with fine forceps and the coleoptile bisected longitudinally prior to freezing. The walls were extended for approximately 30 min in 50 mM sodium acetate, pH 4.5, before the buffer was exchanged for the same one containing various concentrations of partially purified cucumber expansins (the active fraction from the C3 HPLC separation). Extension activity is calculated as the increase in extension rate in the first 15–20 min after addition of C3 protein and is expressed as % increase in length per h). The average extension rate prior to addition of protein in these experiments was 2% per h for oat walls and 1.7% per h for cucumber walls.

(B) Response of oat and cucumber walls to added oat protein. Heat-inactivated oat and cucumber walls were prepared as in (A) and extended in sodium acetate, pH 4.5, for about 30 min prior to exchange of buffer for the same one containing 73 μ g of crude oat coleoptile protein (ammonium sulfate precipitate) per sample holder (about 400 μ L). The extension curves shown illustrate two examples with responses close to the mean (2.8% h⁻¹ increase for the cucumber wall and 1.1% h⁻¹ increase for the oat wall). The mean responses (n=4) of oat and cucumber walls to added oat protein are shown in the inset and are calculated as the increase in extension rate, expressed as % per h. The baseline extension rates prior to addition of protein averaged 2.4% per h (oats) and 1.1% per h (cucumber).

FIG. 13. Effect of 1M NaCl extraction on acid-induced extension of oat coleoptile walls.

Coleoptiles were abraded prior to freezing, then either directly clamped in the extensometer or extracted for 16 h in 1M NaCl containing 10 mM Hepes, pH 6.8, 3 mM EDTA and 2 mM sodium bisulfite prior to measurement. Walls were rinsed briefly in water, clamped at 20 g tension, then incubated in 50 mM Hepes, pH 6.8, with 2 mM DTT. At about 30 min the buffer was exchanged for 50 mM sodium acetate, pH 4.5, with 2 mM DTT. The curves shown are representative of eight trials for each treatment. The inset shows the average extension response (SE, n=8) of each treatment. Response is calculated as the rate at 1.5 to 2 h minus the rate prior to pH 4.5 buffer, and is expressed as % increase in length per h.

FIG. 14. pH dependence of the extension activity reconstituted by oat wall proteins.

Heat-inactivated walls from cucumber hypocotyls were clamped in an extensometer as in FIG. 8 and placed in 50 mM sodium acetate buffers at pH 3.5, 4.0, 4.5, 5.0 or 5.5.

After 20 min, the solutions were replaced with 0.4 mL of the corresponding buffer containing 10 mg oat-coleoptile wall proteins partially purified by DEAE-chromatography. The extension activity was calculated by subtracting the baseline rate without proteins from the linear rate (5–30 min) after the addition of the proteins, and expressed as % increase in length per h above the baseline rate. The data represent the means \pm S.E. (n=5 to 8). The average baseline rate prior to addition of protein ranged between 2.52 and 2.96 $\mu\text{m min}^{-1}$ (or 3.0 to 3.55% per h) for all pH groups.

FIG. 15. Purification of oat expansin by high pressure liquid chromatography (HPLC) on a carboxymethyl (CM) cation exchange column.

(A) Elution of proteins (absorbance at 280 nm) from a CM-column. Proteins were solubilized from the cell walls of etiolated coleoptiles by 1M NaCl and then sequentially fractionated by ammonium sulfate precipitation, DEAE-chromatography, and ConA chromatography, prior to CM-HPLC.

(B) Wall extension activity of HPLC fractions. The extension activity was assayed by addition of fraction samples (equal volumes, typically 20 μL) to sample cuvettes containing 400 μL of 50 mM sodium acetate buffer, pH 4.5. Activity is expressed as increase in extension rate after addition of protein to a 5-mm, heat inactivated, abraded oat coleoptile. A single peak of activity eluted at about 15 min or 3.5% of 1M NaCl. Similar results were obtained in six independent trials.

FIG. 16. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from different purification steps.

Active protein fractions were separated on a SDS-PAGE gradient gel (4–20%) and stained with Coomassie Blue R-250. Lane 1, 1M NaCl extraction (20 mg); lane 2, ammonium sulfate precipitate (20 mg); lane 3, active DEAE-fraction (10 mg); lane 4, proteins passing through Con A column (5 mg); lane 5, active fraction from CM-HPLC (0.3 mg). The protein with apparent molecular mass (by SDS-PAGE) of about 29 kD was designated as oat expansin (oat Ex29). Similar results were obtained in five trials, except in some of these an additional protein band at about 35 kD appeared in lane 5.

FIG. 17. Western analysis of oat coleoptile proteins probed with antiserum against cucumber Ex29.

Comparison of lane 1 (crude wall protein) with lane 2 (crude protoplasmic protein) shows that the antiserum recognizes an Ex29-like protein (arrow) specifically bound to the coleoptile cell wall. Comparison of lane 3 (crude wall protein) with lane 4 (purified protein with extension activity) shows that the Ex29-like protein copurifies with the extension activity. Methods: Lane 1 was loaded with 15 μg of crude wall protein (ammonium sulfate precipitate of 1M NaCl extract). Lane 2 had 15 μg of the soluble protoplasmic protein fraction. These samples were separated on the same 4–20% gradient SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with rabbit antiserum against cucumber Ex29. Lane 3 was loaded with 0.2 μg of crude wall protein and Lane 4 had 0.2 μg of active oat wall-extension protein purified sequentially by DEAE Sephadex and CM-HPLC. These samples (3–4) were separated on the same 14% SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with antiserum. This assay, with minor variations, was carried out four times with similar results.

FIG. 18. Extension induced in isolated cucumber walls by proteins from snails.

Cucumber walls were prepared as in the description for FIG. 11. Proteins prepared by dissolving snail acetone

powder (10 mg/mL) in 50 mM sodium acetate, pH 4.5, is capable of inducing extension of isolated cucumber walls. The immediate response and the linearity of the extension are unique characteristics that indicate an expansin-like protein may be involved.

FIG. 19. Extension induced in isolated cucumber walls by proteins from snails feces.

The feces of *Helix aspersa* (snail) induced extension in cucumber walls. The feces were dissolved in 50 mM sodium acetate buffer, pH 4.5. The procedure was as described in FIG. 11.

FIG. 20. Western blot analysis of active HPLC-separated proteins fraction from snail.

Acetone powder solution was probed with antibody which was raised against cucumber expansin-29. The analysis was performed as described in FIG. 17. The active fractions show a striking band at about 26 kD, which is similar to cucumber expansin-29. These results provide strong evidence that the wall extension activity found in the snail acetone powder is due to a protein of similar size and similar antigenic determinants as cucumber expansin-29.

FIG. 21. Western blot of the protein from *Helix aspersa* feces, probed with the antibody PA-1.

Feces from *Helix aspersa* were dissolved as described in FIG. 19 and Western blot analysis was performed as in FIG. 20. The single band indicates the presence of an protein antigenically similar to cucumber expansin-29.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes the first evidence of a new class of proteins named expansins, as well as their purification, sequencing, expression and use. The proteins can be characterized as catalysts of the extension of plant cell walls and the weakening of the hydrogen bonds in pure cellulose. During the course of detailed studies of plant cell growth, our laboratory isolated first from cucumber two novel proteins which we named expansins. Two proteins have been isolated by fractionation techniques from washed wall fragments of cucumber hypocotyls, referred to as "cucumber expansin-29" and "cucumber-expansion 30" (abbreviated cEx-29 and cEx-30, with respect to their apparent relative masses as determined by SDS-PAGE. Further research found expansins in Arabidopsis, rice and oat coleoptile as well as in the proteins obtained from the digestive tract of snails. An important aspect of expansin technology is the identification of the defining characteristics of the expansin protein at a molecular level, and the incorporation of complementary gene sequences into appropriate expression systems for large-scale manufacture of the desired expansin proteins.

Several detailed embodiments of the present invention are disclosed herein, including cucumber, Arabidopsis, rice and oat proteins, as well as an expansin-like protein from snail digestive tract. Another expansin protein has been isolated from oat coleoptiles (oat expansin oEx-29), while three additional expansin sequences have been identified in Arabidopsis and an additional two in rice. Expansins appear to be broadly distributed throughout the plant kingdom and can be identified in stem and leaf vegetables (i.e., broccoli, cabbage), fruit and seed vegetables (i.e., tomato), fiber crops and cereals (i.e., corn), and forest and ornamental crops (i.e., cotton). However it should be understood that these embodiments are merely illustrative of the invention, which may be embodied in various forms and with predictable divergence of amino acid sequence within the class of expansins

described herein. Generally speaking, a particular amino acid sequence (SEQ. ID. NO:7), or greater than about 60% sequence similarity and preferably greater than about 70% sequence similarity with that sequence, characterizes the individual members of the class of purified proteins which constitute the inventive expansin genus. Ultimately, however, the dispositive factor in defining an expansin is whether it behaves as an expansin, or exhibits the characteristics of an expansin, as discussed throughout this specification and the parent applications identified above.

We herein present evidence that the walls of growing cucumber seedlings possess extractable proteins which can induce extension of isolated walls. Additionally, we identify proteins having this activity and provide the relevant amino acid sequence. We propose the name "expansin" for this class of proteins, defined as endogenous cell wall proteins which restore extension activity to inactivated walls held under tension. We further propose the specific names "expansin-29" and "expansin-30" (abbreviated Ex-29 and Ex-30, with respect to their relative molecular masses apparent from SDS PAGE; McQueen-Mason et al. *Plant Cell*, 4:1425-1433, 1992) for the two proteins isolated from cucumber. Moreover, three peptide fragments from the purified cEx-29 protein were sequenced, then oligonucleotide primers were designed to amplify a portion of the expansin cDNA using polymerase chain reaction with a cDNA template derived from cucumber seedlings, and then the PCR fragment was used to screen a cDNA library to identify full length clones. We have also identified an oat coleoptile wall protein that induced extension in isolated dicot walls (Z. C. Lee et al., 1993, *Planta*, 191:349-356). The oat protein has an apparent molecular mass of 29 kD as revealed by SDS-PAGE. For clarity we will refer to the cucumber proteins as cEx, and to the oat proteins as oEx. New data demonstrate that an expansin-like protein may be found in proteins obtained from the digestive tract of snail and its feces. We refer to the snail protein as sEx.

During studies of the biochemical mechanism of action of expansins, we found that they had the ability to weaken the hydrogen bonding between plant cell wall polysaccharides (such as cellulose fibers). These findings allow us to propose the following commercial uses of these novel proteins, including paper treatment, agricultural uses, and a variety of use in food and industrial markets.

The following abbreviations have been used in the specification: ConA=Concanavalin A; CM=carboxymethyl; DEAE=diethylaminoethyl; Hepes=4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DTT=dithiothreitol; Ex29=29-kD expansin; Mes=2-(N-morpholino) ethanesulphonic acid; HPLC=high-pressure liquid chromatography; PBST=phosphate buffered saline with Tween-20; and SDSPAGE sodium dodecyl sulfate-polyacrylamide gel electrophoresis, along with the standard abbreviations for amino acids incorporated in amino acid sequences.

Our basic approach was to solubilize and to purify ionically bound proteins from growing cell walls and assay their ability to restore the endogenous extension activity in heat-inactivated walls. Extension was assayed using a constant load extensometer, in which tissue samples were damped under constant force and their extension recorded using an electronic displacement transducer (Cosgrove, D. J. *Planta*, 177:121-130, 1989). The first two proteins possessing the ability to restore the extension activity in heat-inactivated wall were solubilized from wall fragments isolated from the growing hypocotyls of dark-grown cucumber seedlings. After various unsuccessful attempts at reconstitution, we obtained a crude salt-solubilized fraction with the ability to

induce extension in inactivated walls, shown in FIG. 1A. The extension induced by this extract mimicked the native extension activity in magnitude and kinetics, i.e., initially high rates decayed over a period of 2 hours to more stable rates of about 2% per hour. These rates are lower than elongation rates of the living stem, but the stress applied to the isolated walls was only one-fifth of the equivalent longitudinal stress imposed on the walls by cell turgor. Like the endogenous extension activity of isolated cell walls, the reconstituted activity required an acid pH and was irreversible (i.e. upon removal of load, the segments did not return to their original length). The addition of protein to native cell walls did not substantially enhance extension, suggesting that endogenous activity was saturating or that binding sites were not accessible to the added material.

The active material appeared to be restricted to the growing region of the hypocotyl. When wall fragments from basal (non growing) stem tissue or from the cotyledons (which expand negligibly in our conditions) were extracted, proteins solubilized and purified in the same way did not induce wall extension (FIG. 1A). These results suggest that non-growing tissues lack the active material; however, we cannot exclude the possibility that the active material was present in the tissue but more firmly bound, inactivated during extraction, or lost during wall isolation. Soluble cytoplasmic proteins (collected as expressed cell sap or as homogenate) showed no activity, while proteins which were salt solubilized in an equal volume from cleaned wall residues taken from the same tissue showed clear extension inducing activity, indicating an association of the extension activity with the wall.

The wall extract from growing cucumber walls did not induce extension of walls from the basal (non-elongating) stem (FIG. 1A). Evidently, during maturation the wall is biochemically modified so that it is not susceptible to extension by this material. Perhaps peroxidative cross-linking of lignin or structural proteins such as extensin is involved in this loss of sensitivity.

The extension activity showed an interesting pattern of species specificity. FIG. 1B shows that the cucumber wall extract was active on various dicot seedlings (pea, radish, cucumber and tomato) and on monocots of the Amaryllidaceae (onion and zephyr lily). In contrast, the extract had a much smaller effect on the coleoptile wall of graminaceous monocots (maize and barley). As graminaceous monocot cell walls differ from those of dicots by having less pectin and-hydroxyproline-rich glycoprotein, and also by having a different type of hemicellulose, it may be that the active fraction extracted from cucumber walls interacts with one or more of these components to induce extension. It may also be that these grass cell walls are cross-linked (not necessarily covalently) in a manner that renders them immune to the cucumber extract. In contrast monocots of the Amaryllidaceae have a cell wall composition more similar to dicots than to the gramineae, and this probably explains their susceptibility to the cucumber derived activity.

The cucumber wall extract was separated by ammonium sulfate precipitation followed by sequential HPLC, shown in FIG. 2, first using a hydrophobic interactions column, where a single peak of activity desiphated as the C3 proteins was obtained, and then using a cation exchange column from which the activity was eluted as two distinct peaks. These fractions we have designated S1 and S2 with respect to their order of elution. FIG. 3 shows the analysis of the active fractions by SDSPAGE which revealed a major band of relative molecular mass 29 kD associated with S1, while S2 contained a major band at 30 kD (FIG. 3). Active extracts

have also been separated by native PAGE and by liquid chromatography with hydroxyapatite, gel filtration media, and DEAE anion exchangers, where activity was consistently associated with these two bands (data not shown). The S1 fraction required only 0.3–1.0 micrograms of protein to reconstitute extension rates similar to that of native extension, while it required 1.0–2.0 micrograms of S2 to do this.

Cucumber expansins induced extension of walls from several dicot and monocot species, but had little effect when assayed with coleoptile walls from maize and barley. Grass coleoptiles have been a favored object of growth studies since Darwin's analysis of phototropism and have been instrumental in many discoveries about plant growth, including the discovery of auxin. The wall composition of grass coleoptiles is notably different from that of dicots and other monocots nevertheless, coleoptile walls, like dicot walls, do exhibit a strong acid-induced extension *in vitro* and *in vivo*. These observations led us to postulate that a wall protein, with functions analogous to the cucumber expansins, might mediate the endogenous acid-induced extension of coleoptile walls. Therefore, in further study we attempted to identify oat coleoptile proteins that could induce wall extension, using the wall extraction and reconstitution approach that proved successful with cucumber walls.

To identify the hypothetical oat proteins responsible for wall extension, we used 1M NaCl to extract proteins from cell walls of etiolated oat coleoptiles. Proteins in this crude extract were precipitated with ammonium sulfate, desalted, and fractionated on a DEAE Sephadex anion exchange column. The unbound proteins passing through this column possessed the ability to induce extension in heat inactivated walls from oat coleoptiles (FIG. 11A). Moreover, this active fraction also induced extension of-heat-inactivated cucumber hypocotyl walls and pea epicotyl walls (FIG. 11A). This result was surprising because earlier results led us to expect poor cross-reactivity between extension proteins and walls from dicots and grass coleoptiles.

FIG. 8 shows an example of acid-induced extension of oat coleoptile walls. When native walls were clamped under constant tension at neutral pH, the extension rate was low 30 min after application of the load and could be greatly increased by replacing the neutral buffer with a buffer of pH 4.5. The wall extension rate decreased continuously with time after the change to acid buffer (FIG. 8B). Addition of 10 mM dithiothreitol, a sulfhydryl reducing agent, increased the extension rate and stabilized it (FIG. 9). This effect is similar to that previously found with cucumber walls (Cosgrove 1989). Inclusion of dithiothreitol throughout the extension period resulted in a simpler decay to a constant extension rate (FIG. 10). Pretreatment of the coleoptile wall with boiling water for 15 sec to inactivate wall proteins eliminated the acid-induced extension (FIG. 8A), suggesting that extension of these walls may be due to a protein-mediated reaction. These observations are consistent with previous reports of acid-induced extension in oat and cucumber walls.

To pursue this point further, we tested the ability of cucumber expansins to induce extension of oat coleoptile walls. As shown in FIG. 11B, they indeed induced extension in oat coleoptile walls, but with less effectiveness than when assayed with cucumber walls. This last point is quantified in FIG. 12A, which compares responsiveness of cucumber and oat walls to a partially purified cucumber expansin fraction. The maximal response of cucumber walls was about three times higher than that of oat walls. To obtain the same

extension response in oat walls as was elicited in cucumber walls by $2 \mu\text{g mL}^{-1}$ of cucumber protein, ten times as much protein was required. FIG. 12B shows that cucumber walls were similarly more sensitive to the active oat extract than were oat walls. The protein-induced extension was stabilized by inclusion of 1–10 mM dithiothreitol in the incubation buffer (data not shown). We also confirmed that cucumber expansins caused very little extension of barley coleoptiles (data not shown). Thus, we conclude that oat coleoptile walls are substantially more responsive in these reconstitution assays than barley coleoptile walls, but less responsive than cucumber walls.

Despite the quantitative differences, these results show that oat and cucumber proteins can induce qualitatively similar extension responses in oat and cucumber walls. We thus infer that a similar biochemical mechanism is involved in acid-induced wall expansion in grasses as in dicots.

One might expect that extraction of frozen/thawed coleoptiles with 1M NaCl might remove most or all of the acid-extension response. FIG. 13 shows that overnight extraction of coleoptiles indeed diminished their acid-extension response. This loss could be due to extraction of the expansins; alternatively, the walls or their proteins may have been modified in some other way so that they lost the acid-extension response. Cucumber walls were previously found to lose their native extension response when they were pre-incubated in neutral pH (Cosgrove 1989). Further work will be needed to differentiate between these and other possible explanations.

To assay the pH dependence of the extractable expansin activity from oats, we used cucumber hypocotyl walls as the "substrate" for measuring the extension activity of oat proteins. Cucumber walls were used because they were easier to prepare, broke less often, had lower baseline extension rates, and proved to be a more sensitive substrate for extension assays than did the walls from oat coleoptiles. The active oat fractions from the DEAE-column had a pH optimum between 4.5 and 5.0 (FIG. 14). At pH 3.5 or 5.5, the activity was reduced by about 50%. In contrast, cucumber expansins displayed a broader pH optimum, with high activity maintained at pH 3.5.

When the active proteins from the DEAE-column were passed through a Concanavalin A (Con A) column, the majority of the extension activity did not bind to the lectin column, suggesting that the activity was not associated with glucosyl- or manosyl- glycoproteins. When the active fractions which passed through the Con A column were further fractionated by HPLC on a carboxymethyl cation exchange column, extension activity was eluted as a single major peak at about 15 min (FIG. 15). The activity of this protein fraction had an acid optimum similar to that shown in FIG. 14 (data not shown). SDS-polyacrylamide gel electrophoresis revealed that a protein with molecular mass of about 29 kD was associated with this active fraction (FIG. 16). We designate this protein as oat expansin-29 (oat Ex29, oEx-29). Generally, expansins are about 25–30 kD in size.

A summary of the purification steps for the oat Ex29 is shown in Table 1. This protein was purified sequentially by ammonium sulfate precipitation, DEAE chromatography, Con A affinity chromatography and CM cation exchange chromatography. If the extension activity in ammonium-sulfate-precipitated proteins is taken as 100%, a purification of S1 fold with 69% yield was obtained after two steps of ion exchange chromatography. Because Con A affinity chromatography did not significantly purify oEx-29, this step was often omitted without significant effect on the purification.

To examine whether expansin activity was also present in the protoplasm of oat coleoptiles, we fractionated the soluble protoplasmic proteins (all proteins in the coleoptile homogenate not bound to the wall) by ammonium sulfate precipitation and DEAE-column chromatography. Little or no activity was detected in any fractions (data not shown), suggesting that the responsible protein was bound to the cell walls of oat coleoptiles.

Because oat Ex29 was capable of catalyzing extension of walls from cucumber, we wished to test whether it is immunologically related to the (apparently) 29-kD cucumber expansin cEx-29. FIG. 17 shows that the oat Ex-29 was specifically recognized by an antibody raised against cucumber Ex-29. Pre-immune serum under the same conditions did not label Ex-29 (not shown). Little signal was detected in the soluble protoplasmic fraction of oat coleoptiles, which is consistent with our other evidence that the Ex-29 is a wall-bound protein. When equal amounts of crude and purified wall protein were assayed by Western analysis, the purified protein gave a much greater signal (FIG. 17). These results demonstrate that the-extension-inducing activity co-purifies with the protein that is antigenically related to cucumber Ex-29.

Our results show that oat coleoptile walls possess a protein that can mediate acid-induced extension of grass coleoptile walls and dicot walls. This protein resembles the cucumber 29-kD expansin in that it induces extension at acid pH but not at neutral pH, its activity is stabilized by dithiothreitol, it has a similar size as judged by SDS-PAGE, and it is specifically recognized by an antiserum raised against the cucumber protein. Because of these similarities between the oat and cucumber expansins, we propose that expansins are evolutionarily conserved proteins that underly at least part of the acid-extension response common to the walls of many plant species.

The similarity between oat and cucumber expansins surprised us because we found that cucumber expansins caused little extension of maize coleoptile walls and no extension of barley coleoptile walls. We confirmed that barley walls were unresponsive to cucumber expansins, but our positive results with oat coleoptiles show that this insensitivity is not a general property of grass coleoptiles. We do not know why barley walls failed to extend in the presence of cucumber or oat expansins.

The similarity between oat and cucumber expansins is also surprising because the matrix components of the wall are believed to be important for wall loosening and extension, yet these components are quite different for grass walls and dicots. The major matrix polysaccharides of the coleoptile wall are (1→3, 1→4)-β-D-glucans and arabinoxylans whereas dicots contain principally xyloglucans and pectins. Dicot walls contain hydroxyproline-rich glycoproteins of the extensin family whereas grass walls contain much lower amounts of such proteins. It may be that expansins interact directly with wall components common to both types of walls (e.g. cellulose or a minor matrix component) or that they can act on different glycans with similar functions, e.g. xyloglucans and (1→3, 1→4)-β-D-glucans.

Although oat Ex29 can induce an acid-dependent extension in coleoptile walls, there are notable differences between the native and reconstituted acid-extension responses in coleoptiles. First, the acid response of native walls includes a large, but transient, burst in extension, which is mostly decayed away by 60–90 min. This burst is largely lacking in oEx29-reconstituted extensions, which

resemble more closely the steady extensions which outlast the transient (e.g. FIGS. 8 and 10). Second, the pH dependence of reconstituted extension (FIG. 14) does not exactly match the pH dependence reported for acid-extension responses of isolated coleoptile walls. The reconstituted extensions displayed a maximum at pH 4.5 and fell off at lower pH values, whereas acid extensions of native coleoptile walls did not fall off at lower pH values. Third, the maximum extension rate inducible with exogenous Ex29 was substantially less than acid-extension responses of native coleoptile walls (i.e. about 2% h⁻¹ for reconstituted extensions versus about 5% h⁻¹ for the stable component of native wall extensions or 20–30% h⁻¹ for the immediate response of native walls).

These differences between the reconstituted and native acid-extension responses of coleoptile walls could indicate that oat coleoptiles possess additional acid-extension processes, other than the one mediated by oEx-29. On the other hand, these differences might also result from inadequacies in our reconstitution methods. For example, heat inactivation of the coleoptile wall by treatment with boiling water may modify the wall's structure so that it is not as susceptible to oEx-29 action. There may also be differences due to poor accessibility of exogenous Ex-29 to its site of action in the wall, or with need for ancillary wall enzymes that are inactivated by heat treatment. Hence, we believe that, at this stage, caution is warranted in interpreting the differences between native and reconstituted extensions.

From the characteristics of oEx-29-induced extension of coleoptile walls and the above considerations, we propose that oat Ex29 is responsible for at least part of the long-term (>1 h) acid-induced extension responses of oat coleoptiles. This view is strengthened by the findings that reconstituted wall extension and endogenous wall extension exhibit similar sensitivities to biochemical activators and inhibitors. In cucumber, exogenous expansins can restore extension rates as high or higher than the long-term extension rates in native walls exposed to acid pH. Although we believe there to be good reasons for thinking that expansins mediate the long-term acid-induced extension of isolated walls, their role in the growth of living tissues has not been directly addressed. This assessment will require experiments in which the action of expansins are specifically inhibited or enhanced. Further studies of the biochemical action of expansins and the genes that encode them may provide the tools for this assessment.

The digestive tract of many animals contain enzymes able to catalyze reactions weakening various chemical bonds. We postulated that the digestive tract of animals which consume green parts of plants may contain proteins of action similar to expansins. We found expansin-like proteins in the digestive tract and feces of the snail *Helix aspersa*. Western blot analysis of the snail proteins probed with the antibody raised against cucumber expansins cEx-29 indicates the presence of an protein antigenically similar to cucumber expansin.

The expansin-like proteins in the snail digestive tract may be synthesized by the snails or by an endosymbiont living in the digestive system of the snail. Such symbionts might be bacteria, fungi, protists or some other microbe.

We assayed the effects of expansins on the mechanical properties of pure cellulose paper, which derives its strength principally from hydrogen bonding between cellulose microfibrils. Because paper is much simpler in composition and structure than plant cell walls, the action of expansins could be much easier to interpret. S1 protein showed clear effects on the mechanical properties of Whatman number 3 filter paper in both extensometer and stress relaxation

assays. As our experiments show expansins are also able to disrupt slick paper (FIG. 7).

Expansins appeared to weaken cellulose paper in a manner different from that effected by cellulase. While both expansins and cellulase led to breakage of the paper in extensometer assays, only expansins showed a clear enhancement of stress relaxation in this material. Furthermore, incubations of paper with expansins did not lead to the release of reducing sugars, as was seen in incubations with cellulase. Because expansins did not hydrolyze the paper, and because hydrogen bonding accounts for the tensile strength of paper we conclude that expansins disrupt the hydrogen bonding between cellulose fibrils. Expansins probably do not catalyze cell wall loosening by disrupting hydrogen bonds directly between microfibrils, as these structures appear to be separated from one another by matrix polysaccharides which are thought to coat the outside of microfibrils in the wall. However, it is possible that they could induce wall extension by disrupting hydrogen bonding between the matrix polysaccharides and the microfibrils, thus reducing the resistance to movement of the microfibrils.

Stress relaxation assays performed to test the ability of the snail expansin-like protein to weaken the bonds in the paper indicate that this ability is attributed to all expansins and expansin-like proteins.

In addition to the above, we have cloned, isolated and fully sequenced a cDNA coding for the cucumber cEx-29 (S1 fraction) expansin protein. The sequence is disclosed both as the nucleic acid sequence SEQ ID NO: 1 and as the corresponding amino acid sequence SEQ ID NO: 7. Five other amino acid sequences are also disclosed, two from rice and three from *Arabidopsis*, SEQ ID NO: 2 through SEQ ID NO:6, respectively. DNA sequences can be used in expression systems to obtain large quantities of expansin(s) for commercial applications. Moreover, the DNA sequences may be modified to obtain expansins with improved properties such as different pH dependence, stability, substrate specificity, activity, and so on. The DNA sequences may be used to identify and to isolate other naturally-occurring expansin genes with improved or novel properties, and the corresponding proteins expressed by such genes.

An expansin, according to the present invention, is a purified protein generally having greater than about 60% sequence similarity and preferably greater than about 70% sequence similarity with the amino acid sequence disclosed in SEQ. ID. NO:7. In a corresponding manner, the applicable DNA sequence the expresses an expansin will have about 60% sequence similarity, and preferably greater than about 70% sequence similarity, with the nucleic acid sequence disclosed in SEQ. ID. NO:1. The amino acid sequences identified in SEQ. ID. NO:2 through SEQ. ID. NO:6 are examples of sequences which meet the sequence similarity definition and thus are examples of expansins. Expansins equivalent to those described by this definition can be identified by their expansin characteristics, as explained throughout this specification, and all expansins are thus believed to be patentable equivalents.

EXAMPLE 1

Preparation of Plant Materials

Seeds of cucumber (*Cucumis sativus* cv Burpee Pickler) were sown on Seed Germination Kimpack Paper K-22 (Seedburro Equipment Corporation, Chicago, Ill.) soaked with distilled water, in flats, 50x25x6 cm, with lids of the

same dimensions. Seedlings were grown in the dark for 4 days at 27° C. The apical 3 cm of hypocotyl was excised and frozen at -20° C. for no more than 5 days and prepared for creep measurements as previously described (Cosgrove 1989). For bulk wall extractions, the apical 3-cm hypocotyl regions were collected on ice water and homogenized with 25 mM sodium acetate, 2 mM EDTA, pH 4.5 in a Waring blender. The wall fragments were collected and washed twice by filtration through Miracloth and subsequently used for protein extraction. Basal walls were from the lower 6 cm of the (15 cm long) hypocotyls.

Oat seedlings were grown in moist vermiculite in complete darkness at 27° C. Except as noted, coleoptiles were from 4-day old oat seedlings (*Avena sativus* L. cv. Olge, from Carolina Biological Supply, Burlington, N.C., USA).

Seedlings were quickly harvested under room lights. For wall extension assays, the apical 2 cm region of the growing stem or coleoptile was excised, sealed in aluminum foil and frozen at -20° C. prior to use. Coleoptiles were separated from primary leaves. Cuticles were abraded by rubbing the coleoptiles or stems between two fingers coated with a slurry of carborundum (320 grit, well washed prior to use; Fisher Scientific, Fair Lawn, N.J.). For oat coleoptiles, the cuticle was generally abraded prior to freezing, whereas for cucumber and pea stems the frozen segment was quickly abraded. In some instances as noted, coleoptile cuticles were removed by stripping the epidermis from the tissue with fine forceps and the remaining coleoptile cylinder was bisected longitudinally prior to freezing. Tissues were thawed, pressed under weight for 5-10 min to remove tissue fluids and clamped in an extensometer (5 mm between the clamps, corresponding to the apical 3-8 mm of the stem or coleoptile).

Seeds of pea (*Pisum sativum* cv Alaska), radish (*Raphanus sativus* cv Crimson Giant), tomato (*Lycopersicon esculentum* cv Rutgers), onion (*Allium cepa* cv Snow White), maize (*Zea mays* cv B73xMo17), and barley (*Hordeum sativum* cv Barsoy) were sown on vermiculite wetted with distilled water and grown for 4 days in the dark at 27° C. Hypocotyls of pea, radish and tomato; primary leaves of onion; and coleoptiles of maize and barley were excised and frozen for subsequent extension experiments. Young growing leaves of zephyr lily (*Zephyranthes candida*) were removed from a greenhouse-grown colony of plants which were kept in the dark at 27° C. for 12 hr prior to harvest, and frozen for later extension assay.

EXAMPLE 2

Isolation of Expansins cEx-29 and cEx-30 from Cucumber Protein Extraction

Washed cucumber cell wall fragments (from 150-200 g of tissue) were extracted overnight in 20 mM Hepes, pH 6.8, 1M NaCl at 4° C. Cell wall fragments were filtered on Miracloth and the salt-solubilized fraction precipitated with ammonium sulfate (the activity precipitated between 20 and 60% saturation with $[(\text{NH}_4)_2\text{SO}_4]$). The precipitate was desalted on a 7-mL column of Bio-Gel P2 (BioRad Laboratories) into 50 mM sodium acetate, pH 4.5. Protein concentration was 2 to 4 mg/mL, estimated by Coomassie Protein Assay Reagent (Pierce, Rockford, Ill.).

For the comparison of soluble and wall associated proteins, 100 g of tissue was harvested and homogenized with 100 mL of 25 mM sodium acetate, pH 4.5, 1 mM EDTA. Wall fragments were filtered out and the remaining solution designated as the soluble fraction. Wall fragments were cleaned as described above, and then extracted in 200

mL of mM Hepes, pH 6.8, 1M NaCl for one hour at 40° C. Aliquots of both solutions were dialyzed against 50 mM sodium acetate pH 4.5 and tested for reconstitution activity, as described below.

Protein Fractionation

The 20 to 60% (NH₄)₂SO₄ precipitate pellet was resuspended in 2 mL of distilled water and insoluble material was removed by centrifugation and filtration through a Centricon 30 microconcentrator (Amicon, Beverly, Mass.) prior to being loaded onto a C3 hydrophobic interactions column (ISCO C-3/6.5 μm 10×250 mm) equilibrated with 50 mM sodium acetate, pH 4.5, 20% (saturation) (NH₄)₂SO₄. Proteins were eluted from the column with a linear gradient from the equilibration buffer into 50 mM sodium acetate, pH 4.5, in 35 min. at a flow rate of 1 mL/min. Fractions were desalted on a 7-mL column of Bio-Gel P-2 (Bio-Rad Laboratories, Richmond, Calif.) into 50 mM sodium acetate and assayed for their ability to reconstitute extension to inactivated cucumber hypocotyl sections.

The active fractions from the C3 column were pooled and subsequently desalted and concentrated on a Centricon 30 microconcentrator (Amicon, Beverly, Mass.), the buffer being exchanged for 15 mM Mes NaOH, pH 6.5. The concentrated sample was then loaded (in a volume of 1.7 mL) onto a sulfoethyl cation exchange column (Bio-Rad HRLC MA7S 50×7.8 mm) equilibrated with 15 mM Mes NaOH, pH 6.5, and proteins eluted with an ascending gradient of NaCl (from 0 to 1.0M over 45 min) in the same buffer at a flow rate of 1 mL/min. A₂₈₀ nm of eluting proteins was measured using a Dionex Variable Wavelength Detector (VDM-2).

Active fractions from ammonium sulfate precipitation, C3 column and sulfoethyl fractions S₁ and S₂ were concentrated, desalted on Centricon 30 Microconcentrators, and then run on SDS-polyacrylamide gels, according to the method of Laemmli. Gels were subsequently stained with Coomassie Brilliant Blue R250. Fractionation by this procedure was repeated more than 10 times with similar results. Results are presented in FIG. 2.

EXAMPLE 3

Isolation of Expansins oEx-29 from Oat Protein Extraction

For oat protein extraction, oat seedlings were rapidly cut under room lights and placed in ice water. The apical 2.5 cm (+1–0.5 cm) of each coleoptile was then cut, separated from the primary leaf, and placed on ice while the other coleoptiles were harvested. About 500 coleoptiles were homogenized in 200 mL of 10 mM sodium phosphate, pH 6.0. In some instances the coleoptiles were collected in lots of 100–200 and frozen (–20° C.) for 1 to 3 d prior to homogenization. The homogenate was filtered through a nylon screen (70 mm mesh), and the cell walls were collected and washed 4 times by resuspending in the homogenization buffer (300 mL) followed by filtration. Ionically-bound proteins were extracted for at least 1 hour at 4° C. with 50 mL of 1M NaCl containing 20 mM Hepes (pH 6.8), 2 mM EDTA and 3 mM sodium metabisulfite. Wall fragments were removed by filtration or centrifugation and the wall proteins in the supernatant were precipitated with ammonium sulfate (0.4 g added to each mL). Precipitated proteins were dissolved in 1.5 mL of water and desalted on an Econo-Pac 10DG desalting column (BIO-RAD Laboratories, Richmond, CA, USA) which was equilibrated with 20 mM

Tris-HCl, pH 8.0, containing 100 mM NaCl. Without NaCl the active proteins tended to bind to the desalting column, resulting in a lower recovery.

Protein Fractionation

Protein solution from the desalting column was centrifuged in a microcentrifuge for 3 min to remove precipitates. Proteins were then loaded onto a DEAE-column (Sephadex A-25, Sigma) equilibrated with 20 mM Tris-HCl, pH 8.0, containing 100 mM NaCl at 25° C. The proteins bound to the DEAE-column were eluted by 1M NaCl in 20 mM Tris-HCl buffer, pH 8.0.

A 1-mL Concanavalin A column (Sigma) was equilibrated with 200 mM NaCl containing 1 mM each of Mg²⁺, Ca²⁺, and Mn²⁺. Proteins from the DEAE-column were passed through this column. After the column was washed extensively with the same solution, the bound proteins were eluted with the same solution containing 6.5M α-methyl mannoside. Extension activity was associated with the fractions that did not bind to the column.

Active fractions were further separated by HPLC using a carboxymethyl (CM) cation exchange column (4.6×250 mm, CM30016.5 mm, ISCO, Nebraska) equilibrated with 10 mM Mes, pH 5.5. Before the protein sample (1 mL) was loaded on the column, the sample buffer was exchanged for 10 mM MES, pH 5.5, by use of a 30-kD Centricon microconcentrator (Amicon, Beverly, MA, USA). Proteins were eluted from the CM column at a flow rate of 1 mL/min with a gradient of 0 to 0%, 0 to 4%, 4 to 6% and 6 to 100% of 1.0M NaCl in 10 mM MES, pH 5.5, from 0 to 5 min, 5 to 10 min, 10 to 30 min and 30 to 50 min, respectively, and detected by absorbance at 280 nm.

Proteins were quantified colorimetrically using the Coomassie Protein Assay Reagent (Pierce, Rockford, Ill., USA). For SDS-PAGE (Laemmli 1970), proteins were separated on a 14% polyacrylamide gel or a 4–20% gradient polyacrylamide gel (Bio-Rad Ready Gel, Richmond, Calif., USA). For Western analysis, proteins were electrophoretically transferred to a nitrocellulose membrane in a solution of 192 mM glycine, 25 mM Tris, 20% methanol (v/v) at 10 volt/cm for 3 h or in some cases 16 h. After the membrane was blocked with 3% bovine serum albumin in phosphate buffered saline containing 0.05% Tween-20 (PBST), it was incubated for 2 hr in PBST containing antiserum (1:3000 dilution). The membrane was washed 4 times with PBST and then incubated for 1 h with goat anti-rabbit IgG-conjugated alkaline phosphatase (Sigma; dilution of 1:4000) in PBST. The Western blot was developed using bromochloroindolyl phosphate/nitro blue tetrazolium and the reaction was stopped with 10 mM EDTA.

Table 1. Purification of oat Ex29 from etiolated oat coleoptiles. Activity was assayed as described in FIG. 4 and expressed as the initial increase in the extension rate of isolated cucumber cell walls upon addition of the protein fraction (e.g. 5 to 30 min after protein addition). Total activity was calculated by dividing the activity (measured in 1–3 extension assays) by the fraction of protein used for each assay. Specific activity was calculated by dividing the total activity by the total protein.

| Purification Step | Total Protein | Total Activity units ($\mu\text{m}/\text{min}$) | Specific Activity (units/mg protein) | Purification (fold) | Yield (%) |
|--|---------------|---|--------------------------------------|---------------------|-----------|
| $(\text{NH}_4)_2\text{SO}_4$ precipitation | 444 | 123 | 278 | 1 | 100 |
| DEAE | 190 | 104 | 546 | 2 | 84 |
| CM-HPLC | 6 | 85 | 14278 | 51 | 69 |

Soluble protoplasmic proteins were obtained by homogenizing oat coleoptiles in 10 mM sodium phosphate buffer (in some cases, 10 mM Hepes, pH 6.8) and centrifuging at 4° C. at 26,000 g for 10 min to remove particulate matter.

EXAMPLE 4

Preparation of Expansin-like Proteins from Snails and Snail Feces

Acetone powders of the visceral humps of *Helix pomatia* were purchased from Sigma Chemical Company. Live snails (*Helix aspersa*) were purchased from NASCO. Protein solution was made by dissolving snail acetone powder (10 mg/ml) in 50 mM sodium acetate buffer, pH 4.5. Feces of *Helix aspersa* were dissolved in the same buffer.

EXAMPLE 5

Extension Measurements

Extension measurements were performed using a constant load extensometer as described by Cosgrove (1989). Briefly, frozen/thawed tissues were abraded for a short time with carborundum to disrupt the cuticle, boiled in water for 15 sec (for reconstitution assays), and secured between two clamps (with about 5 mm between the clamps) under a constant tension of 20 g force (for assays with radish, tomato and onion tissues the tension was reduced to 10 g). Plastic cuvettes were fitted around the walls and filled with bathing solution. For reconstitution assays the bathing solution was first 50 mM sodium-acetate pH 4.5 for 30 min., followed by the protein fraction to be assayed in the same, or equivalent pH buffer. Movement of the lower clamp was detected with a position transducer and recorded on a microcomputer. All extension assays reported here were repeated at least five times, except for the heterologous reconstitutions (e.g. with walls from pea, tomato and corn), which were performed three to five times.

Cucumber Expansins Activity

Extension induced by cucumber expansins is presented in FIGS. 1, 11, 12.

Oat Expansins Activity

Extension induced by oat expansin is presented in FIGS. 11 and 12.

Snails Expansins Activity

Extension induced by snail expansin-like proteins are presented in FIGS. 18 and 19. The experiment was performed as described for cucumber and oat proteins.

The protein solution obtained from snail acetone powder by dissolving snail acetone powder (10 mg/mL) into 50 mM sodium acetate, pH 4.5, is capable of inducing extension of isolated cucumber walls.

EXAMPLE 6

Effects of DTT and Metal Ions on Endogenous Acid Induced Wall Extension

Endogenous acid-induced extension was previously shown to be sensitive to a number of exogenously applied factors (Cosgrove D.J., *Planta* vol. 177, pp 121-130, 1989). The thiol reducing compound dithiothreitol (DTT) was shown to stabilize or even enhance the extension of isolated cucumber cell walls at acid pH. To see if this was a characteristic of our extension inducing proteins, we first reconstituted wall specimens with an active C3 fraction from cucumber, as shown in FIG. 4a, stems were allowed to extend for one hour at which point sufficient DTT was applied to bring the bathing solution to 10 mM. Almost immediately the rate of extension is increased and thereafter remained constant, whilst reconstituted sections without the addition of DTT typically show a gradual decline in the rate of extension.

In contrast heavy metal ions including copper and aluminum were shown to be strongly inhibitory to endogenous acid-induced extension (Cosgrove 1989). Again, in FIG. 4a, boiled stem tissue reconstituted with C3 proteins showed very clearly the same sensitivity to an application of these ions, the rates of extension falling rapidly to the rate seen in the inactivated tissue before reconstitution.

EXAMPLE 7

Effects of Boiling in Methanol or Water on the Activity of Cucumber Expansins

An unusual property of the endogenous cell wall extension activity is its ability to survive boiling in methanol (Cosgrove, 1989). We found that methanol-boiled walls retained extractable activity, whereas extracts from cucumber tissues boiled in water do not exhibit this activity, shown in FIG. 4a. Fractionation of the active extract from methanol boiled walls by the methods given above showed it to contain the 29- and 30-kD proteins in the S1 and S2 fractions (data not shown). The ability of the activity to survive boiling methanol may be due to the small size of these proteins and to protection within their carbohydrate-rich sites within the cell wall.

Protein concentrations were estimated using Coomassie Protein Assay Reagent (Pierce, Rockford Illinois) using a standard curve constructed using BSA (Pierce). Wall specimens inactivated by boiling were preincubated with S1 and S2 proteins at estimated concentrations of 5 micrograms per mL and then extended in the extensometer, as described above, in 50 mM sodium acetate pH 4.5. After 1 hr enough dithiothreitol (DTT), from a 100 mM stock, was added to bring the bathing solution to 10 mM DTT.

To study the effect of heavy metal ions wall specimens were extended initially in 50 mM sodium acetate, pH 4.5. After 20 minutes the bathing solution was exchanged for one containing an estimated 50 micrograms of active C3 proteins. One hour after the start of the experiment $\text{AlCl}_3 \cdot 6\text{H}_2\text{O}$ or $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$, from 100 mM stocks, were supplied to give a final concentration of 1 mM.

For both treatments 100 g (fresh weight) of apical tissue was harvested and either boiled for 30 seconds in 1 L of distilled water, or for three minutes in 1 L of Methanol. Methanol boiled tissue was allowed to dry before being resuspended in 50 mM sodium acetate, pH 4.5. Both sets of tissue were then homogenized, cleaned and salt extracted, as described above. Salt extracts were precipitated with (NH_4)

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$_2\text{SO}_4$, resuspended and desalted, and tested for reconstitution activity as described above.

EXAMPLE 8

Effect of Protease Treatments on the Activity of
Cucumber Expansins

Cosgrove (1989) also showed that endogenous wall extension was eliminated by incubation with proteases, one of the initial clues that extension was catalyzed by proteins. The proteinaceous nature of our extension inducing factors was indicated by the sensitivity of reconstituted extension to protease incubations. This data is presented in FIG. 4b; 4-hr incubations with chymotrypsin, papain, pronase and trypsin all reduced extension to rates seen in inactivated tissues.

Inactivated stem sections were incubated with active C3 proteins, at an estimated concentration of 50 micrograms per mL, for 30 min at 25° C. Reconstituted stems were then incubated with 1000 units of trypsin or 1000 units of chymotrypsin in 50 mM Hepes-NaOH, pH 7.3 for 4 hr at 30° C., and with pronase (2 mg/mL), or papain (2 mg/mL) in 50 mM sodium acetate, pH 5.0, 1 mM DTT for 4 hr at 30° C. For a control C3 proteins were incubated at 30° C. for 4 hr without the addition of proteases. After incubation the stem sections were assayed for extension, initially in 50 mM Hepes, pH 6.8, after 30 min the buffer was switched to 50 mM sodium acetate, pH 4.5. The difference in the rates of extension (pH 4.5–pH 5.5) was calculated. All experiments were repeated four times.

EXAMPLE 9

Effects of pH on Reconstituted Extension Induced
by Cucumber Expansins

Reconstitution experiments were carried out at a range of values of pH, results presented in FIG. 5 show that there is little or no induction of wall extension by the C3 proteins until between pH 5.5 and pH 5.0 and that extension activity has a maximum somewhere between pH 4.5 and pH 3.5. The region of highest response to changes in pH lies in the range pH 5.5 and pH 4.5. These values are within the range of apoplastic pH values reported in elongating tissues, and supports the possibility that these proteins could play an important role in the acid-growth response.

Buffers in the range pH 3.0 to pH 6.5 were prepared by titrating 50 mM citric acid with 1M K_2HPO_4 . Inactivated stem sections were suspended in the extensometer in buffer of appropriate pH. After 30 min of extension the bathing solution was replaced by a 1:1 dilution of C3 proteins (estimated final concentration 50 micrograms per mL) with the appropriate buffer, where necessary the pH was adjusted using either 1M citric acid or 1M K_2HPO_4 . Change in rate of extension was calculated as the difference in extension rates before and after addition of proteins. All experiments were repeated eight times.

EXAMPLE 10

Effects of Cucumber Expansins on Cellulose Filter Paper Strips of Whatman number 3 filter paper (Whatman Lab Sales, Hillsboro Oreg.) (2 mm by 10 mm) were cut and were clamped in the constant load extensometer as described for cucumber hypocotyl sections. Extension was measured in 50 mM sodium acetate and in the same buffer containing protein fractions. Additionally, effect of expansins on the loss of mechanical strength of paper was measured by stress relaxation assay. For stress relaxation measurements, the

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paper was incubated in various pretreatment solutions and assayed while still wet, in the standard method.

In extensometer assays S1 protein induced a brief period of extension prior to rapid breakage of the paper, an effect not seen in incubations with buffer alone or with boiled protein. Incubations of paper strips with cellulase showed a similar effect. However, it required at least 100 mg of cellulase to weaken the paper sufficiently to be detected in this assay, whereas only 5 mg of S1 protein produced similar effects. In parallel experiments, cellulase (100 mg/mL) was shown to release 25 mmol reducing equivalents per mL during a five h incubation with Whatman No. 3 paper. In contrast, no sugar release was detectable in similar assays using S 1 and S2 protein (both 5 mg/mL). The results are presented in FIG. 6.

S1 protein (5 mg/mL) caused a marked increase in the relaxation rate of paper strips, while cellulase (100 mg/mL) had almost no effect. In both extensometer assays and stress relaxation tests, S1 protein which had been boiled for two minutes did not induce the effects apparent with native S1 proteins, indicating that the effects were associated with the activity of the protein. Similar results were obtained in assays with S2 proteins (data not shown).

These data indicate that expansins substantially weaken cellulose paper in a manner which does not involve the hydrolysis of cellulose. Since the structural integrity of cellulose paper appears to result from hydrogen bonding between cellulose fibrils, it seems likely that these proteins weaken paper by disrupting these associations.

EXAMPLE 11

Effect of Cucumber Expansins on Slick Paper

In order to test whether a protein preparation containing cucumber expansins can disrupt slick paper, in a similar fashion as it acts on pure cellulose paper the following experiments were conducted. Slick paper was obtained from three sources: (a) the pages of "Nature" magazine (unprinted margins were used); (b) a commercial catalog for chromatography equipment; and (c) a colored advertising insert (Hills) in the Sunday paper. Paper strips of 2–3 mm width were cut and hung in our extensometer, such that a 5-mm long strip was put under 20-g force. The buffer contained 50 mM sodium acetate, pH 4.5 with or without added C3 protein (about 20 ug per paper strip was used). The length of the paper strips was recorded for up to 5 h. Four samples of each paper were used with each treatment (+1-protein). The untreated (control) paper showed very little change in length, and maintained its mechanical strength throughout the test period. In contrast, the treated papers began to extend and quickly broke once extension began. The time for breakage after addition of protein varied from 45 min to 2.5 h, but all samples broke. FIG. 7 illustrates the typical extension.

EXAMPLE 12

Effect of Snail Expansin-like Proteins on Cellulose
Paper

A stress relaxation assay was performed as described in Example 10. Increased relaxation in time domains of 10–100 msec (faster relaxation in comparison with controls) proved the ability of snail expansin-like proteins to weaken the mechanical strength of paper.

EXAMPLE 13

Preparation of Antibodies to Cucumber Expansin
cEx-29

Antiserum with specific recognition of this protein was raised in a female New Zealand White rabbit by subcuta-

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neous injections of cucumber Ex29 with Freund's adjuvants (Harlow and Lane 1988). Serum dilutions in the range of 2000:1 to 4000:1 proved optimal for Western analyses of cucumber and oat proteins.

EXAMPLE 14

Western Blot Analysis of Immunoreactivity
between Cucumber and Oat Expansins

We performed western analysis of oat coleoptile proteins probed with antiserum against cucumber Ex29. Results are presented in FIG. 17. Comparison of lane 1 (crude wall protein) with lane 2 (crude protoplasmic protein) shows that the antiserum recognizes an Ex29-like protein (arrow) specifically bound to the coleoptile cell wall. Comparison of lane 3 (crude wall protein) with lane 4 (purified protein with extension activity) shows that the Ex29-like protein co-purifies with the extension activity. Lane 1 was loaded with 15 μ g of crude wall protein (ammonium sulfate precipitate of 1M NaCl extract). Lane 2 had 15 μ g of the soluble protoplasmic protein fraction. These samples were separated on the same 4–20% gradient SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with rabbit antiserum against cucumber Ex29. Lane 3 was loaded with 0.2 μ g of crude wall protein and Lane 4 had 0.2 μ g of active oat wall-extension protein purified sequentially by DEAE Sephadex and CM-HPLC. These samples (3–4) were separated on the same 14% SDS polyacrylamide gel, blotted onto nitrocellulose, and probed with antiserum. This assay, with minor variations, was carried out four times with similar results.

EXAMPLE 15

Western Blot Analysis of Immunoreactivity
between Expansin-like Protein from Snail and
Cucumber Expansin cEx-29.

FIG. 20 presents a Western blot of active HPLC-separated protein fractions from snail acetone powder, probed with antibody PA1 which was raised against cucumber expansin-29. The active fractions show a striking band at about 26 kD, which is similar to (though slightly smaller than) cucumber expansin-29. This results provide strong evidence that the wall extension activity found in the snail acetone powder is due to a protein with similar antigenic determinants as cucumber expansin-29.

FIG. 21 presents a Western blot of the proteins from *Helix aspersa feces*, probed with the antibody PA-I. The single band indicates the presence of a protein antigenically similar to cucumber expansin-29.

EXAMPLE 16

Effects of Urea on Plant Cell Wall Extension

This example shows the effects of 2M urea on native cell walls, walls reconstituted with expansin, and heat inactivated walls. Native cell walls or heat-inactivated cell walls from cucumber hypocotyles were obtained as earlier described and clamped in the extensometer in 50 mM sodium acetate at pH 4.5. At about 20 minutes, an S1 fraction of cucumber expansin (10 μ g/mL) was added to one set of heat-inactivated walls. At about 45 minutes the incubation solutions were replaced with 2M urea buffered with 50 mM sodium acetate, pH 4.5. The aforesaid solutions also contained 2 mM dithiothreitol to stabilize the expansin activity. Results of this experiment are presented in FIG. 22.

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Data are representative of four (4) trials for each respective treatment. The dotted line of FIG. 22 represents native cucumber cell walls.

These data show that 2 mM urea acts synergistically with expansins to enhance the extension rate of native cucumber cell walls and heat-inactivated cell walls reconstituted with purified expansins. As indicated in the trace labeled "heat-inactivated" in FIG. 22, urea alone (without active expansins in the cell walls) has little effect.

These results support the contention that expansins disrupt hydrogen bonding between cell wall polymers.

EXAMPLE 17

Identification of Expansins in Various Plant
Materials

Using the methods earlier described herein for extraction of expansins from cucumber and oat materials, quantities of the following plant materials were treated:

- (i) broccoli flower stalks (*Brassica oleracea italica*);
- (ii) celery petioles (*Apium graveolens*);
- (iii) tomato leaves (*Lycopersicon esculentum*);
- (iv) cotton fibers (*Gossypium*); and
- (v) corn coleoptiles (*Zea mays*).

Expansins were identified and confirmed by use of the antibody assay described herein (having common antigenic epitomes with the antibody against Ex29), and the extension activity analysis described herein.

Summary and Conclusions

In summary, cucumber expansins appear to associate with the cellulose fraction of the cell wall. They do not exhibit polysaccharide hydrolysis under a number of assay conditions and they do not cause a regressive weakening of the wall. Expansins also appear to disrupt hydrogen bonds as particularly noted with cellulose paper.

Isolation of new proteins with new activities often opens new possibilities of application of same. Although the potential applications in paper industry have been emphasized in this disclosure, numerous other directions of use can be imagined. For example expansins could be used for processing of polysaccharides for control of physical properties. Hydrogen bonding is an important determinant of many physical properties of commercial products containing polysaccharides. Expansins may be incorporated into the polysaccharide products to modify hydrogen bonding and thereby modify the physical characteristics of the products. Examples include control of the viscosity and texture of polysaccharide thickeners used in foods and chemical products; control of stiffness and texture of paper products; and control of mechanical strength (e.g. tear strength) of paper products.

The present invention is believed broadly applicable to alteration of various physical properties of polysaccharides. While plant polysaccharides represent a preferred embodiment of the invention, expansins of the invention are believed useful catalytic proteins for the treatment polysaccharides from a variety of sources (i.e., synthetic, bacterial or other microbial system, etc.). While it is reasonable to make a strong claim that expansins primarily operate by disruption of hydrogen bonds, the invention is not necessarily limited to this mode of action. Results concerning altered physical properties of polysaccharides are nonetheless produced by use of the novel expansins of the invention.

Expansins may be used for de-inking paper, which is a significant limitation in current paper recycling operations.

In this application, expansins may help loosen the bonding between surface polymers, which are stuck to the ink, and the remainder of the cellulose fibers. Also, in the paper industry expansins may prove useful for large scale paper dissolution, or perhaps for alteration of the mechanical properties of dry paper. Treatment of dry paper could produce paper with novel properties.

Expansins may be used in combination with other chemicals or enzymes to improve various processes. For example, a major limitation in ethanol production from biomass is the degradation of cellulose. Expansins in concert with cellulose may act synergistically in the breakdown of cellulose. If expansins help celluloses gain access to glucan chains that make up microfibrils, then they could speed cellulose action. Also, delignification is a major problem in industrial uses of many plant fibers. To solve this concern, expansins may be used with lignases (peroxidases) for synergistically de-lignifying plant fibers. Expansins may also be useful in various bioremediation applications, either above or in combination with other biological or chemical materials.

Expansins have been found in cotton fibers and are probably found in native cell walls, but are likely not to be present in processed cotton fiber walls. Accordingly, expansins may be useful to the textile industry by virtue of alteration of either the wet or dry mechanical properties of cotton flax, or other natural fibers.

It is known that certain fungal cell walls (i.e., *Phycomyces*) bear chemical resemblance to the structure of plant cell walls. Also, certain insects have chitin structures or walls (i.e., *Manduca* cuticle) that resemble fungal walls in some of their biochemistry. Expansins may modify chitin properties. Accordingly, expansins may be useful for insect control or as anti-fungal agents, either alone or in combination with other insecticidal or fungicidal agents.

A further use of expansins could be for altering the mechanical strength of gels or otherwise affecting the gelling or other properties of gels (i.e., gelatin, gellum-gum/phytagel, agar, agarose, etc.). Such material are useful agents in foods, cosmetics, and other similar materials. Since hydrogen bonding is important for such gels and in view of the belief that expansins alter hydrogen bonding of wall glycons, expansins may alter the gelling properties of various gels.

An additional use of expansins may involve alteration of aggregation of hemicellulose and solubilized cellulose. In the event that expansins appropriately affect such aggregation, they may prove useful for industrial processes involving these materials, including cellulose processing and film making.

Isolation of a novel protein allows one to attempt the cloning of the gene coding for this protein using the standard approach of establishing amino acids sequence of a fragment of the protein and designing oligonucleotides to screen the CDNA library. When cloned, the gene for one or more expansins will need to be expressed in a bacterial or other system to obtain sufficient quantities for the commercial usefulness of the ideas listed above. Cloning will also be a necessary first step for the commercial uses requiring genetic manipulation of the protein in transgenic plants.

Engineering of plant cell growth for agricultural uses can become reality. We have evidence that expansins are important for endogenous growth of plant cells. One can envision several commercial applications of expansins. One such application includes increase of the growth of specific organs and tissues by selective enhanced expression of expansins during plant development. Control of selective

expression would be by any means such as application of specific chemicals that promote transcription of expansins or by insertion into the genome of plants specific artificial genetic constructs (i.e. appropriate promoters, enhancers, structural genes and associated elements) to effect organ-specific, tissue-specific, and/or chemical-specific enhancement of expression of the introduced or endogenous genes for the expansin proteins. Examples of use might include enhancement of fruit production (grapes, citrus fruits, etc.), enhancement of leaf growth (lettuce, spinach, cabbage), enhancement of stem or petiole growth (sugarcane, celery, flower stalks), and enhancement of root growth. Tissues engineered to grow by expression of expansins might have enhanced desirable traits (size, succulence, texture, durability).

Another direction of controlled growth would include decrease of the growth of specific organs and tissue by reducing the expression or effectiveness of endogenous expansins. The control of expression would be by any means such as application of specific chemicals that reduce transcription of expansins or by insertion of antisense genetic constructs that reduce mRNA levels of endogenous expansin genes, or by manipulation of the genetic control elements that regulate expression of endogenous expansin genes. Examples of such use might include dwarfing of stems for enhanced mechanical stability and genetic pruning, stunting, or elimination of undesirable plant organs.

Similar approaches could be utilized to control the cell size in plant cell tissue or cell cultures used in bioreactors or for production of useful chemical agents. Control might be effected by addition of exogenous expansins or inhibitors of expansin action, by genetic regulation of endogenous expansin genes and their products, or by regulation of artificially inserted genes for expansin proteins, for antisense constructs against endogenous expansin mRNAs, or for proteins that regulate or modify expansin action.

Thus, while we have illustrated and described the preferred embodiment of our invention, it is to be understood that this invention is capable of variation and modification, and we, therefore, do not wish or intend to be limited to the precise terms set forth, but desire and intend to avail ourselves of such changes and alterations which may be made for adapting the invention of the present invention to various usages and conditions. Accordingly, such changes and alterations are properly intended to be within the full range of equivalents and, therefore, within the purview of the following claims. The terms and expressions which have been employed in the foregoing specification are used therein as terms of description and not of limitation, and thus there is no intention in the use of such terms and expressions of excluding equivalents of features shown and described or portions thereof, it being recognized that the scope of the invention is defined and limited only by the claims which follow.

Thus is described our invention and the manner and process of making and using it in such full, clear, concise, and exact terms so as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same.

SEQUENCE LISTING

<160> NUMBER OF SEQ ID NOS: 7

<210> SEQ ID NO: 1
 <211> LENGTH: 681
 <212> TYPE: DNA
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: cDNA
 cucumber expansin

<400> SEQUENCE: 1

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gactacggtg gctggcagag cggccacgcc accttttatg gtgggtgtga cgcactctggc      60
accatgggtg gagcttctgtg gtatgggaat ttatacagcc aagggtatgg cacgaacacg      120
gtggcgctga gcactgcgctt atttaacaat ggattaagtt gtgggtgcttg cttcgaaatg      180
acttgtaaca acgaccctaa atgggtgcctt ccgggaacta ttagggtcac tgccaccaac      240
ttttgccttc ctaactttgc tctccctaac aacaatggtg gatggtgcaa ccctcctctc      300
caacactctg acatggctga gcctgccttc cttcaaatcg ctcaataccg agctgggtatc      360
gtccccgtct cctttcgtag ggtaccatgt atgaagaaag gtggagttag gtttacaatc      420
aatggccact catacttcaa cctcgttttg atcacaacac tgggtggcgc aggcgacgctc      480
cactctgtgt cgataaaggg gtctcgaact ggatggcaat ccatgtctag aaattggggc      540
caaaactggc aaagcaacaa ctatctcaat ggccaaggcc tttcctttca agtcactcct      600
agtgatggtc gcactctcac tgcctataat ctcgttcctt ccaattggca atttgccaa      660
acctatgaag gcctcaatt c                                     681
    
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<210> SEQ ID NO: 2
 <211> LENGTH: 228
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: rice
 expansin
 <220> FEATURE:
 <221> NAME/KEY: UNSURE
 <222> LOCATION: 211
 <223> OTHER INFORMATION: Xaa is unknown or other.

<400> SEQUENCE: 2

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Ala Gly Gly Gly Trp Val Asn Ala His Ala Thr Phe Tyr Gly Gly Gly
  1           5           10          15
Asp Ala Ser Gly Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr
  20          25          30
Ser Gln Gly Tyr Gly Thr Asn Thr Ala Ala Leu Ser Thr Ala Leu Phe
  35          40          45
Asn Asn Gly Leu Ser Cys Gly Ala Cys Phe Glu Ile Arg Cys Gln Asn
  50          55          60
Asp Gly Lys Trp Cys Leu Pro Gly Ser Ile Val Val Thr Ala Thr Asn
  65          70          75          80
Phe Cys Pro Pro Asn Asn Ala Leu Pro Asn Asn Ala Gly Gly Trp Cys
  85          90          95
Asn Pro Pro Gln Gln His Phe Asp Leu Ser Gln Pro Val Phe Gln Arg
  100         105         110
Ile Ala Gln Tyr Arg Ala Gly Ile Val Pro Val Ala Tyr Arg Arg Val
  115         120         125
Pro Cys Val Arg Arg Gly Gly Ile Arg Phe Thr Ile Asn Gly His Ser
    
```

-continued

130 135 140

Tyr Phe Asn Leu Val Leu Ile Thr Asn Val Gly Gly Ala Gly Asp Val
 145 150 155 160

His Ser Ala Met Val Lys Gly Ser Arg Thr Gly Trp Gln Ala Met Ser
 165 170 175

Arg Asn Trp Gly Gln Asn Trp Gln Ser Asn Ser Tyr Leu Asn Gly Gln
 180 185 190

Ser Leu Ser Phe Lys Val Thr Thr Ser Asp Gly Gln Thr Ile Val Ser
 195 200 205

Asn Asn Xaa Ala Asn Ala Gly Trp Ser Phe Gly Gln Thr Phe Thr Gly
 210 215 220

Ala His Val Arg
 225

<210> SEQ ID NO: 3
 <211> LENGTH: 222
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: rice
 expansin
 <220> FEATURE:
 <221> NAME/KEY: UNSURE
 <222> LOCATION: (14)..(58)
 <223> OTHER INFORMATION: Xaa is unknown or other.

<400> SEQUENCE: 3

His Met Gly Pro Trp Ile Asn Ala His Ala Thr Phe Tyr Xaa Xaa Gly
 1 5 10 15

Asp Ala Xaa Xaa Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr
 20 25 30

Ser Gln Gly Tyr Gly Leu Glu Thr Ala Ala Leu Ser Thr Ala Leu Phe
 35 40 45

Asp Gln Gly Leu Ser Cys Gly Ala Cys Xaa Glu Leu Met Cys Val Asn
 50 55 60

Asp Pro Gln Trp Cys Ile Lys Gly Arg Ser Ile Val Val Thr Ala Thr
 65 70 75 80

Asn Phe Cys Pro Pro Gly Gly Ala Cys Asp Pro Pro Asn His His Phe
 85 90 95

Asp Leu Ser Gln Pro Ile Tyr Glu Lys Ile Ala Leu Tyr Lys Ser Gly
 100 105 110

Ile Ile Pro Val Met Tyr Arg Arg Val Arg Cys Lys Arg Ser Gly Gly
 115 120 125

Ile Arg Phe Thr Ile Asn Gly His Ser Tyr Phe Asn Leu Val Leu Val
 130 135 140

Thr Asn Val Gly Gly Ala Gly Asp Val His Ser Val Ser Met Lys Gly
 145 150 155 160

Ser Arg Thr Lys Trp Gln Leu Met Ser Arg Asn Trp Gly Gln Asn Trp
 165 170 175

Gln Ser Asn Ser Tyr Leu Asn Gly Gln Ser Leu Ser Phe Val Val Thr
 180 185 190

Thr Ser Asp Arg Arg Ser Val Val Ser Phe Asn Val Ala Pro Pro Thr
 195 200 205

Trp Ser Phe Gly Gln Thr Tyr Thr Gly Gly Gln Phe Arg Tyr
 210 215 220

<210> SEQ ID NO: 4

-continued

<211> LENGTH: 227
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Arabidopsis
 expansin
 <220> FEATURE:
 <221> NAME/KEY: UNSURE
 <222> LOCATION: (2)..(227)
 <223> OTHER INFORMATION: Xaa is unknown or other.

<400> SEQUENCE: 4

Lys Xaa Ser Val Ala Gln Ser Ala Phe Ala Thr Phe Tyr Gly Gly Lys
 1 5 10 15
 Asp Gly Ser Cys Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr
 20 25 30
 Asn Ala Gly Tyr Gly Leu Tyr Asn Ala Ala Leu Ser Ser Ala Leu Phe
 35 40 45
 Asn Asp Gly Ala Met Cys Gly Ala Cys Tyr Thr Ile Thr Cys Asp Thr
 50 55 60
 Ser Gln Thr Lys Trp Cys Lys Pro Gly Gly Asn Ser Ile Thr Ile Thr
 65 70 75 80
 Ala Thr Asn Leu Cys Xaa Pro Asn Trp Ala Leu Pro Ser Asn Ser Gly
 85 90 95
 Gly Trp Cys Asn Pro Pro Leu Xaa His Phe Asp Met Ser Gln Pro Ala
 100 105 110
 Trp Glu Asn Ile Ala Val Tyr Gln Ala Gly Ile Val Pro Val Asn Tyr
 115 120 125
 Lys Arg Val Pro Xaa Gln Arg Ser Gly Gly Ile Arg Phe Ala Ile Ser
 130 135 140
 Gly His Asp Tyr Phe Glu Leu Val Thr Val Thr Asn Val Gly Gly Ser
 145 150 155 160
 Gly Val Val Ala Gln Met Ser Ile Lys Gly Ser Asn Thr Gly Trp Met
 165 170 175
 Ala Met Ser Arg Asn Trp Gly Ala Asn Trp Gln Ser Asn Ala Tyr Leu
 180 185 190
 Ala Gly Gln Ser Leu Ser Phe Ile Val Gln Leu Asp Asp Gly Arg Lys
 195 200 205
 Val Thr Ala Trp Asn Xaa Ala Pro Xaa Asn Trp Leu Xaa Xaa Xaa Xaa
 210 215 220
 Xaa Xaa Xaa
 225

<210> SEQ ID NO: 5
 <211> LENGTH: 225
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Arabidopsis
 expansin

<400> SEQUENCE: 5

Asp Asn Gly Gly Trp Glu Arg Gly His Ala Thr Phe Tyr Gly Gly Ala
 1 5 10 15
 Asp Ala Ser Gly Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu His
 20 25 30
 Ser Gln Gly Tyr Gly Leu Gln Thr Ala Ala Leu Ser Thr Ala Leu Phe
 35 40 45
 Asn Ser Gly Gln Lys Cys Gly Ala Cys Phe Glu Leu Thr Cys Glu Asp

-continued

| 50 | 55 | 60 |
|--|---------------------------|--|
| Asp 65 | Pro Glu Trp Cys Ile 70 | Pro Gly Ser Ile Ile Val Arg Tyr Asn Leu 75 80 |
| Ala Asn Phe Ala Leu Ala Asn Asp Asn Gly Gly Trp Cys Asn Pro Pro 85 90 95 | | |
| Leu Lys His Phe Asp Leu Ala Glu Pro Ala Phe Leu Gln Ile Ala Gln 100 105 110 | | |
| Tyr Arg Ala Gly Ile Val Pro Val Ala Phe Arg Arg Val Pro Cys Glu 115 120 125 | | |
| Lys Gly Gly Gly Ile Arg Phe Thr Ile Asn Gly Asn Pro Tyr Phe Asp 130 135 140 | | |
| Leu Val Leu Ile Thr Asn Val Gly Gly Ala Gly Asp Ile Arg Ala Val 145 150 155 160 | | |
| Ser Leu Lys Gly Ser Lys Thr Asp Gln Trp Gln Ser Met Ser Arg Asn 165 170 175 | | |
| Trp Gly Gln Asn Trp Gln Ser Asn Thr Tyr Leu Arg Gly Gln Ser Leu 180 185 190 | | |
| Ser Phe Gln Val Thr Asp Ser Asp Gly Arg Thr Val Val Ser Tyr Asp 195 200 205 | | |
| Val Val Pro His Asp Trp Gln Phe Gly Gln Thr Phe Glu Gly Gly Gln 210 215 220 | | |
| Phe 225 | | |

<210> SEQ ID NO: 6
 <211> LENGTH: 226
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: Arabidopsis
 expansin

<400> SEQUENCE: 6

| | |
|--|--|
| Asp 1 | Tyr Ser Ser Trp Gln Ser Ala His Ala Thr Phe Tyr Gly Gly Gly 5 10 15 |
| Asp Ala Ser Gly Thr Met Gly Gly Thr Cys Gly Tyr Gly Asn Leu Tyr 20 25 30 | |
| Ser Thr Gly Tyr Thr Asn Thr Ala Ala Leu Ser Thr Val Leu Phe Asn 35 40 45 | |
| Asp Gly Ala Ala Cys Arg Ser Cys Tyr Glu Leu Arg Cys Asp Asn Asp 50 55 60 | |
| Gly Gln Trp Cys Leu Pro Gly Ser Val Thr Val Thr Ala Thr Asn Leu 65 70 75 80 | |
| Cys Pro Pro Asn Tyr Ala Leu Pro Asn Asp Asp Gly Gly Trp Cys Asn 85 90 95 | |
| Pro Pro Arg Pro His Phe Asp Met Ala Glu Pro Ala Phe Leu Gln Ile 100 105 110 | |
| Gly Val Tyr Arg Ala Gly Ile Val Pro Val Ser Tyr Arg Arg Val Pro 115 120 125 | |
| Cys Val Lys Lys Gly Gly Ile Arg Phe Thr Ile Asn Gly His Ser Tyr 130 135 140 | |
| Phe Asn Leu Val Leu Val Thr Asn Val Ala Gly Pro Gly Asp Val Gln 145 150 155 160 | |
| Ser Val Ser Ile Lys Gly Ser Ser Thr Gly Trp Gln Pro Met Ser Arg 165 170 175 | |

-continued

Asn Trp Gly Gln Asn Trp Gln Ser Asn Ser Tyr Leu Asp Gly Gln Ser
 180 185 190
 Leu Ser Phe Gln Val Ala Val Ser Asp Gly Arg Thr Val Thr Ser Asn
 195 200 205
 Asn Val Val Pro Ala Gly Trp Gln Phe Gly Gln Thr Phe Glu Gly Gly
 210 215 220
 Gln Phe
 225

<210> SEQ ID NO: 7
 <211> LENGTH: 227
 <212> TYPE: PRT
 <213> ORGANISM: Artificial Sequence
 <220> FEATURE:
 <223> OTHER INFORMATION: Description of Artificial Sequence: cucumber
 expansin

<400> SEQUENCE: 7

Asp Tyr Gly Gly Trp Gln Ser Gly His Ala Thr Phe Tyr Gly Gly Gly
 1 5 10 15
 Asp Ala Ser Gly Thr Met Gly Gly Ala Cys Gly Tyr Gly Asn Leu Tyr
 20 25 30
 Ser Gln Gly Tyr Gly Thr Asn Thr Val Ala Leu Ser Thr Ala Leu Phe
 35 40 45
 Asn Asn Gly Leu Ser Cys Gly Ala Cys Phe Glu Met Thr Cys Thr Asn
 50 55 60
 Asp Pro Lys Trp Cys Leu Pro Gly Thr Ile Arg Val Thr Ala Thr Asn
 65 70 75 80
 Phe Cys Pro Pro Asn Phe Ala Leu Pro Asn Asp Asp Gly Gly Trp Cys
 85 90 95
 Asn Pro Pro Leu Gln His Phe Asp Met Ala Glu Pro Ala Phe Leu Gln
 100 105 110
 Ile Ala Gln Tyr Arg Ala Gly Ile Val Pro Val Ser Phe Arg Arg Val
 115 120 125
 Pro Cys Met Lys Lys Gly Gly Val Arg Phe Thr Ile Asn Gly His Ser
 130 135 140
 Tyr Phe Asn Leu Val Leu Ile Thr Asn Val Gly Gly Ala Gly Asp Val
 145 150 155 160
 His Ser Val Ser Ile Lys Gly Ser Arg Thr Gly Trp Gln Ser Met Ser
 165 170 175
 Arg Asn Trp Gly Gln Asn Trp Gln Ser Asn Asn Tyr Leu Asn Gly Gln
 180 185 190
 Gly Leu Ser Phe Gln Val Thr Leu Ser Asp Gly Arg Thr Leu Thr Ala
 195 200 205
 Tyr Asn Leu Val Pro Ser Asn Trp Gln Phe Gly Gln Thr Tyr Glu Gly
 210 215 220
 Pro Gln Phe
 225

What is claimed is:

1. An isolated polynucleotide comprising a nucleotide sequence of SEQ ID NO: 1, and which encodes a protein having expansin activity.
2. An isolated polynucleotide comprising a DNA sequence encoding a polypeptide selected from the group consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6 and SEQ ID NO: 7.

3. An isolated polynucleotide having 90% sequence similarity to SEQ ID NO: 1, and which encodes a protein having expansin activity.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,255,466 B1

Page 1 of 1

DATED : July 3, 2001

INVENTOR(S) : Daniel J. Cosgrove, Simon McQueen-Mason, Mark J. Guiltinan, Tatyana Shcherban
and Jun Shi

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

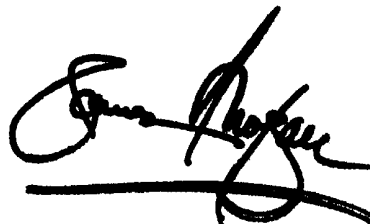
Title page, Item [54], and Column 1, line 1,

Correct to read -- [54] **PURIFIED PLANT EXPANSIN PROTEINS AND DNA
ENCODING SAME** --

Signed and Sealed this

Sixth Day of August, 2002

Attest:

A handwritten signature in black ink, appearing to read "James E. Rogan", written over a horizontal line.

Attesting Officer

JAMES E. ROGAN
Director of the United States Patent and Trademark Office