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Induction of Hydrolytic Enzymes in *Brassica campestris* in Response to Pathovars of *Xanthomonas campestris*[1]

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ABSTRACT

Inoculation of mature leaves of turnip (*Brassica campestris*) with the incompatible *Xanthomonas campestris* pv *vitians* resulted in the induction of β-1,3-glucanase and chitinase/lysozyme (CHL) activity. No increase in the basal activity of β-1,3-glucanase was observed after inoculation of leaves with heat- or rifampicin-killed *X. c. vitians*, *Escherichia coli*, or sterile water. Inoculation with the compatible *X. campestris pv campestris* resulted in a slower induction of glucanase than that seen with *X. c. vitians*. In contrast, all bacteria caused an induction of CHL activity. One major β-1,3-glucanase (molecular mass 36.5 kilodaltons, isoelectric point [pI] ~8.5) was purified from both inoculated and untreated leaves by ion-exchange chromatography. The enzyme degraded laminarin by an *endo*-glycolytic mechanism. Two major CHL isozymes (CHL 1 and CHL 2, molecular mass 30 kilodaltons and pI 9.4 and 10.2, respectively) were purified from *X. c. vitians* inoculated leaves by affinity chromatography on a chitin column followed by ion-exchange chromatography. Both enzymes degraded chitin by an *endo*-glycolytic mechanism although the ratio of lysozyme to chitinase specific activities for CHL 1 and CHL2 were different. The induction of CHL 1 was associated with the hypersensitive reaction caused by *X. c. vitians* whereas all other treatments induced largely CHL 2.

The induction of the hydrolytic enzymes β-1,3-glucanase and chitinase has been studied in a number of plants in response to infection, heat-killed pathogens, pathogen cell walls, or wounding (2, 3, 11). Some of the PR[2] proteins induced in several different plants in response to infection have now been identified as β-1,3-glucanases and chitinases (12, 13, 18). These hydrolases are of interest as plant defence products because of their ability to directly attack specific structures of pathogens. Plant chitinases are potent inhibitors of fungal growth (22) and in combination with β-1,3-glucanase are able to attack a number of fungi *in vitro* (3, 17). This antifungal action is probably due to the hydrolysis of the noncrystalline chitin present at the apex of the growing fungal hypha (26). Bean and pea chitinases also possess lysozyme activity that is capable of hydrolyzing the peptidoglycan in bacterial cell walls (4, 16). Thus, these hydrolases are potentially effective in defence reactions against bacterial pathogens. All plant lysozymes so far described have been shown to act as endochitinases (3). β-1,3-Glucanase and chitinase are coordinately induced in a number of plant tissues by pathogen attack and elicitors (25).

We are interested in the mechanisms of resistance of *Brassica* spp., in particular turnip (*Brassica campestris*) to incompatible pathovars of the Gram-negative phytopathogen *Xanthomonas campestris*. *X. campestris* pv *campestris* (hereafter referred to as *X. c. campestris*) is an important worldwide pathogen of almost all cultivated brassicas and noncrop crucifers (27) whereas *X. c. vitians*, a pathogen of lettuce (*Lactuca sativa*) is incompatible with brassica and triggers HR characterized by tissue collapse (6). As part of our study on defence mechanisms in *Brassica*, we have investigated the induction of CHL and β-1,3-glucanase in response to *X. c. campestris*, *X. c. vitians*, and *Escherichia coli* (a nonpathogen). Lysozyme activity in turnip was originally described by Fleming (10) and the enzymes from turnip root (1) and cauliflower (9) have been partially characterized. In this paper we report on the characterization of the β-1,3-glucanase and of isoforms of CHL that show differential regulation in response to the different bacteria.

MATERIALS AND METHODS

Biological Materials

The following strains of bacteria were used in this work: *Xanthomonas campestris* pv *campestris*, strain 8004 a spontaneous rifampicin resistant mutant of NCPPB 1145; *Xanthomonas campestris* pv *vitians*, strain 9000, NCPPB 1839; *Escherichia coli*, strain ED8767; *Xanthomonas* cultures were grown in NYGB medium, *E. coli* cultures in L broth (8). Bacteria were resuspended in sterile distilled water to a final concentration of 10^7 or 10^8 per mL before inoculation into mature leaves of 4 week old turnip plants cv Just Right as described previously (6). Bacteria were heat-killed by autoclaving at 121°C for 20 min. For rifampicin treatment, the antibiotic was added to growing cultures to a final concentration of 50 μg/mL for 1 h, then the bacteria were harvested by centrifugation and washed with water before inoculation.

Enzyme Assays

Relative activities of β-1,3-glucanase were assayed at 30°C by a radial diffusion assay into an agar plate containing the β-1,3-glucan substrate laminarin as described by Wood (28). The plates contained 0.5% (w/v) laminarin (Sigma) in 50 mM phosphate buffer pH 6 with 2% (w/v) agar. The substrate was

[1] The work described in this paper was supported by the Gatsby Foundation.

[2] Abbreviations: PR: pathogenesis-related; HR: hypersensitive reaction; HRGP: hydroxyproline-rich glycoprotein; CHL: chitinase/lysozyme.

detected with Congo red. One unit of activity was defined as that causing a zone of substrate breakdown 18 mm in diameter around a 5 mm diameter central well in 24 h.

Lysozyme activity was measured by following the reduction of turbidity at 600 nm of a suspension of *Micrococcus lysodeikticus* cells at 30°C. Assays contained 0.9 mL bacterial suspension (0.125 mg/mL in 50 mM acetate buffer pH 5) and up to 100 μL of enzyme solution in a total volume of 1.0 mL. One unit of activity was defined as that causing a change in apparent absorbance of 0.001 per min. This assay was used for the routine screening of HPLC fractions, for induction profiles and for purification (see below).

Chitinase activity was measured by the release of soluble radioactive products from tritiated chitin (19) essentially as described by Boller *et al.* (4). Assays contained 4 mg of radioactive chitin (670,500 cpm), enzyme, and 50 mM acetate buffer pH 5 in a total volume of 1 mL. At different time points, aliquots were taken and the reaction was stopped by the addition of an equal volume of 1 M TCA. After centrifugation, the radioactivity in the supernatant was determined by liquid scintillation counting.

Purification of CHLs and Glucanase

Leaf tissue was homogenized in a blender with 100 mM Tris buffer pH 7.5 containing 1 mM EDTA, 1 mM phenylmethylsulphonylfluoride, and 1% (w/v) polyvinylpolypyrrolidone. After filtration through muslin, particulate material was removed by centrifugation at 20,000*g* for 15 min and the samples were dialyzed overnight against 10 mM Tris buffer (pH 8).

CHLs were separated from the dialysed extract by affinity chromatography on a column of regenerated chitin (19) at pH 8, as described by Mauch *et al.* (16). After washing with 10 mM Tris buffer (pH 8), the enzymes were eluted from the column with 20 mM acetic acid (pH 3), and the eluate was adjusted to pH 4.3 by addition of 1 M Tris (pH 7.5). More than 70% of the activity was recovered from the column. Individual CHL isozymes were separated by ion-exchange chromatography on SP-5PW, a cation exchanger, using a Gilson HPLC system. The ion-exchange column was equilibrated with 20 mM acetate (pH 4.3). At this pH, all the CHL activity bound to the column. The column was washed and eluted with a linear gradient of sodium chloride in 20 mM acetate buffer (pH 4.3). Protein elution was monitored by absorbance at 254 nm.

For purification of β-1,3-glucanase, the dialyzed extract was first passed through a DEAE-Sephadex column equilibrated with 10 mM Tris (pH 8) to remove acidic proteins and pigmented material. The nonretained fraction was adjusted to pH 4.3 by addition of 1 M acetate buffer (pH 4.3). The precipitate which formed was removed by centrifugation before ion-exchange chromatography on SP-5PW under the same conditions used for the separation of CHL. In some experiments the supernatant was additionally purified by ion-exchange on SP-Sephadex before HPLC. The SP-Sephadex column was equilibrated with 20 mM acetate (pH 4.3) and was eluted with a linear gradient of sodium chloride in the same buffer. Active fractions were pooled and dialyzed overnight against 20 mM acetate (pH 4.3) before HPLC. At this pH, all the glucanase activity bound to the SP-5PW column.

Other Analytical Methods

SDS-PAGE was performed on 11% separation gels or 10 to 20% gradient gels essentially as described by Laemmli (14). Isoelectric focusing was performed in a vertical minigel system as described by Robertson *et al.* (20). For analysis of reaction products from the enzymic digestion of laminarin, the enzyme assay contained 0.5 mg laminarin, 5 mM acetate buffer (pH 6), and enzyme in a total volume of 0.5 mL. After various times of incubation at 30°C, the reaction was stopped by boiling and 10 μL samples applied directly to TLC silica gel plates. Products were separated by three developments in acetonitrile:water (4:1, v/v). Standards were monomeric glucose and laminarin hydrolyzed with 0.1 N H_2SO_4 for 5 h at 100°C. For analysis of the reaction products from the enzymic digestion of chitin, the reaction mixture contained 9 mg chitin, 10 mM acetate buffer (pH 5), and enzyme in a total volume of 1 mL. After stopping the reaction by boiling, the undissolved chitin was removed by centrifugation and the supernatant concentrated 12-fold before application of 10 μL samples to the TLC plate. The solvent was propan-2-ol:water:ammonia, 70:30:1 v/v. Chitooligosaccharide standards were generated by hydrolysis of regenerated chitin in 11 M HCl for 1.5 h at 40°C. Reaction products from both enzyme activities were detected using N-naphthylethylenediamine (5).

RESULTS AND DISCUSSION

Time Course of Induction of Hydrolytic Enzyme Activity in Turnip Leaves

The specific activities of CHL and β-1,3-glucanase were determined in leaves inoculated in two slightly different ways. Bacterial suspensions were introduced through the stomata on the underside of the leaves using a syringe (with no needle) either in a patchwise fashion or over the whole leaf area. The patchwise method of inoculation with *X. c. vitians* lead to collapse of the tissue within the area of inoculation alone so that each collapsed area was surrounded by 'healthy' tissue comprising at least 50% of the leaf area. This was done in order to mimic 'natural' HR processes in which tissue both directly in contact with and distant to the triggering pathogen may contribute to the overall response (15). Leaves inoculated over their whole surfaces might be expected, in contrast, to show largely the responses of tissue directly in contact with the bacteria.

Differences were seen in the time course and magnitude of induction of β-1,3-glucanase in response to *X. c. campestris* and *X. c. vitians* (Fig. 1, C and D); *X. c. vitians* caused a larger and more rapid change in glucanase activity than *X. c. campestris.* This effect was seen with both methods of inoculation and at all concentrations of bacteria used. Inoculation with *Escherichia coli*, heat- or rifampicin-killed *X. c. vitians* (treatments which do not elicit HR; 6) or sterile water caused no change in the basal level of glucanase found in uninoculated leaves. Similar increases in glucanase specific activity were detected in response to *X. c. vitians* in both the inoculated

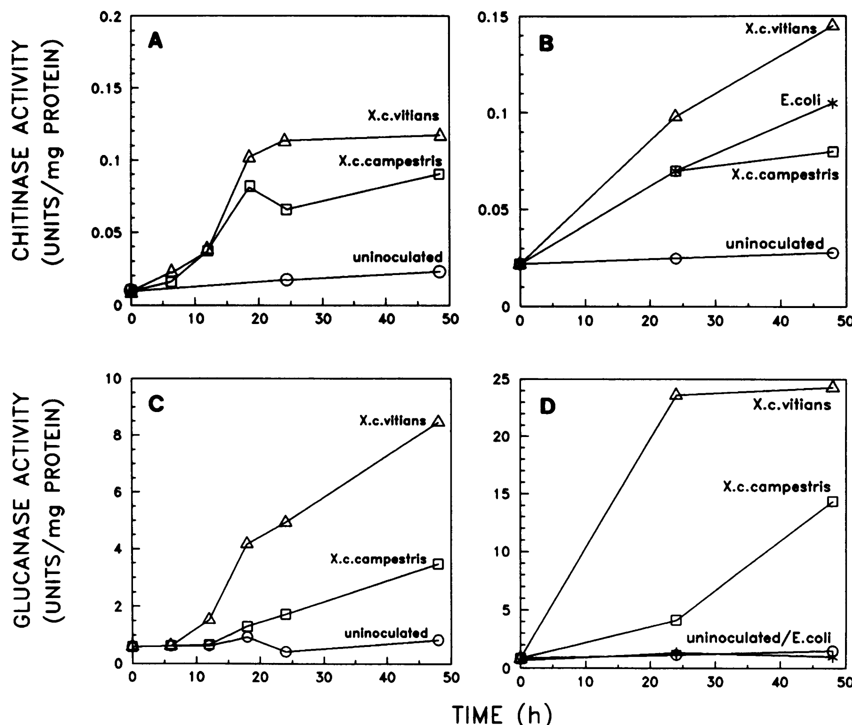

Figure 1. Induction of chitinase and β-1,3-glucanase in turnip leaves by bacteria. A and C, Patchwise method of inoculation with 10^8 bacteria per mL; B and D, full leaf inoculations with 10^7 bacteria per mL.

(collapsed) areas and in the surrounding tissue in patchwise inoculated plants.

Differences between the time course of CHL induction by the two pathovars were less pronounced than those seen with β-glucanase (Fig. 1, A and B). With the patchwise method of inoculation (Fig. 1A), no distinct differences in CHL levels were seen between leaves inoculated with the two pathovars within the first 12 h, although CHL levels were higher than in uninoculated plants. After 24 h, levels of chitinase in *X. c. vitians* inoculated leaves were higher than in leaves inoculated with *X. c. campestris*. With the full leaf inoculations (Fig. 1B), both bacteria induced changes in CHL levels although, again, levels were slightly higher in *X. c. vitians* inoculated leaves. Although inoculation with sterile water had no effect on CHL levels, inoculation with heat- or rifampicin-killed *X. c. vitians* or with *E. coli* caused an increase in CHL activity of similar magnitude to that seen with *X. c. campestris*. This is in distinct contrast to the results with β-glucanase induction. In plants inoculated in a patchwise fashion with *X. c. vitians*, increases in CHL activity were seen in both inoculated, collapsed areas and in the surrounding, apparently healthy, tissue. In contrast, changes in CHL activity in plants inoculated with *E. coli* were only seen in the inoculated areas; the specific activity of CHL in the surrounding tissue was the same as that of uninoculated leaves.

Characterization of β-1,3-Glucanase and Isozymes of CHL and Their Differential Induction in Response to Pathogens and Nonpathogens

Multiple isoforms of chitinase and β-1,3-glucanase have been described in a number of plants and in some cases these isoforms show differential regulation in response to pathogens or with aging (16). Accordingly, we wished to determine the number of isoforms of β-glucanase and CHL in healthy plants and whether different isoforms were specifically induced by the different treatments employed.

CHL and β-glucanases were purified from turnip leaves 24 h after inoculation with the various bacteria as described in "Materials and Methods." Almost all the β-1,3-glucanase activity passed through the DEAE-Sephadex column, suggesting that acidic β-1,3-glucanases do not make a significant contribution to the overall β-1,3-glucanase activity. One major peak of β-1,3-glucanase activity was found on HPLC ion-exchange chromatography of extracts of plants inoculated with *X. c. vitians* (Fig. 2). The active fractions were pooled, diluted and, rechromatographed with a shallower salt gradient at the same pH. Again, only one peak of activity and a coincident peak of A_{254} was evident. A single peak of activity with the same chromatographic properties was found in the extracts from uninoculated plants (data not shown). SDS-PAGE of the active fractions showed a single molecular mass species of 36.5 kD from both uninoculated and *X. c. vitians* inoculated plants (Fig. 3A). Isoelectric focusing again showed one species of pI about 8.5 (data not shown). These results suggest, but do not prove, that glucanases from healthy and HR-triggered plants are identical. The purified enzymes degraded laminarin by an endomechanism; analysis of the products of digestion by TLC showed that even after long incubation times, monomeric glucose was not a product; the trimer (laminaritriose) predominated, with smaller amounts of the dimer (laminaribiose) and higher oligomers.

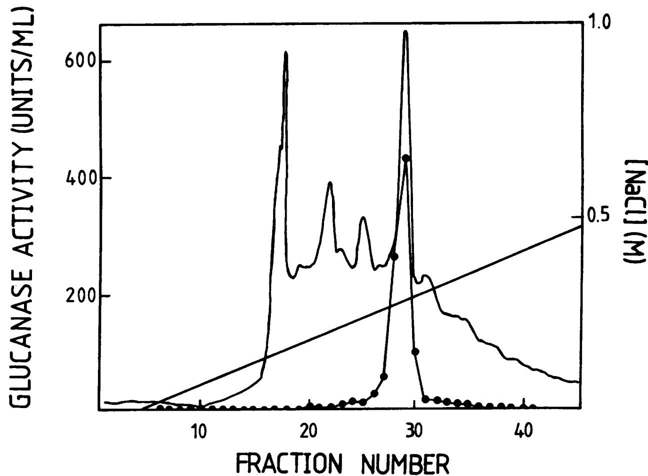

Figure 2. HPLC ion-exchange chromatography on SP-5PW of β-1,3-glucanase from turnip leaves inoculated with *X. c. vitians*. (——), Absorbance at 254 nm; (●), β-1,3-glucanase activity. Infected leaf tissue (15 g) were harvested 24 h after patchwise inoculation with 10^8 bacteria per mL and were processed before HPLC ion-exchange chromatography as described in "Materials and Methods" with inclusion of the SP-Sephadex chromatography. Fraction size = 1 mL.

In contrast to the situation with β-1,3-glucanase, several isozymes of CHL were resolved by ion-exchange HPLC (Fig. 4). In addition, the pattern of CHL isozymes induced by treatment with *X. c. vitians* (Fig. 4A) was different from that induced by *X. c. campestris* or *E. coli* (Fig. 4, B and C) or by heat- or rifampicin-killed *X. c. vitians* (data not shown). The former treatment induced largely an earlier eluting isoform (CHL 1) with significant amounts of a later eluting form (CHL 2), whereas all the other treatments induced largely CHL 2. These changes in isozyme activity are summarized in Table I. The ion-exchange profiles of extracts from uninoculated plants showed another minor peak of activity (fraction 47) which did not increase after inoculation.

SDS-PAGE of the active fractions showed a single molecular mass species of 30 kD for both CHL 1 and CHL 2 (Fig. 3B). The estimated pIs were 9.4 and 10.2 for CHL1 and CHL 2, respectively (data not shown). Both enzymes were capable of degrading regenerated chitin to give soluble reaction products. Apart from a small activity associated with the minor peak of activity, no other fraction in the chromatograms demonstrated chitinase activity. The ratio of lysozyme to chitinase specific activity was approximately 15-fold higher for CHL 2 than CHL 1 (Table II). Both enzymes produced a similar pattern of chitobiose, chitotriose, and higher oligomers as soluble reaction products as revealed by TLC. Monomeric GlcNAc was not a product suggesting that the enzymes are endo-chitinases.

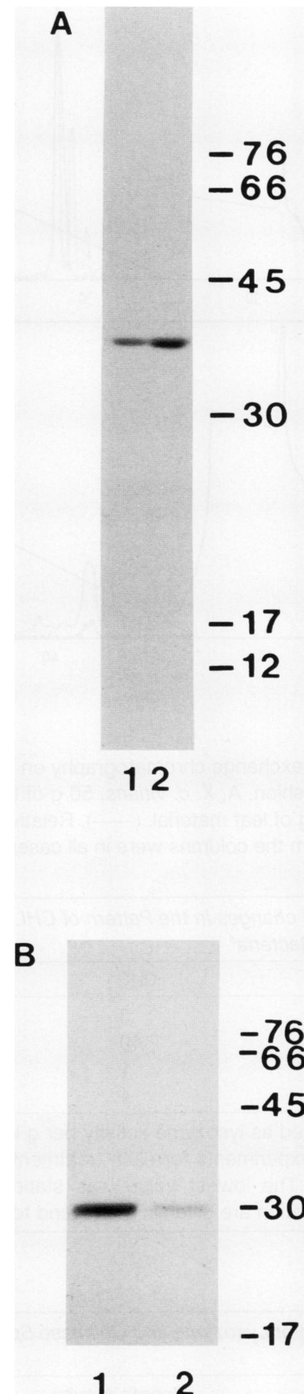

Figure 3. SDS-PAGE of purified enzymes from turnip leaves. A, β-1,3-Glucanase from uninoculated (lane 1) and *X. c. vitians* inoculated (lane 2) leaves; 25 μL aliquots from the most active fraction on HPLC ion-exchange chromatography derived from 15 g of leaves were used. B, CHL 1 (lane 1) and CHL 2 (lane 2) from *X. c. vitians* inoculated leaves; 10 μL aliquots of the most active fractions (33 and 52, respectively) from the purification shown in Figure 4A were used. Molecular mass markers in kD are given on the right.

CONCLUDING REMARKS

The results presented here suggest that the induction of β-1,3-glucanase and one CHL isoform (CHL 1) are specifically associated with the HR reaction induced in turnip leaves by living incompatible bacterial pathogens and occur both in directly inoculated and surrounding tissue. Another isoform

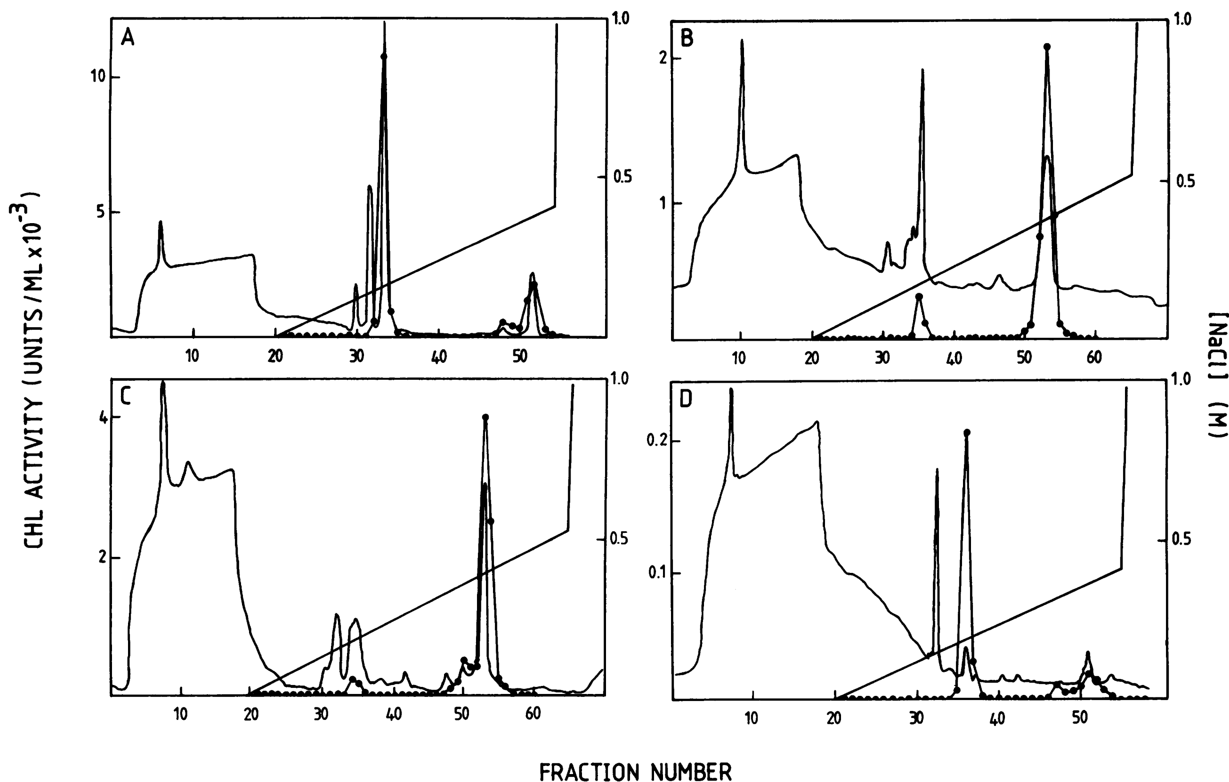

Figure 4. HPLC ion-exchange chromatography on SP-5PW of CHL from turnip leaves harvested 24 h after inoculation with bacteria at 10^8 per mL in a patchwise fashion. A, *X. c. vitians*, 50 g of leaf material; panel B, *X. c. campestris*, 50 g of leaf material; C, *E. coli*, 82 g of leaf material; D, uninoculated, 25 g of leaf material. (——), Relative absorbance at 254 nm, the highest value is set to 100%; (●), CHL activity. Recoveries of enzyme activities from the columns were in all cases greater than 75%. Fraction size was 1 mL.

Table I. *Summary of changes in the Pattern of CHL Isozymes Induced by Various Bacteria*[a]

Treatment[b]	CHL 1	CHL 2
Uninoculated	3	1
X. c. vitians	40	4
X. c. campestris	1	32
E. coli	4	32

[a] Activities calculated as lysozyme activity per g leaf tissue are an average from three experiments for each treatment of which one is shown in Figure 4. The lowest value was standardized to one. [b] Conditions of inoculation are given in the legend to Figure 4.

Table II. *Comparison of Lysozyme and Chitinase Specific Activities of CHL 1 and CHL 2*

Enzyme	Specific Activity	
	Lysozyme[a]	Chitinase[b]
	units/mg × 10^{-6}	*cpm/min/mg* × 10^{-6}
CHL 1	0.11	1.63
CHL 2	0.15	0.14

[a] Lysozyme activity was measured by a reduction in turbidity of a suspension of *Micrococcus lysodeikticus* as described in "Materials and Methods." [b] Chitinase activity was measured as the rate of release of radioactivity from tritiated regenerated chitin which was linear for up to 45 min.

(CHL 2) with a predominantly lysozyme activity is induced by a range of living and dead bacteria including nonpathogens such as *E. coli*, but in a localized fashion. Differential activation of members of small multigene families encoding defence related products under different stress conditions have been described for HRGP and chalcone synthase in *Phaseolus vulgaris* (7, 21). In addition, two chitinases and two β-glucanases showing differential regulation during development and in response to fungal infection have been described in pea tissue (16). Transcripts of some defence genes (HRGP, PR 1) have been shown to accumulate in areas distant to the site of infection whereas others may be more localized (23, 24). It is our intention to investigate spatial and temporal aspects of the regulation of expression of the genes for the hydrolytic enzymes in *Brassica* which we believe to underlie the changes in activity reported here.

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