ACCUMULATION OF TRYPSIN INHIBITORS IN TOMATO LEAVES INDUCED BY PECTIN AND CHITOSAN DERIVATIVES

A. Falcón, J. C. Cabrera, R. Pombo and A. Gutiérrez

ABSTRACT. Synthesis and accumulation of proteinase inhibitors, renowed defenses of plants against insects, can be induced by oligosaccharides released from arthropod exoskeleton and plant cell walls. The first results of our laboratory on the induction of trypsin inhibitors in tomato plant leaves from Campbell-28 cv. are reported here. These plants were previously supplied through its petioles for an hour with buffers containing chitosan, chitosan hydrolysates and pectin obtained from national raw materials. Results showed percentages of inhibitor activities in different treatments, which were higher than the basal level of the inhibiting activity present in control plant leaves, chitosan being the most effective treatment to induce the accumulation of these defensive proteins.

Key words: proteinase inhibitors, trypsin, tomato, chitosan, pectin

RESUMEN. La síntesis y acumulación de inhibidores de proteinasas, conocidas defensas de las plantas contra insectos, puede ser inducida por oligosacáridos liberados de paredes celulares de plantas y exoesqueletos de artrópodos. Se informan al respecto los primeros resultados de nuestro laboratorio en la inducción de inhibidores de tripsina en hojas de plántulas de tomate variedad Campbell-28, previamente tratadas con quitosana, hidrolizados de quitosana y pectina, obtenidos a partir de materias primas nacionales. Los resultados mostraron porcentajes de acumulación de inhibidores en los distintos tratamientos por encima del valor basal de actividad inhibidora presente en las plantas control, siendo el tratamiento con quitosana el más efectivo en inducir la acumulación de estas proteínas defensivas.

Palabras clave: inhibidores de proteinasas, tripsina, tomate, quitosana, pectina

INTRODUCTION

Proteinase inhibitors are widespread in plant kingdom, mainly in sexual and asexual reproductive organs from plants. They have the quality of selectively inhibiting animal and microbial proteolytical enzymes (Ryan, 1973); therefore, they have been considered as protective proteins against pathogens and pests.

This protective function has been confirmed by diverse experimental results in different crops. In tomato, for instance, protease inhibitor accumulation in leaf tissues is directly correlated with the varietal resistance against the attack of *Phytophthora infestans* (Peng and Black, 1976; Cleveland and Black, 1983). They proved to have the capacity of inactivating proteases from *Fusarium solani* and *Colletotrichum lindemuthianum*, phytopathogenic fungi of potato and bean, respectively (Mosolov *et al.*, 1976 and 1979). Finally, the systemic accumulation of these proteins provoked by a localized mechanical damage suggests the defensive role of protecting healthy tissues from depredating insect attack (Green and Ryan, 1972).

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Researchers of Pullman University found proteinase inhibitor inducing factor (PIIF) derived from pectic polisaccharides constitute, at least, the first wound signal released as induced by the synthesis and accumulation of proteinase inhibitors in other parts of the plant (Bishop *et al.*, 1984; Mc Farland and Ryan, 1974). That synthesis in some plants can also be induced by oligosaccharides, constituents of arthropod exoskeleton and microorganism cell walls, like the soluble chitin and chitosan derivatives (Mary Walker-Simmons and Ryan, 1984; Mary Walker-Simmons *et al.*, 1984).

The objective of this paper is to experimentally evaluate oligosaccharides derived from national raw materials for their potential provoking a systemic accumulation of proteinase inhibitors in tomato plants.

MATERIALS AND METHODS

A partially deesterified pectin concentrate was supplied by IIIA (Research Institute of Food Industry); the lobster (*Panulirus argus* sp.) exoskeleton-extracted chitin was obtained in "Mario Muñoz" Laboratory from the Ministry of People's Health.

Pectin hydrolysis was carried out by means of Pectinex Ultra SPL (Novo Nordisk A/S). Proteinase inhibitors were measured through using cristalized trypsin (Merk) and BAEE (Benzoyl-Arginyl ethyl ester) as a substrate (SIGMA).

Reducing sugars in the trial were employed to determine the optimum time of pectin hydrolysis by using Nelson's method (1944).

How to obtain pectin hydrolysates. A partially deesterified pectin concentrate was purified for successive precipitations of pectic substances in ethylic alcohol 70 %.

Pectin enzymatic hydrolysates were obtained through a modified technique of Endred, Omran and Gierschner (1991). A purified pectin solution 1 % was incubated at 30°C with a diluted solution 1:1 000 v/v of Pectinex Ultra SPL (26 000 u/mL) enzymatic preparation for 60 minutes. The mixture reaction was stopped by boiling to 100°C for 15 minutes.

This hydrolysate was centrifuged to 3 000 g for 20 minutes and the supernatant concentrated in a vacuum rotatory evaporator and lyophilized.

How to obtain chitin derivatives. Chitosan was obtained by a modified method of Hirano, Kondo and Fujii (1985) through desacetilating process from lobster exoskeleton chitin with sodium hydroxide (NaOH) at 40 % and sodium borohydride ($NaBH_4$) at 120°C for three hours, besides successive washes with distilled water until a neutral pH. Finally, it was dried to 40°C in a vaccuum stove.

The methodology of Kauss, Jeblick and Domard (1989) was followed for the hydrolysis process. The resulting chitosan was dissolved in acetic acid at 6 %, also adding sodium nitrite ($NaNO_2$) by stirring, dissolved in distilled water for 30 minutes, to provoke *in situ* synthesis of nitrous acid (HNO_2), then a soluble hydrolysate appears.

The mixture was neutralized with sodium hydroxide (NaOH), finally rotoevaporated and lyophilized.

Induced accumulation of trypsin inhibitors in tomato leaves. Tomato seedlings (*Lycopersicon esculentum* Mill, Campbell cv.) were grown in banks for 12-hour light (10 000 lux and 28°C) and 12-hour night (22°C). When they were 20 days old (10 cm high and two fully developed true leaves as well as an apical leaflet), they were used for the experiments, following Ryan's methodology (1974).

Ten plants per treatment were selected for induction process; the control and experiment were replicated three times. Plants were carefully excised with a razor blade just above primary leaves; the cut surface of its petioles was immersed into the inducer dissolved with sodium phosphate buffer (NaH_2PO_4) and distilled water -control-, according to Ryan's methodology (1974). After an hour of incubation, the excised plants were transferred to water and held under constant light (11 000 lux) for 24 hours at 28°C. Then, leaves were detached from their petioles, collected and placed in a freezer at -20°C. Subsequently, leaves were lyophilized, reducing dried particles into a fine powder with a mortar and pestle.

The treatments were: pectin hydrolysate concentrations of 1 and 2 mg/mL; chitosan 0.1 mg/mL and chitosan hydrolysate 1 mg/mL.

Extraction and inhibitory activity. Protein was extracted from lyophilized leaves by homogenization with 0.5 M Tris/HCl pH 8.5 buffer (0.25 g/8 mL) with a small mortar and pestle. Homogenates were transferred to centrifuged tubes with Pasteur pipette and they were centrifuged for 15 minutes at 31 000 g, 4°C in a Beckman LC-65 ultracentrifuge with Sw-30 rotor. The inhibitory activity was determined in the supernatant by means of modified Schwert and Takenaka's method (1955).

Residual trypsin inhibitory activity was measured after preincubating leaf extracts with trypsin in this buffer for 15 minutes. The assay was conducted in 1-cm thick cells at 25°C, by adding BAEE substrate to the preincubated mixture.

Results were presented as the increasing percentage of trypsin inhibitory activity in different treatments and control, compared to trypsin and BAEE assay.

RESULTS AND DISCUSSION

Pectic substances are polyuronic compounds, mainly made up of unions of α-1-4-galacturonic acid -or its methylic ester- in association with neutral sugars. They are most of the components from primary cell wall and middle lamella of plant tissues, specifically fruit tissues (Rouan and Thibault, 1984).

Pectic fragments between 3 and 20 polymerization degree are proved to induce proteinase inhibitor accumulation in tomato plants (Ryan and Farmer, 1991; Fernández-Bolaño and Heredia, 1993). Reducing carbohydrate releasing dynamics along pectin enzymatic degradation present in pectinex Ultra SPL commercial preparation is shown in Figure 1. 60 minutes after the enzymatic hydrolysis, mid-polymerization degree oligopectates are more probable to be present; thus, this value is considered the right pectin hydrolysis time to obtain hydrolysates futher used for the biological assay.

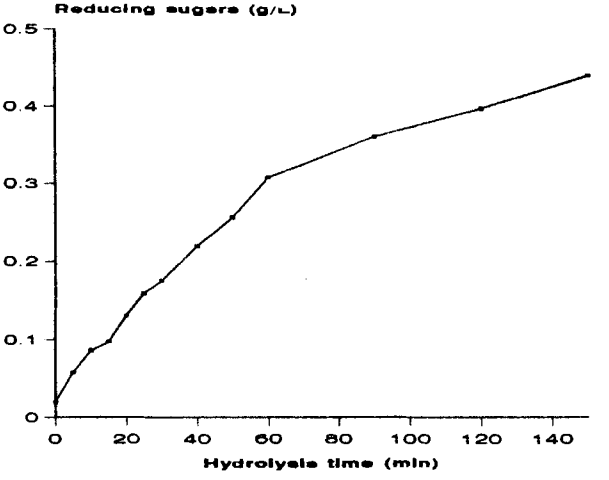

Figure 1. Course of pectin enzymatic degradation

Figure 2 shows how pectic hydrolysate treatments trypsin inhibitor accumulation in tomate leaves. This response depended upon hydrolysate concentration, which has to do with some other results (Ryan, 1974). Similar inhibition percentages are observed between

pectic hydrolysate (1 mg/mL) treatment and that of buffer used for dissolving; therefore, a low active fragment concentration is suggested in the mixture maybe as a consequence of a wide dispersion of molecular weights and charge of pectic substances present in the partially deesterified pectin concentrate (Cabrera, 1993, personal communication). Consequently, high-purity pectic acid is recommended to be used as enzymatic substrate for obtaining pectic hydrolysates with proteinase inhibitory inducing activity, since it assures a greater chemical homogeneity of degradation products and higher yields of active species.

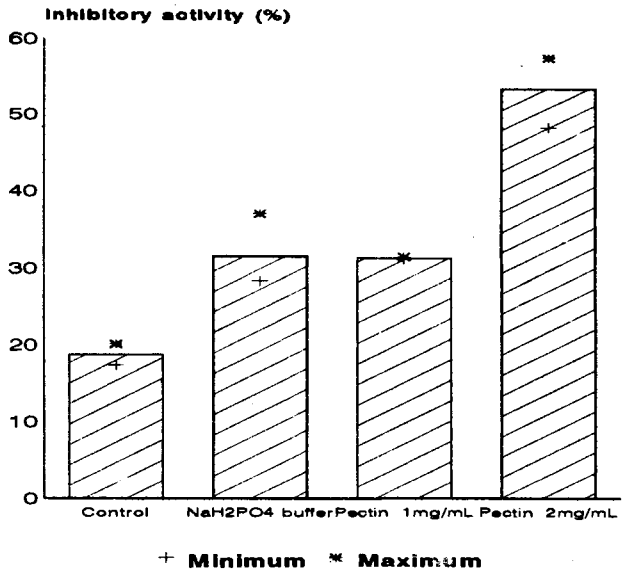

Figure 2. Trypsin inhibitory activity induced by pectin derivatives

Inhibitory activity induction by the buffer used to disolve inducers does not disagree with other authors' results (Ryan, 1974). However, taking into account the possible analogy existing between the systemic induction of proteinase inhibitor accumulation and the systemic resistanse induced by applying phosphate solution to plants (Gottstein and Kuc, 1989), selecting another salt solution as bioessay buffer is advisable.

Chitosan is derived from chitin desacetilation. Chitosan oligomeric fragments resulting from nitrous (HNO_2) or clorhydric (HCl) acid hydrolysis, having DP between 6 and 11 are reported as powerful inducers of proteinase inhibitors in tomato leaves (Mary Walker-Simmons and Ryan, 1984).

Chitosan treatment provoked a higher effect than that of chytosan hydrolysate (Figure 3). It can be explained when taking into consideration the drastic conditions under which chytin desacetylation experiment was carried out either because of using a strong base (NaOH, 40 %) or the time (three hours) and temperature (120°C) in the process; therefore, besides the desired effect, there was a polymer degradation which gave birth to polysaccharide and oligosaccharide fractions and agree with some other authors' results (Muzzarelli, 1977; Domard and Rinaudo, 1983). Among these oligomers existing at chitosan treatment, there are some in charge of the activity recorded (Mary Walker-Simmons and Ryan, 1984).

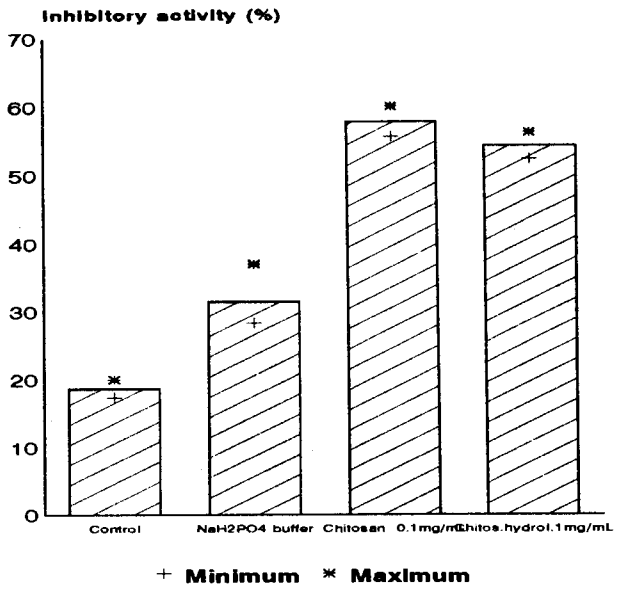

Figure 3. Trypsin inhibitory activity induced by chitin derivatives

In case of chitosan hydrolysate, even though there is a positive effect with regard to the control, the continuous breakdown of either polymeric chain or different oligomers decrease active fragments. Also, when considering chains with DP < 3 are not reported as proteinase inhibitor inducers (Mary Walker-Simmons and Ryan, 1984), it could account for a tenfold lower effect of hydrolysate related to chitosan.

In brief, it is essential to deepen upon the characterization of all oligosaccharide mixtures used in this paper as well as on the constituting species purification, so as to obtain higher specific induction values and know higher-activity chemical species in the tested products.

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