INDUCTION AND DISTRIBUTION OF AMYLOLYTIC ACTIVITY IN *CUCUMIS SATIVUS* L. IN RESPONSE TO VIRUS INFECTION

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Summary. – Inoculation of cucumber (*Cucumis sativus* L. cv. Laura) cotyledons with tobacco necrosis virus (TNV) causes both qualitative and quantitative changes in the total and fractionated protein extracts as well as in amylolytic activity. Using a specific test it was demonstrated that the virus infection strongly enhances a major band (R_f 0.0645) of amylolytic activity, predominantly located in apoplast space. The accumulation of this extracellular amylolytic activity is regulated by a time-dependent manner and is correlated with the development of necrotic lessions. The amylolytic activity may be related to degradation of starch shown to be accumulated in the immediate vicinity of necrotic lesions associated with the hypersensitive response (HR). The possible biological function of the identified amylolytic activity in the term of „pathosmosis" is also discussed.

Key words: cucumber; tobacco necrosis virus; starch; electrophoresis; immunoblot analysis

Introduction

Far the most effective mechanism of plant resistance to viruses is HR, *i.e.* the localization of the virus through a blocking of its movement in the host from the site of infection. HR is a multifaceted defense mechanism which is correlated with diverse cell biological and biochemical changes.

The early macroscopic event of HR is a rapid and often programmed cell death thought to be a mechanism of inhibition of virus spread (Keen, 1990; Greenberg, 1997). Besides a plethora of endogenous signals produced during the onset of HR, an activation of a battery of defense-related genes and accumulation of antimicrobial substances also occur (Bowles, 1990; Hammond-Kosack and Jones, 1996). These include various enzymes of secondary metabolism, PAL, LOX, GST, defensins, thionins, antivirus factors, glucanases, and chitinases (Fritig *et al.*, 1987; Lamb *et al.*, 1989; Pellegrini *et al.*, 1994; Broekaert *et al.*, 1995; Chang *et al.*, 1995; Repka *et al.*, 1997; Repka, 1997).

An extensive analysis of HR-associated events in model system of cucumber – TNV has previously been conducted. Using high-resolution two-dimensional polyacrylamide gel electrophoresis (PAGE) accompanied with ultrasensitive silver staining of proteins we demonstrated reproducible alterations in the pattern of soluble proteins extracted from intercellular fluid (ICF) of hypersensitively reacting cucumber cotyledons (Repka *et al.*, 1993). A large set of proteins comprising at least 10 major host-encoded products has been identified, and betweenwhiles the potential biological function (s) of a vast majority of them was elucidated (Repka and Slováková, 1994; Repka, 1996, 1997; Repka *et al.*, 1997). Although the exact biological role of the other components still remains unknown, preliminary experiments with various substrates directly incorporated into the gel revealed some amylolytic activity. Such an

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Abbreviations: HR = hypersensitive reaction; ICF = intercellular fluid; PAGE = polyacrylamide gel electrophoresis; PAT = plate activity test; PBS = phosphate-buffered saline, PVP = polyvinylpyrrolidone; SDS = sodium dodecyl sulfate; TNV = tobacco necrosis virus

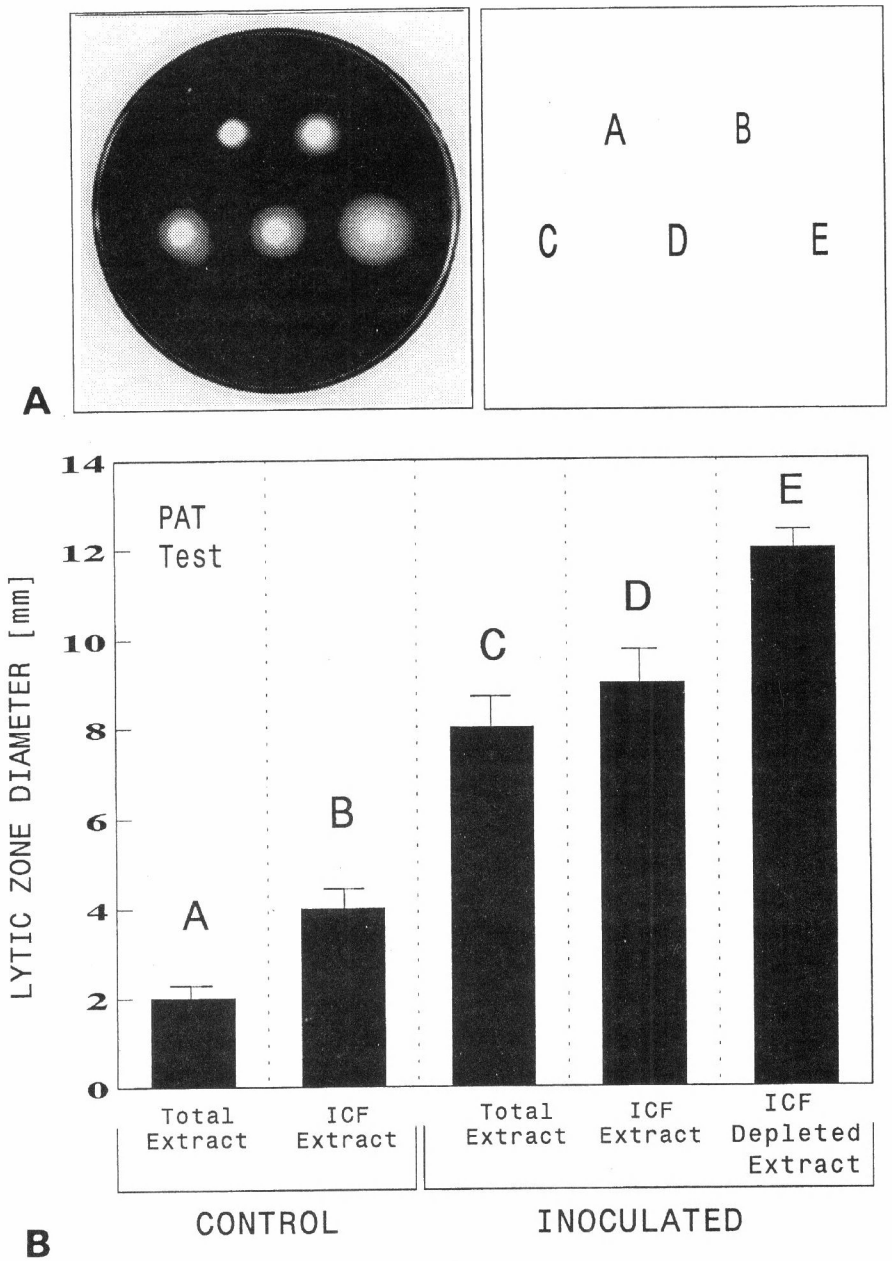

Fig. 2

Amylolytic activity in fractionated plant extracts from healthy and virus-infected cucumber cotyledons assayed by PAT

A. Samples corresponding to total plant extract (A,C), ICF-extract (B,D), and ICF-depleted extract (E) and containing equal amounts of protein (50 μg) were loaded to holes in PAT plate and developed with iodine solution.

B. Video-densitometric quantification of the lytic zones indicating starch degradation activity on PAT plate. Starch degradation activity is expressed as a lytic zone diameter. Values are means of three independent determinations and vertical bars represent maximum S.E. values.

no α-amylase-corresponding signal was detected during five consecutive days in the apoplast space of the virus-inoculated cotyledons, while an immunopositive signal of chitinase p28, HR-associated marker protein, strongly accumulated in the same ICF extract in time-dependent manner (Fig. 4).

Zymogram analysis of the amylolytic activty in plant extracts

We used PAGE in non-denaturing conditions to detect the amylolytic activity in both healthy and virus-infected plant

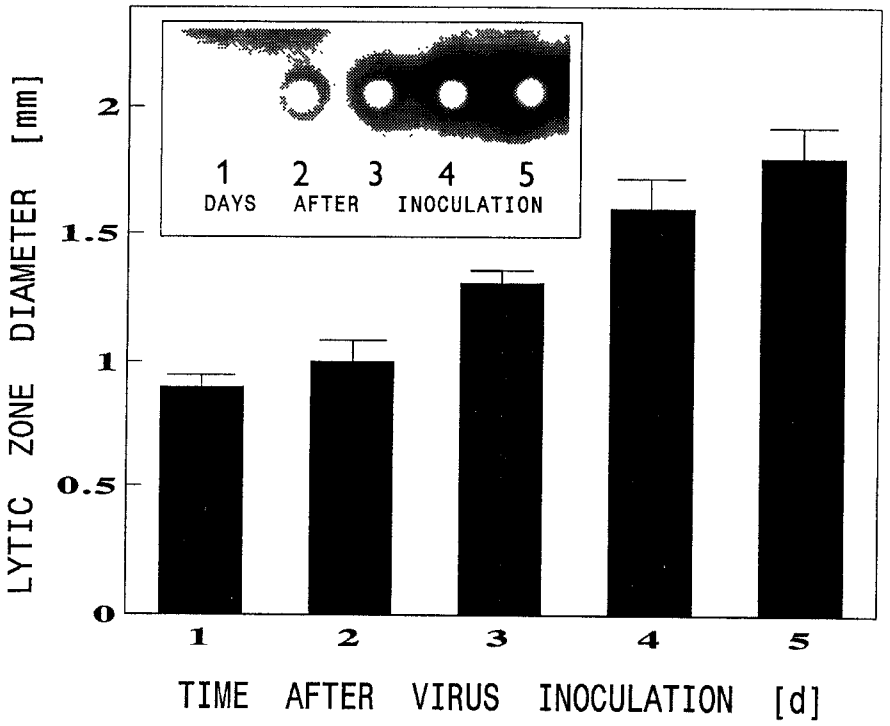

Fig. 3

Amylolytic activity in ICF extracts from virus-infected cucumber cotyledons assayed by PAT

Equal amounts of protein (40 μg) were isolated from cotyledons at days 1–5 p.i. and assayed for amylolytic activity on PAT plates (insert). Relative amounts of amylolytic activity on PAT plates were quantified videodensitometrically. Vertical bars represent maximum S.E. and the data are means of three separate measurements.

extracts in a non-quantitative manner. Incubation of gels at an appropriate pH after anodic PAGE followed by *in gel* activity staining with iodine solution revealed a single band with amylolytic activity (Fig. 5B). The isoenzyme band at R_f value of 0.0645 migrated very slowly and occupied the upper part of the gel. Although a very slight constitutive expression of this isoenzyme was detected in ICF extracts of healthy plants, a massive accumulation of the enzyme in the ICF from virus-infected plants was evident. Comparing the signal intensities using densitometric tracing of the gel showed that the signal present in the ICF from the virus-infected cotyledons was roughly 25 times more intense than that from control plants. More interestingly, we were surprised to find that the intensity of amylolytic signal from ICF-depleted extracts obtained from the virus-infected cucumber cotyledons did not correlate with that observed on PAT plate. This may indicate the presence of other protein (s) with a putative amylolytic activity non-separable in anodic PAGE system. As shown in Fig. 5A, the R_f value comparison enabled us to identify the protein band with a putative amylolytic activity on the silver-stained gel loaded with corresponding plant extracts and run under identical conditions with those used for activity staining.

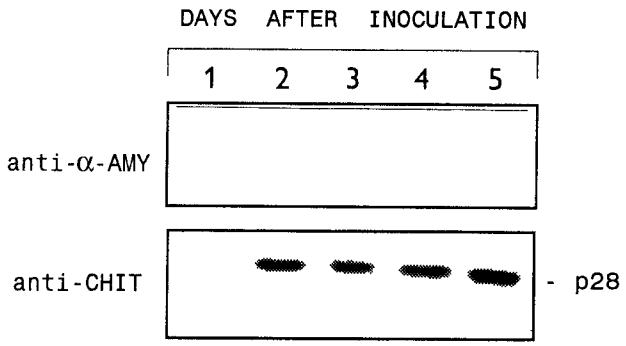

Fig. 4

Western blot analysis of the time course of accumulation of α-amylase and p28 chitinase in virus-infected cucumber cotyledons

Equal amounts of ICF-protein (50 μg/lane) were loaded on a non-denaturing 10% polyacrylamide gel. The blots were probed with the anti-mung bean α-amylase serum and the anti-cucumber chitinase (p28) serum.

In situ histochemical analysis of starch accumulation in healthy and virus-infected cucumber cotyledons

To investigate whether the stimulated amylolytic activity correlates with the development of macroscopic symptoms

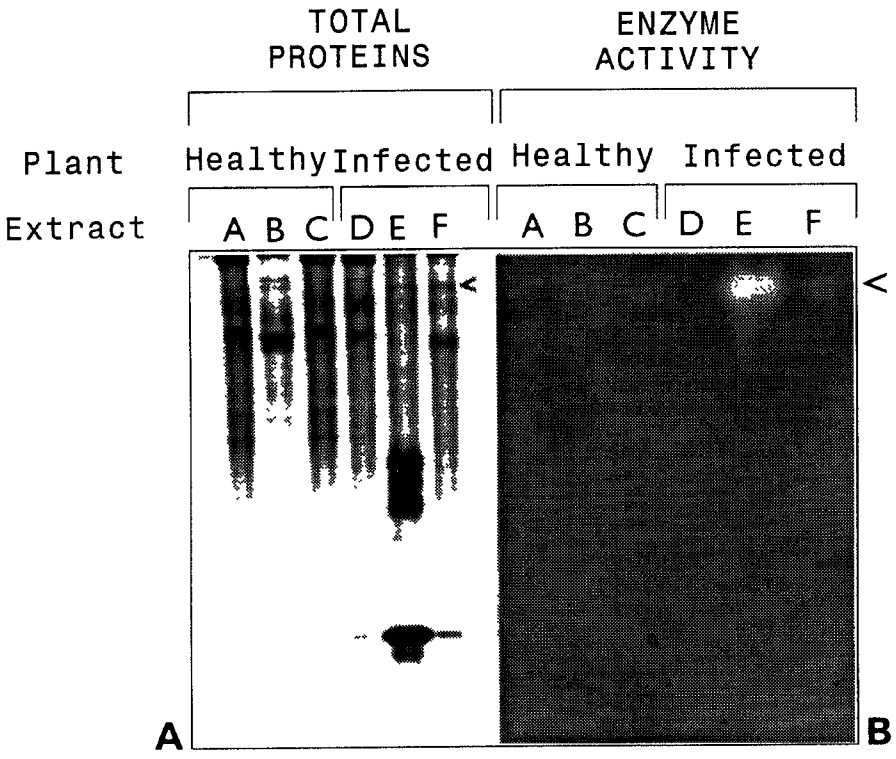

Fig. 5

Protein and amylolytic enzyme profiles from healthy and virus-infected cucumber cotyledons assayed by PAGE

Samples corresponding to total plant extract (A,D), ICF-extract (B,E), and ICF-depleted extract (C,F) and containing equal amounts of protein (10 μg/lane) were loaded on a 10% non-denaturing polyacrylamide gel and stained with silver (A). The identical samples but containing 50 μg of protein were loaded on a paralel gel and, after the electrophoresis was completed, the gel was stained for amylolytic activity with iodine solution (B) Arrows denote the position of protein with amylolytic activity

of HR we conducted an *in situ* histochemical analysis of starch metabolism in both healthy and virus-infected cotyledons. The intact cotyledons were first photographed and then processed for histochemical determination of starch using staining with iodine solution. The results of this experiment (Fig. 6A,B) demonstrate that the leaf transitory starch in the control and virus-infected cotyledons was degraded to almost the same extent. In contrast, the histochemical staining of starch revealed a strong positive signal in virus-infected cotyledons which spatially correlated with the symptoms of HR. Moreover, at higher magnifications (Fig. 6C), it was clearly evident that the starch was strictly localized in the vicinity of necrotic lesions.

Discussion

In the present study we investigated the effect of virus infection on induction and accumulation of amylolytic activity in cucumber cotyledons as a first step toward elucidating the role of HR-associated defense-related genes.

As revealed by the highly sensitive PAT, the amylolytic activity strongly accumulated in the apoplast space of virus-inoculated cucumber cotyledons although the spectrophotometrical quantitation indicated, albeit at a very low level, a constitutive accumulation of putative amylolytic activity also in apparently healthy plants. It is interesting that the PAT did not confirm such an accumulation pattern of amylolytic activity in uninfected leaves. Thus, these contradictory results may be viewed in the light of the inherent principles of both assays used in this work. Since both the tests were performed in different buffer systems it is possible that the total amylolytic activity reflects different amylolytic enzymes, and that particular amylolytic enzymes have different optimal conditions.

The increased accumulation of amylolytic activity is not an inherent characteristic of virus-induced HR but, on the contrary, it may be generally related to stress. The induction and/or modulated expression of amylolytic activity

(especially for α-amylases) has been demonstrated in barley leaves by water stress (Jacobsen *et al.*, 1986), in osmotically stressed cucumber cotyledons (Wagih and Coutts 1982), virus-infected tobacco leaves (Heitz *et al.*, 1991), and wounded mung bean leaves (Koizuka *et al.*, 1995). Moreover, the anoxia was found to be modulating the expression of α-amylase in cereal seeds (Perata *et al.*, 1993).

Based on the time course experiments of accumulation of amylolytic activity in the apoplast space as monitored by PAT it is clearly evident that the kinetics of its induction is very similar to that described for other defense-related proteins, *e.g.* chitinase and β-1,3-glucanase (Repka, 1997; Repka *et al.*, 1997). In contrast to this, the Western blot analysis in the present study demonstrated that the time course of virus-induced increase in the level of protein for the amylolytic activity and stress-related chitinase (p28) were different from each other. There are two possible explanations for the absence of α-amylase-specific signal on the Western blots. One explanation may reflect the fact that the amylolytic activity did not serologically cross-react with the serum prepared against the mung bean α-amylase. Interestingly, we have previously shown that neither anti-cucumber peroxidase nor anti-cucumber chitinase and β-1,3-glucanase sera serologically cross-reacted with the corresponding proteins in the *Fabaceae* family (Repka, 1996). Another explanation may respect the situation that the amylolytic activity is rather other starch-degrading enzyme than an α-amylase. In this context it is important to note that besides the α-amylase activity there are other routes for starch degradation. The starch may be degraded also by starch debranching enzymes (DBEs) or by a phosphorolytic attack on starch granules mediated by some pyrophosphorylase (Journet and Douce, 1985).

In this study, we used an *in gel* activity staining technique to identify and visualize the position of protein (s) with a putative amylolytic activity on non-denaturing polyacrylamide gels. We demonstrated that a cucumber protein with R_f of 0.0645 displays high amylolytic activity in ICF extracts from virus-infected leaves and very low activity also in ICF from apparently healthy counterpart. Accumulation of α-amylases in virus-infected or osmotically-stressed cucumber plants has previously been reported (Wagih, 1992; Wagih and Coutts, 1982). It is interesting that both the α-amylase isoforms detected had a much higher R_f values (0.7 and 0.1) as compared to that found by us. Furthermore, there was no information available about a compartmentalization of either of these isoforms since only total plant extracts were analyzed by these authors. Therefore, we suppose that differences between these two observations are rather cultivar-specific than pathogen-specific, or more broadly, stress-specific.

One of the major questions which underline the main objective of the present study is the physiological significance of the enhanced accumulation of amylolytic activity by stress. Stress, such as pathogen infection or

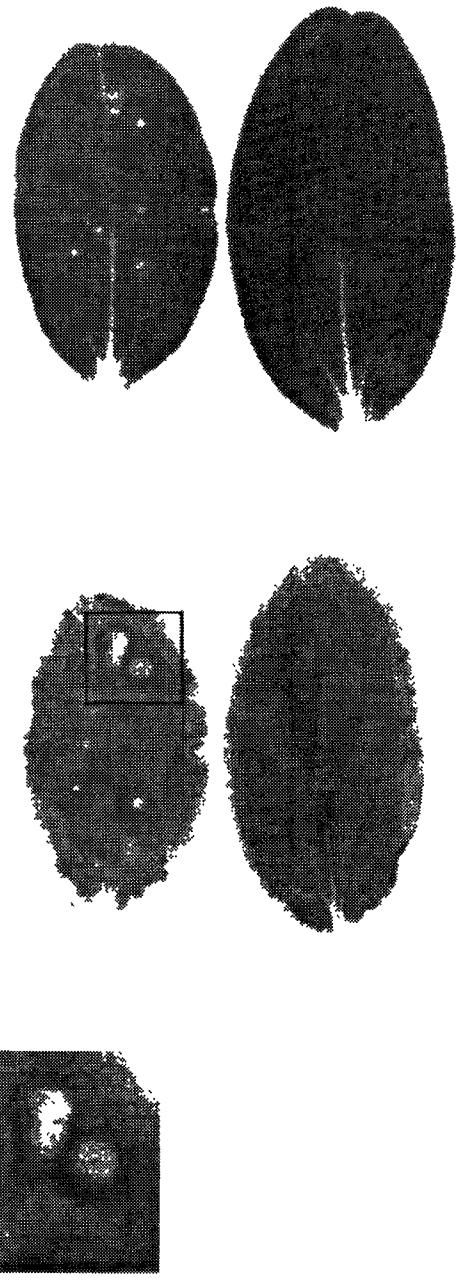

Fig. 6
Starch distribution in healthy and virus-infected cucumber cotyledons
Cotyledons were harvested from plants at the end of the night and photographed immediately to show necrotic lesions (A), and the same leaves were stained with iodine solution for the presence of starch (B). Part C represents a higher magnification (4x) of the upper part of the virus-infected cotyledon showed in part B to demonstrate a more detailed view of colocalization of starch with the edge of necrotic lesion.

wounding, is supposed to switch cellular metabolic activities from a less-active state to a highly active state. It is to be expected that, in stress situations, cells may have strong demand for energy and carbon to fulfill the needs of defense response mechanism. Thus, the process of active starch degradation which may occur in infected tissues provides the hydrolysis products available for the metabolic activities in stressed cells. A similar possibility has been suggested by Sturm and Chrispeels (1990) who observed an enhanced expression of extracellular β-fructosidase in carrot roots in response to pathogen infection or wounding. Additionally, Benhamou *et al.* (1991) reported an accumulation of β-fructosidase (cwbF) in roots of tomato plants infected with the necrotrophic fungus *Fusarium oxysporum* in the transition zone where the walls of both partners of interaction contacted each other. This pattern of enzyme distribution suggests that the induction of extracellular β-fructosidase and perhaps the amylolytic activity are parts of the plant´s defense response.

To relate the putative amylolytic activity to the process of starch degradation we performed the *in situ* histochemical analysis of starch distribution in healthy and virus-infected cotyledons. By doing so we were surprised that the starch-positive signal tightly correlated with the shape and distribution of necrotic lesions. Thus it seems that the starch was either synthesized *de novo* or only partially degraded rather than completely hydrolyzed. Because the histochemical test for starch was performed on cotyledons harvested at the end of night, *i.e.* in the period when transitory starch synthesized during the previous day was degraded at the night, its *de novo* synthesis is less probable. Therefore, a more acceptable alternative is a partial degradation of the starch located around the lesions. Two distinct types of starch hydrolyzing enzyme activities may be considered in this phenomenon. One is an α-amylase activity which hydrolyzes the α-1,4-linear linkage between glucosyl units. Other is related to some starch DBEs, *e.g.* iso-amylase which hydrolyzes only the α-1,6-branch linkage in branched glucans and has no activity at all toward the α-1,4-linkage. To be more precise in understanding the role of amylolytic activity identified in the present work the tests of substrate specficity should be performed. The partial degradation by at present unknown mechanism(s) of starch colocated with necrotic lesions is supported by a finding that the color of starch stained by iodine solution appeared as purple-red. Kuipers *et al.* (1994) reported that the red color is typical of amylose-free starches, while the starch containing amylose stains with iodine dark blue. The pathogen-induced process of partial degradation of transitory starch fits the hypothesis of „pathosmosis" first coined by Wagih (1981). Similarly, we suggest that partial cleavage of starch into free sugars (glucose and maltose) may double the osmotic pressure with the subsequent development of „pathosmotic" stress resulting in HR symptoms.

In conclusion, the present study indicates important function (s) for the amylolytic activity in a pathogen-triggered stress and perhaps in other physiological processes. These findings will constitute the basis for future, more detailed studies, such as analysis of the substrate specificity of identified amylolytic activity and immunochemical identification of its expression in the course of development of the HR.

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