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Inactivation of *Rhizoctonia solani* toxin by a putative α-glucosidase from coconut leaves for control of sheath blight disease in rice

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Summary

Inactivation of a host-specific toxin, *RS*-toxin, induced by *Rhizoctonia solani*, the cause of rice sheath blight disease was investigated. A putative α-glucosidase identified based on enzyme assay and Western blot analysis was purified from coconut (*Cocos nucifera*; the only known non-host of *R. solani*) leaves and tested for its efficacy in degrading *RS*-toxin. SDS–PAGE analysis showed the appearance of a 97 kDa protein, which appeared in proteins extracted from coconut leaf bits during 48 and 96 h after *RS*-toxin-treatment and the protein eventually disappeared. A comparison of the u.v. spectra read at 150–300 nm revealed conspicuous disturbances in the absorbance at 24 h of incubation of *RS*-toxin with the coconut leaf protein extracts as compared to that at 12 h, indicating the possible degradation of *RS*-toxin by coconut leaf α-glucosidase during incubation. Incubation of rice leaf sheath bits with coconut leaf protein extracts significantly reduced electrolyte leakage due to *RS*-toxin 30 min after the toxin treatment. Simultaneously, there was a significant reduction in sheath blight symptoms when the incubation of rice leaf sheaths with the coconut leaf protein extracts was extended up to 96 or 120 h. This appears to be the first report of purification and characterization of a putative plant α-glucosidase.

Introduction

α-Glucosidase (EC 3.2.1.20) is an exo-carbohydrase which catalyzes the splitting of α-glucosyl residues from the non-reducing terminus of substrates to liberate α-glucose (Yamasaki & Suzuki 1980; Chiba 1997). Such enzymes are widespread among microorganisms, plants and animals. Yamasaki *et al.* (1973) purified and crystallized an α-glucosidase protein from the mycelium of *Mucor javanicus*. Yamasaki *et al.* (1995) reported an α-glucosidase protein of 67 kDa molecular weight with a pI value of 5.0 from *Trichoderma viride*. Though α-glucosidase is considered an important enzyme in the food industry, its ability to control plant pathogens by possible inactivation of their phytotoxins has only recently been realized. Rice sheath blight caused by *Rhizoctonia solani* Kühn is a serious disease in all rice-growing countries (Roy 1993). The pathogen induces a glycoproteinaceous host-specific toxin, designated as *RS*-toxin, which reproduces all the symptoms of the disease and is associated with the virulence of the pathogen (Vidhyasekaran *et al.* 1997a). Sriram *et al.* (2000) reported a high molecular weight (110 kDa) *RS*-toxin-degrading protein identified as α-glucosidase from a *T. viride* isolate, TvMNT7 when *RS*-toxin was provided as sole carbon source. We report here the isolation and purification of an α-glucosidase protein from coconut leaves, the non-host of *R. solani* that degrades *RS*-toxin.

Materials and methods

Isolation and purification of RS-*toxin*

Isolation and purification of toxin was carried out from rice sheaths inoculated with *R. solani* as follows: the fungus was inoculated on rice sheaths (cv. IR 50) of 40–45 day-old plants. After 1 week, *R. solani*-infected sheaths along with the necrotic lesions were collected and cut into small pieces (0.5 cm) and ground with distilled water (5 ml/g). The extract was filtered and centrifuged (12,096 × g, 15 min) to remove the chlorophyll and the centrifugation was repeated till all the chlorophyll materials had sedimented. The water fraction containing the toxin was concentrated to one-tenth of its original volume by flash evaporation in vacuum at 50 °C. An equal volume of methanol was added to the concentrated culture filtrate and incubated overnight at 5 °C. The precipitate was removed by filtration and

methanol was evaporated in vacuum at 50 °C using a vacuum flash evaporator. The water fraction was partitioned with three volumes of ethyl acetate, hexane and chloroform in sequence using a separating funnel. The partitioned material was concentrated to half its volume by flash evaporation and subjected to column chromatography on Sephadex G-75 (Sigma, USA). The toxin was eluted with distilled water and fractions of 5 ml volume were collected from the outlet after allowing 15 ml of void volume. From the graph obtained based on absorbance (230 nm) of each fraction in the recorder attached to the u.v. monitor of the chromatography unit, fractions coinciding with peak absorbance were collected and used for further studies.

Precipitation of RS-*toxin-degrading protein from detached leaves consequent to treatment with* RS-*toxin and inoculation with pathogen*

Rice sheaths of uniform thickness and length (7 cm) collected from 40–50 day-old rice plants (cv. IR 50) grown under glass house conditions and young coconut leaves of size 5 × 5 cm were cut and surface sterilized in 0.1% mercuric chloride for 30 s and washed with repeated changes of sterile distilled water. Two sheaths/leaf bits were placed on sterile glass slides kept on two layers of moist filter paper inside a Petri dish. Five millimeters culture disc of the pathogen, *R. solani* and 10 μl (50 μg glucose equivalents) of purified *RS*-toxin were inoculated separately on leaf sheaths/leaf bits and incubated for 24, 48, 72, 96 and 120 h at room temperature (28 ± 2 °C). Sterile water inoculation served as control. The samples were collected after the incubation periods and immediately homogenized in a pestle and mortar with liquid nitrogen. One gram of powdered sample was extracted with 2 ml of 0.1 M sodium phosphate buffer pH 7.0. After centrifugation (12,096 × g, 15 min) at 4 °C, the supernatant was taken and added to an equal volume of ice-cold acetone and incubated at 4 °C for 3 h. The protein in the extract was pelleted by centrifugation and the pellets were dissolved in sodium phosphate buffer (pH 7.0, 0.1 M).

Assay for the α*-glucosidase activity of* RS-*toxin-degrading protein from detached coconut leaves*

The assay for the α-glucosidase activity was carried out in the protein extract as described by Nakao *et al.* (1994) by monitoring the *p*-nitrophenolate released from *p*-nitrophenyl α-D-glucopyranoside at 55 °C. The assay mixture contained 1 ml of 21 μM *p*-nitrophenyl α-D-glucopyranoside, 1 ml of 21 μM sodium phosphate buffer (pH 7.2) and 1 ml protein extract in triplicate. The reaction mixtures (before the addition of protein extract) were brought to 55 °C by keeping them in an incubator for 30 min. The change in absorbance of the reaction mixture at 405 nm after the addition of protein extract was recorded. One set of reaction mixture heated in boiling water bath for 15 min was used as blank. The extinction coefficient for *p*-nitrophenolate under these conditions was 13,400 l/mol/cm (Nakao *et al.* 1994). One unit of the enzyme was defined as the amount of enzyme that catalyzed the formation of 1 μmol *p*-nitrophenolate per minute at 55 °C.

SDS–PAGE analysis of the putative α*-glucosidase protein extracted from* RS-*toxin-treated coconut leaf bits*

SDS–PAGE analysis of *RS*-toxin-inactivating protein precipitated from detached coconut leaves inoculated with *RS*-toxin (5 g glucose equivalents/l) and incubated for 0, 24, 48, 72, 96 and 120 h was carried out in polyacrylamide slab gels consisting of 4% stacking gel and 12% separating gel using a Sigma Aldrich Techware system (Sigma, USA) following the method suggested by Laemmli (1970). Protein content of the sample was estimated by the Bradford method and the sample was loaded at the rate of 50 μg per well. Electrophoresis was carried out at a constant 65 V and the gel was stained with Coomassie brilliant blue. Molecular weights were determined based on the R_f value of the protein bands.

Purification of the putative α*-glucosidase protein of coconut leaves*

The putative α-glucosidase protein (97 kDa) of coconut leaves treated with *RS*-toxin was purified by native gel electrophoresis. All the wells in the gel were loaded with the same sample, and after electrophoresis, one single lane was cut out using a sterile blade and stained in Coomassie brilliant blue. The remaining part of the gel was soaked in soaking buffer (3.16 g ammonium bicarbonate dissolved in 100 ml distilled water). Based on the position of the high molecular weight protein band in the stained lane, the corresponding area of the gel in soaking buffer was cut with a sterile blade and transferred separately to a dialysis bag containing an elution buffer (1.98 g ammonium bicarbonate dissolved in 500 ml distilled water). The protein in the gel was subjected to electroelution with an electrode buffer (without SDS) at 50 V for 1 h (Salas *et al.* 1995). The protein eluted into the elution buffer was dialysed against distilled water overnight at 4 °C and lyophilized. The purified protein was loaded into separate wells and subjected to SDS–PAGE. Presence of a single band confirmed the purification of putative α-glucosidase protein of coconut leaves.

Antifungal activity of the putative α*-glucosidase protein against* R. solani

Effect on sclerotial germination

Sclerotia of *R. solani* with uniform size grown on PDA were soaked in a preparation containing the purified form of the putative α-glucosidase protein dissolved in sodium phosphate buffer pH 7.0. The protein was estimated by the Bradford method and 10, 20 and 50 μg

concentration were used. Sclerotia soaked in sterile distilled water served as control. Each treatment contained three replications and five sclerotia were maintained for each replication. The treated sclerotia kept in microfuge tubes were incubated on potato dextrose agar (PDA). The number of sclerotia germinated after 24, 48 or 72 h was counted.

Effect on mycelial growth

The antifungal activity of the purified α-glucosidase protein of coconut leaves was studied on the basis of the inhibition of mycelial growth of *R. solani*. Three millimeter diameter paper discs from Whatman No. 1 filter paper were cut and sterilized by baking at 150 °C for 6 h. In sterile 90 mm Petri plates of three numbers, 18 ml of PDA medium was poured separately and three discs were placed equally spaced on the outside periphery of each plate. Five microgram of purified α-glucosidase protein dissolved in sodium phosphate buffer pH 7.0 was added to the discs using sterile pipettes. Sterile distilled water served as control. Fungal discs (8 mm) were punched from 5 day-old culture of *R. solani* and placed in the middle of the Petri plate. The plates were incubated at room temperature (28 ± 2 °C) and the inhibition of mycelial growth was observed after 3 days of incubation.

Changes in electrolyte leakage from rice leaf sheath bits due to RS-*toxin upon incubation with coconut α-glucosidase*

The protein extracts of coconut leaves inoculated with *RS*-toxin for 0, 24, 48, 72, 96 and 120 h were lyophilized separately (SENTRY, The Virtis Company Inc, NY, USA) and 1.5 ml of each of the protein sample (100 μg/ml) was incubated with 1.5 ml *RS*-toxin (100 g glucose equivalents/ml) separately at 37 °C for 12 and 24 h. *RS*-toxin incubated with sterile water and healthy leaf extract separately served as control. Three replications were maintained for the treatment and control. After the incubation period, the electrolyte leakage by *RS*-toxin was assessed. Rice leaf sheaths from 40-day-old plants were cut into small pieces (2 mm) and tied in washed muslin cloth. The sheath bits in the cloth (100 mg leaf bits/cloth bag) were placed into 3 ml (5 mg/ml) of *RS*-toxin. The sheath bits were vacuum infiltrated with toxin for 30 min; cloth bags were washed in repeated changes of sterile water and placed into 10 ml sterile water. Electrical conductivity was measured at 15 min interval up to 45 min using conductivity meter (Orion Research, Inc, Boston, Mass.) and the electrolyte leakage was expressed as μSiemens/100 mg of rice leaf tissue.

U.v. spectral analysis of the effect of coconut leaf α-glucosidase on RS-*toxin activity*

RS-toxin incubated with protein extracts of coconut leaves and healthy plant extract as described earlier were collected and subjected to u.v. spectrum analysis separately in a spectrophotometer (ECIL) with wavelength range from 150 to 300 nm and with a maximum absorbance of 2.0.

Changes in ratings of sheath blight due to RS-*toxin upon incubation with coconut leaf α-glycosidase protein*

The protein extracts of coconut leaves inoculated with *RS*-toxin for 0, 24, 48, 72, 96 and 120 h were lyophilized separately and incubated with *RS*-toxin for 12 and 24 h as described for the studies on changes in electrolyte leakage from rice leaf sheath bits due to *RS*-toxin upon incubation with coconut α-glucosidase. Similar type of control treatments were also maintained and each treatment consisted of three replications. After the incubation period, symptom development on detached rice sheaths was studied for the treatments as follows: Ten microliters (50 μg glucose equivalents) of each of the *RS*-toxin treatment was placed on the sheaths separately and incubated for 7 days at room temperature (28 ± 2 °C). Symptoms developed were graded using 0–5 scale (Ahn *et al*. 1986).

Production of polyclonal antibodies against coconut leaf α-glucosidase

The polyclonal antibodies to the purified toxin-inactivating α-glucosidase protein (97 kDa) of coconut leaves were produced in rabbit by intramuscular immunization. The protein content was assessed and 100 μg protein was used for each immunization. Adult New Zealand white rabbits weighing about 1.5 kg were used for immunization. One milliliter of Freund's complete adjuvant added to 1 ml of purified protein preparations (100 μg protein/ml) were emulsified in a cyclomixer. The emulsion was taken in a sterile syringe with 22 G needle and administered intramuscularly in the rabbits. A booster injection was given 4 weeks after the first injection with same quantity of antigen emulsified with Freund's incomplete adjuvant. A second booster injection was given 2 weeks later. After bleeding, blood was transferred to sterile glass vials and allowed to stand in a slanting position till the blood clotted. The antisera were transferred to sterile centrifuge tubes and the red blood cells were pelleted by repeated centrifugation (8000 rev/min, 4 °C, 10 min) for three times. The antisera were transferred to sterile microfuge tubes and stored at −70 °C for further studies.

Western blotting

The 97 kDa protein of coconut leaves was subjected to SDS–PAGE and the protein was electroblotted onto 0.45 μm polyvinylidene difluoride (PVDF) membranes (Sigma, USA) as described by Gallagher *et al*. (1995). The electrophoretic transfer of protein was carried out from gel to membrane in a Bio-Rad semi-dry blot transfer apparatus (140 mA, 30 min). The membranes

were then stained with Ponceau S stain (Sigma, USA) for 5 min to check the resolution and transfer quality. Ponceau S stain was destained using Tris-buffered saline containing 0.05% Tween (TBST) for 2 min. After destaining, the membranes were blocked for 1.5 h at room temperature (28 ± 2 °C) in TBST containing 2.5% gelatin (Sigma, USA). The membranes were then soaked in the diluted (1:3000) primary antibodies (anti-rabbit) separately for 3 h in TBST. After incubating with the primary antibodies, the membranes were washed three times with TBST for 10–15 min each time to remove the unbound antibodies. The membranes were then incubated in secondary antibodies for 3 h. Affinity-purified goat anti-rabbit immunoglobulin (IgG) (Bangalore Genei, India) and goat anti-chicken immunoglobulin (IgG) (Sigma, USA) conjugated with alkaline phosphatase were used as secondary antibodies at a dilution of 1:7000. The membranes were then washed three times with TBST for 10–15 min each time. Immunological reactions were visualized by soaking the membranes in alkaline phosphatase colour development reagents (Bangalore Genei, India). After colour development, the membranes were washed in distilled water, dried and stored at 4 °C.

Statistical analyses

The experiments were carried out in completely randomized design and statistical analyses of the experiments were performed using the package, IRRISTAT version 92-1 developed by the International Rice Research Institute Biometrics Unit, The Philippines.

Results

Assay of α-glucosidase activity in detached leaves consequent to inoculation with RS-*toxin or* R. solani

Specific α-glucosidase activity in *RS*-toxin treated or pathogen-inoculated leaf bits recorded a peak at 96 h after inoculation and such a rise was the greatest in the case of *RS*-toxin-treated leaf bits. However, a decreased α-glucosidase activity was observed beyond 96 h irrespective of the treatments (Figure 1).

SDS–PAGE analysis of proteins extracted from RS-*toxin-treated leaf bits*

The SDS–PAGE analysis of proteins extracted from *RS*-toxin-treated leaf bits showed the appearance of a 97 kDa protein at 48 and 96 h after *RS*-toxin-treatment. However, the protein disappeared at 120 h after treatment as observed in detached leaf bioassay (Figure 2).

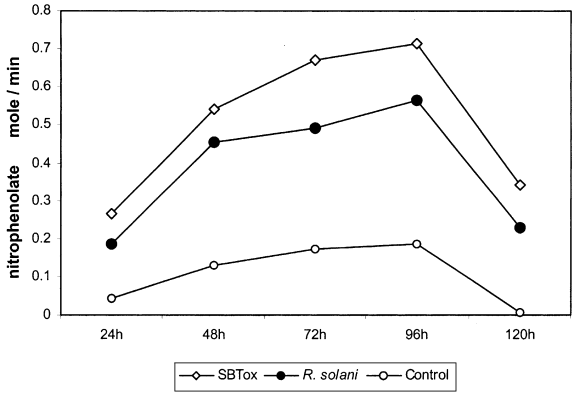

Figure 1. Assay of α-glucosidase activity in detached coconut leaves consequent to inoculation with *RS*-toxin. Each value represents the mean of three replications.

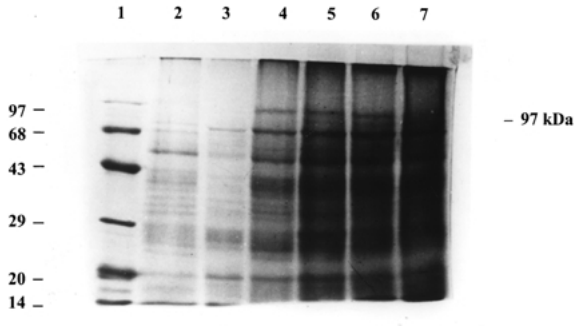

Figure 2. SDS–PAGE analysis of proteins extracted from coconut leaves upon treatment with *RS*-toxin. Lane 1 – Marker; Lane 2 – Control; Lane 3–7 – 24, 48, 72, 96 and 120 h after *RS*-toxin treatment respectively.

Changes in electrolyte leakage from rice leaf sheath due to RS-*toxin upon incubation with leaf α-glucosidase protein*

When compared with control, the *RS*-toxin treated with the leaf α-glucosidase protein for 96 or 120 h, induced lesser electrolyte leakage from rice leaf sheath. Increased electrolyte leakage from rice leaf sheaths was noticed with an increase in the incubation of *RS*-toxin with leaf α-glucosidase protein (Table 1).

U.v. spectrum analysis of the effect of leaf α-glucosidase activity on RS-*toxin*

A comparison of the u.v. spectra read at 150–300 nm revealed conspicuous disturbances in the absorbance, after 24 h of incubation with the leaf α-glucosidase protein as compared to that after 12 h. *RS*-toxin treatment on leaf bits for 120 h however did not show any disturbances in the u.v. spectra when the α-glucosidase protein purified from it was incubated with *RS*-toxin (Figure 3a and b).

Table 1. Susceptibility of rice leaf sheath bits to *RS*-toxin upon incubation with coconut α-glucosidase.

Time after *RS*-toxin treatment in coconut leaves for α-glucosidase induction (h)	Electrolyte leakage (μS)*					
	Incubation period of *RS*-toxin with coconut α-glucosidase (h)					
	12			24		
	15 min	30 min	45 min	15 min	30 min	45 min
0	143[d]	248[b]	319[b]	158[c]	224[b]	293[b]
24	152[b]	189[d]	310[c]	130[d]	174[d]	253[d]
48	134[e]	183[e]	278[e]	127[e]	153[e]	249[e]
72	125[f]	143[f]	207[f]	112[f]	125[f]	187[f]
96	99[g]	129[g]	120[g]	85[g]	102[g]	114[g]
120	90[h]	127[g]	118[g]	71[h]	93[h]	109[h]
Toxin + water	238[a]	276[a]	357[a]	179[a]	234[a]	298[a]
Toxin + healthy leaf	146[c]	231[c]	304[d]	169[b]	212[c]	278[c]

* Mean of three replications; values in a column and row followed by a common letter are not significantly different at 5% level by DMRT; interaction $T \times P$ (12 h) LSD (5%) 2.011; (24 h) LSD (5%) 2.297.

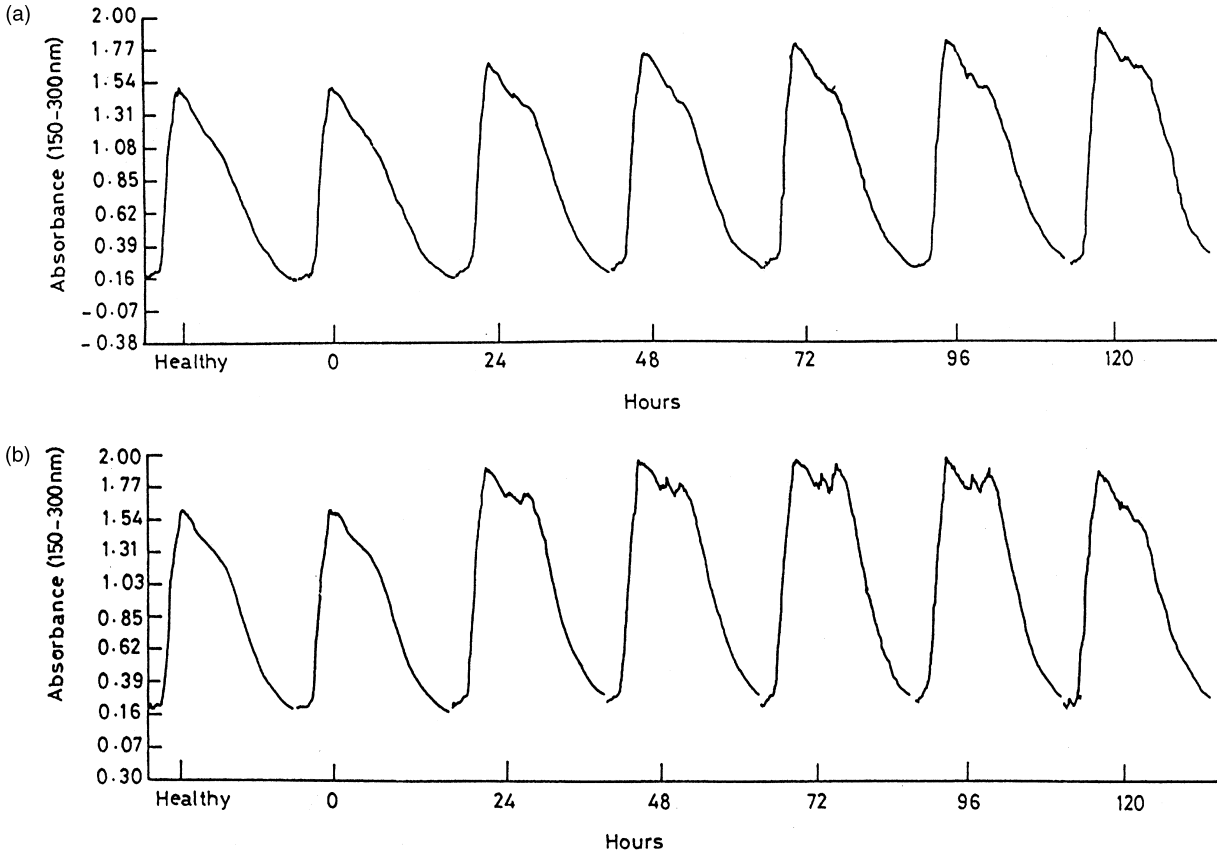

Figure 3. (a) Absorbance spectra of degraded/undegraded *RS*-toxin incubated with coconut α-glucosidase for 12 h. Each value represents the mean of three replications. (b) Absorbance spectra of degraded/undegraded *RS*-toxin incubated with coconut α-glucosidase for 24 h. Each value represents the mean of three replications.

Changes in sheath blight ratings due to RS-*toxin upon incubation with leaf protein extracts*

Significant reduction in disease ratings was noticed, when the incubation of rice leaf sheaths with the leaf α-glucosidase protein was extended up to 96 or 120 h (Table 2).

Western blot analysis

In Western blot analysis with anti-97 kDa putative leaf α-glucosidase antibody raised in rabbit, the 97 kDa putative leaf α-glucosidase and a commercial α-glucosidase (purified from *Bacillus stearothermophilus*; Sigma, USA) were detected as a single band (Figure 4).

Table 2. Sheath blight development on rice sheaths by *RS*-toxin upon incubation with coconut α-glucosidase.

Time after *RS*-toxin treatment in coconut leaves (h) for induction of α-glucosidase	Disease rating*	
	Incubation period of *RS*-toxin with coconut α-glucosidase (h)	
	12	24
0	4.00[a]	4.33[a]
24	3.67[a]	3.33[b]
48	3.00[b]	2.33[c]
72	2.00[b]	1.67[d]
96	1.67[c]	1.33[d]
120	1.67[c]	1.00[d]
Toxin + healthy leaf extract	4.00[a]	4.33[a]
Toxin + water	4.00[a]	4.00[a]

* Mean of three replications; values in a column followed by a common letter are not significantly different at 5% level by DMRT; interaction $T \times P$ LSD (5%) 0.6502.

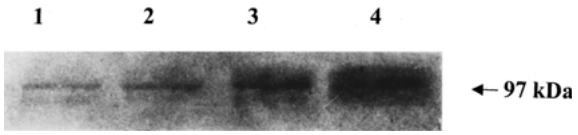

Figure 4. Western blot analysis of the 97 kDa coconut leaf protein. Lane 1 – *B. stearothermophilus* α-glucosidase; Lane 2–4 – Purified 97 kDa protein of coconut leaf at 50, 70 and 90 μg concentrations respectively.

Discussion

Rhizoctonia solani, the causal agent of rice sheath blight induces a host-specific toxin (*RS*-toxin; a glycoprotein) during pathogenesis (Vidhyasekaran *et al.* 1997a). Many microbes, including bacteria and fungi are known to degrade phytotoxins produced by plant pathogens. Sriram *et al.* (2000) reported a putative α-glucosidase protein of 110 kDa molecular mass from the biocontrol agent, *T. viride* which is capable of inactivating *RS*-toxin. Plant α-glucosidases has been purified to homogeneity from rice (Takahashi *et al.* 1971), maize (Chiba & Shimomura 1975), buckwheat (Kanaya *et al.* 1976), sugarbeet (Chiba *et al.* 1978), pea (Sun *et al.* 1995) and spinach (Sugimoto *et al.* 1995). Although plant α-glucosidases have been known for many years and are obtained in homogenous form from various plants, the usefulness of such α-glucosidases in plant disease control particularly towards toxin-producing pathogens have not been explored yet. Earlier, Vidhyasekaran *et al.* (1997b) observed that when coconut leaves were treated with *R. solani* elicitor, three chitinases (28, 35 and 42 kDa), two β-1,3-glucanases (35 and 37 kDa) and a PR-5 protein (28 kDa) appeared at 24 h after treatment and persisted upto 96 h. Of the two chitinases purified from rice and coconut leaves, coconut chitinases inhibited the pathogen even at 1 μg/ml. Such report on the induction of defence enzymes by a fungal elicitor and the ability of the induced enzymes to inhibit the pathogen prompted us to explore the possibility of α-glucosidase induction in coconut leaves (*Cocos nucifera*; the only known non-host of *R. solani*; Vidhyasekaran *et al.* 1997b) by the pathogen as well as its toxin, and to study the efficacy of such induced α-glucosidase protein after purification in inactivating the host-specific phytotoxin produced by the sheath blight pathogen. α-Glucosidase assays on toxin/pathogen-treated coconut leaf bits indicated a spurt in the enzyme activity at 96 h after inoculation and such a rise was maximal in the case of *RS*-toxin-treated leaf bits. Further, SDS–PAGE analysis of the protein extracts of *RS*-toxin-treated coconut leaf bits indicated the appearance of a 97 kDa protein during 48 and 96 h after treatment and the protein eventually disappeared at 120 h after treatment. Earlier, Frandsen & Svensson (1998) reported that most plant α-glucosidases with a few exceptions, are large proteins of 65–150 kDa molecular mass. In Western blot analysis, the polyclonal antiserum raised against the 97 kDa protein reacted positively with both *B. stearothermophilus* α-glucosidase and 97 kDa protein of coconut leaf. These studies clearly established that the 97 kDa protein is a putative α-glucosidase with maximum induction at 96 h after *RS*-toxin treatment. However, any characterization of the 97 kDa protein as an α-glucosidase merely by enzyme assay and Western blot analysis must be conjectural. Since, plant α-glucosidases occur in multiple forms of different isoelectric point, molecular weight and, occasionally, pH optimum, protein chemical and genetic investigations are needed to elucidate the identity of the protein further. Lamb *et al.* (1989) reported that when a pathogen lands on a host surface, it probably activates the defence mechanism of the host by releasing elicitors from its cell walls. Such induced defence mechanisms may be more useful in suppressing the pathogen since their activity is more specific towards the toxin. In the present studies, the pronounced α-glucosidase activity induced by *RS*-toxin, the product of sheath blight pathogen would enable the use of the protein towards inactivating the toxin and subsequently the disease incidence. Hitherto, there have been no reports on the induction of α-glucosidase in coconut leaves and its potential in inactivating the phytotoxin, and the 97 kDa *RS*-toxin inactivating α-glucosidase protein is the first. Interestingly, the purified α-glucosidase protein when studied for its antifungal properties, did not show any inhibition of mycelial growth or sclerotial germination of *R. solani* (data not shown). The results indicated that the α-glucosidase of coconut leaves acted only on the product of the pathogen (*RS*-toxin) and not directly on the growth of the pathogen.

U.v. spectra analyses of *RS*-toxin were studied over the range 150–300 nm to observe any inactivation when incubated with the α-glucosidase protein. The conspicuous disturbances noticed in the absorbance after 24 h of incubation of *RS*-toxin with the putative α-glucosidase protein as compared to that after 12 h, indicated the possible inactivation of *RS*-toxin by the α-glucosidase protein during incubation.

Possibilities of extending the biodegradability of *RS*-toxin by the putative coconut leaf α-glucosidase to rice–*R. solani* interaction with a view to reducing the severity of blighting by *RS*-toxin produced by the pathogen *in vivo*, were also explored in the present study. The following two indicators reporting possible biodegradation of *RS*-toxin were used to assess the efficacy of the putative coconut α-glucosidase against the toxin: (i) electrolyte leakage from rice leaf sheaths, and (ii) sheath blight severity. Electrolyte leakage from rice leaf bits by *RS*-toxin was reported to be an important assay to assess the activity of the toxin (Vidhyasekaran *et al*. 1997a; Samiyappan *et al*. 1996, 1997; Sriram 1997). Incubation of *RS*-toxin inactivated by the α-glucosidase protein with rice leaf sheath bits for 96 or 120 h significantly reduced electrolyte leakage. Simultaneously, there was a significant reduction in disease severity when the incubation of rice leaf sheaths with the coconut leaf α-glucosidase was extended up to 96 or 120 h.

Although α-glucosidase is produced by many microorganisms and plants, such as sugarbeet (Yamasaki *et al*. 1996), cotton (Kishor *et al*. 1992) and spruce (Messner & Boll 1994), it was surprising to observe the absence of α-glucosidase in rice plants.

The forgoing studies clearly indicate that the α-glucosidase from coconut leaves inoculated with *RS*-toxin inactivated the *RS*-toxin, and the inactivated toxin caused significantly less leakage of electrolytes and less necrosis of tissues typical of the sheath blight disease. Since the plant α-glucosidase could inactivate *RS*-toxin, the isolation of the gene that codes for the protein from coconut leaves, and the elucidation of whether rice plants with this gene will show enhanced resistance to the sheath blight pathogen by inactivating the *RS*-toxin produced in infected tissue will be very useful. At present, studies in this regard are under progress in our laboratory.

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