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Identification of an Extracellular Acid Trehalase and Its Gene Involved in Fungal Pathogenesis of *Metarizium anisopliae*

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Trehalose is the main sugar in the haemolymph of insects and is a key nutrient source for an insect pathogenic fungus. Secretion of trehalose-hydrolysing enzymes may be a prerequisite for successful exploitation of this resource by the pathogen. An acid trehalase [EC 3.2.1.28] was purified to homogeneity from a culture of a locust-specific pathogen, *Metarhizium anisopliae*, and its properties were characterized. The gene (*ATM1*) of this acid trehalase was also isolated. The pure enzyme can efficiently hydrolyze haemolymph trehalose into glucose *in vitro*. The new acid trehalase appearing in the haemolymph of *Locusta migratoria* infected with *M. anisopliae* had the same pI and substrate specificity as the purified fungal acid trehalase, and the concentration of trehalose in the haemolymph decreased sharply after infection. RT-PCR also revealed the *ATM1* gene's expression in the haemolymph of the infected insects. Our results indicated that the acid trehalase may serve as an "energy scavenger" and deplete blood trehalose during fungal pathogenesis.

Key words: acid trehalase, *ATM1* gene, identification, fungal pathogenesis, *Metarhizium anisopliae* var. *acridum*.

Unique among microbial pathogens of insects, fungi have the ability to invade, with the aid of cuticle-degrading hydrolases, the external skeleton (=cuticle) of their hosts (*1*). For entomopathogens of the species *Metarhizium anisopliae*, subsequent growth in the host is confined largely to the haemolymph prior to death. The strategy of the fungus during this stage may include the production of toxins to reduce the impact of the cellular immune response. The acquisition of nutrients during the haemolymph phase is little understood. Trehalose [α-D-glucopyranosyl (1-1)–α-D-glucopyranoside] is a non-reducing disaccharide which is rarely found in animals but is present at high concentration in most insects. It is the predominant haemolymph sugar in many insects including *Schistocerca gregaria* and *Manduca sexta* (*2–4*), and the major carbohydrate metabolic fuel used for flight (*5*), which as a consequence must be viewed as a potential nutrient source for insect pathogenic fungi like *Metarhizium anisopliae* once they have infected the haemolymph of their host. Trehalose plays important roles in insects' physiology (*4*), and the depletion of blood trehalose may be detrimental to the insects. Seyoum *et al.* (*6*) showed that an injected trehalose supplement significantly improved the flight performance of desert locusts, *S. gregaria*, infected with *M. anisopliae* var. *acridum*, and the poor flight ability of mycosed desert locusts appears to be due in part to fungal exploitation of host trehalose.

Hydrolysis of trehalose is usually, though not exclusively, carried out by specialist trehalases rather than non-specific α-glucosidases. Trehalases purified from filamentous fungi have been characterized as "acid" or "neutral" depending on their pH optimum. Neutral trehalases are intracellular proteins involved in the catabolism of internal trehalose, while acid trehalases are extracellular or vacuolar glycoproteins that hydrolyze extracellular trehalose (*7*, *8*).

Disaccharides (*e.g.* trehalose) are impermeable substrates for some fungi, and corresponding glucosidases (acid trehalase and/or α-glucosidase) must be outside the cell membrane to hydrolyze them into glucose before use. We have shown previously that *Metarhizium* acquires host trehalose through secretion of hydrolases and absorption of glucose breakdown products using C^{14} labeled trehalose (*9*).

It is reasoned that the secretion of acid trehalase may be a prerequisite for successful exploitation of trehalose in the haemolymph of insects by the pathogen. The aim of the present work was to identify any acid trehalases produced by the locust-pathogenic isolate *M. anisopliae* var. *acridum* CQMa102 which may be responsible for the effects on host carbohydrates. Here we report the identification of the acid trehalase and its gene from *M. anisopliae*, and we also present evidence for the secretion of a fungal acid trehalase during pathogenesis of the specific locust pathogen *M. anisoplie* var. *acridum* CQMa102 for the locust *Locusta migratoria manilensis*. This is the first time that a fungal extracellular enzyme has been shown unequivocally to be secreted *in vivo* in the host and to have an impact on haemolymph biochemistry.

MATERIALS AND METHODS

Fungal Strain and Insect—Metarhizium anisopliae var. *acridum* strain CQMa102, a locust-specific strain isolated

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originally from *Ceracris kiangsu Tsai* by the Genetic Engineering Center of Chongqing University and deposited in the China General Microbiological Culture Collection Center (CGMCC) under access number 0877, was used. The strain was cultured on 1/4 strength Sabouraud's dextrose agar at 28°C under constant light until sporulation (8–10 days). The migratory locusts, *Locusta migratora manilensis*, were provided by our lab and reared on corn leaves according to the protocol of Hunter-Jones (*10*).

Acid Trehalase Assay and Protein Determination—Acid trehalase activitiy was determined as follows: Twenty-five microliters of enzyme was incubated with 25 μl of 0.1 M trehalose in 20 mM 2-(*N*-Morpholino) ethanesulfonic (MES, pH 5.5) for 10 min at 30°C. The reaction was terminated by thermal denaturation (10 min at 100°C), followed by ice cooling for 5 min to prevent protein re-folding. Glucose released was determined using a modification of the glucose oxidase assay (*11*). Absorbance was determined at 490 nm and acid trehalase activity was determined with reference to a calibration curve of standard glucose (Sigma). One unit of enzyme activity was defined as the amount of enzyme required to generate 1 μmol of glucose per min.

Protein was routinely determined by the Bradford method (*12*).

Isoelectric Focusing (IEF) and Acid Trehalase Isozyme Analysis—Vertical isoelectric focusing was carried out in $T = 5\%$, $C = 3\%$ PAGE-IEF gels according to Wang and Fan (*13*). IEF with a narrow range precast gel (Amersham, pH 4.0–5.0) was carried out according to the manufacturer's instructions. An overlay gel method was used to stain for acid trehalase isozyme activity after isoelectric focusing (*14*), and different disaccharides including trehalose, maltose and sucrose were used to analyze the specificity of acid trehalase isozymes in gels.

Purification of Acid Trehalase from M. anisopliae—The culture conditions for production of trehalase were as follows: 250 ml conical flasks containing 100 ml culture medium including 10 g/liter soluble starch and 1 g/liter ammonium sulfate (pH adjusted to 6.0) were inoculated with 3.0×10^7 conidia and then incubated at 26°C with shaking at 150 rpm for 72 h. Mycelia were removed by filtration through a sterile cotton pledget, and the filtrate was dialyzed for 30 h at 4°C against 10 volumes of distilled water, with four changes of the water.

All chromatography procedures were carried out with a Biologic Duo-Flow system (Bio-Rad, USA). Purification was monitored by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and isoelectric focusing polyacrylamide gel electrophoresis (IEF-PAGE). About 2.7 liters of dialyzed filtrate (adjusted to pH 5.8) was loaded onto a 20 ml High Q Sepharose anion column (Bio-Rad, USA) equilibrated with 10 mM MES (pH 5.8), and the resin was washed with the same buffer at a flow rate of 3.0 ml/min. The elution was carried out with a 200 ml 0.0 to 0.5 M NaCl linear gradient containing 10 mM MES (pH 5.8) at a flow rate of 2 ml/min. Fractions containing trehalase activity were collected.

Further purification was achieved by loading the sample onto a 2 ml 25Q Sepharose anion column (Bio-Rad, USA) that had been equilibrated with 15 mM acetate buffer (pH 5.0). Unbound proteins were washed with acetate buffer at a flow rate of 1.0 ml/min. A 100 ml 0.0 to 0.5 M NaCl linear gradient in acetate buffer was then used to remove bound proteins. Fractions with trehalase activity were collected.

The third purification step was carried out on a 2.6 × 70 cm gel filtrate column (Amersham, Sepharcyl-200s HR), which had previously been equilibrated with 15 mM acetate buffer (pH 5.0). 1 milliliter concentrated sample was loaded and the trehalase was eluted with the same buffer at a flow rate of 0.5 ml/min. Fractions containing trehalase activity were pooled (about 10 ml) and concentrated with a 10 kDa molecular cutoff concentration tube (Millipore).

After gel filtration, the enzyme solution was subjected to IEF in a narrow range precast gel (Amersham, pH 4.0–5.0). The band containing trehalase activity was excised and electrically eluted, which resulted in pure acid trehalase.

Deglycosylation of Pure Acid Trehalase—Deglycosylation of acid trehalase was performed in a moisture-free atmosphere using anhydrous trifluoromethanesulfonic acid (TFMS) according to Tams and Welinder (*15*) with some modifications. Fifty microliters anhydrous TFMS (Sigma) was added into a sample of moisture-free trehalase, followed by incubation at –10°C for 30 min. The reaction was terminated by adding 800 μl 2 M Tris-base (Bio-Rad). A 5 ml desalt column (Pharmacia) was used to remove Tris-base and TFMS, and the deglycosylated acid trehalase was concentrated and dissolved in 100 μl solution buffer for SDS-PAGE.

Isolation and Analysis of the ATM1 *Gene*—To isolate *ATM1*, the N-terminal amino acid sequence of the purified acid trehalase was determined by the Edman degradation method (PROCISE® cLC, Applied Biosystems), and the peptide sequence was deduced by MS/MS *de novo* sequencing. Based on the peptide sequence (WVQASGDAFDDPKQVAK), a 51 bp oligonucleotide probe (5′ TGGGTCCAGGCCTCTGGTGATGCCTTTGATGACCCTAAGCAGGTTGCCAAG 3′) was designed to screen the *Metarhizium* Fosmid DNA library and for subcloning. All molecular biology methods were performed using standard procedures (*16*). Nested primer AT631F (5′ AATGTGGACAGCATCTCGGTTGGA 3′) and AT948F (5′ TATCGTTTGTCAACCAACTCGTCGCG 3′) based on the subclone's sequence were subjected to *ATM1* gene 3′ cDNA analysis using a Takara 3′-full RACE core set (*17*). Sequence alignments were performed using the BLAST program at NCBI (*18*) and multiple sequences alignments were carried out by hierarchical clustering (*19*). Signal peptide analysis was performed on the SingalP 3.0 server (www.cbs.dtu.dk/services/singalP/). Hydropathy analysis was conducted according to Kyte and Doolittle (*20*).

Infection of Locusta migratoria manilensis *and Collection of Haemolymph*—Locusts were topically inoculated with fungal spores *viz* 5 μl cotton seed oil containing 1.0×10^5 conidia applied under the pronotum using a microsyringe. Control locusts were treated with 5 μl cotton seed oil alone. Insects were housed at 26 ± 2°C with a 12 h light:12 h dark photoperiod and were fed fresh corn leaves.

The haemolymph was collected from the arthrodial membrane of the hind legs of the locusts. Haemolymph was diluted with the same volume of sterile ice-cold anticoagulant buffer [AC buffer: 0.098 M NaOH, 0.180 M NaCl, 0.017 M EDTA (free acid)], 0.041 M citric acid, pH 5.6) and

distilled water, and then centrifuged at 10,000 rpm for 10 min. The plasma supernatant was pipetted off and stored at –20°C until used.

Determination of Trehalose in Haemolymph—Porcine kidney trehalase (2.5 U/ml; Sigma) was used to determine the trehalose content in haemolymph according to the method of Sato *et al.* (*3*). Glucose released was determined as described above.

RT-PCR Detection of ATM1 *Gene Expression*—Haemolymph of locusts infected by *M. anisopliae* was collected and after centrifugation the debris (including locust hemocyte and infected fungal spores) was used for mixed mRNA extraction (Quick prep micro mRNA purification kit, Amersham) following the manufacturer's instructions, glass beads being used to help release mRNA from the insect hemocyte and fungal spores by violent vortexing. Then oligo(dT)-primed first strand cDNA was prepared from the mRNA using a first strand synthesis system for RT-PCR (Takara). The sense and antisense primers were AT948F (5′ TATCGTTTGTCAACCAACTCGTCGCG 3′) and AT1273R (5′ CCATGTTGCGTTCTTCTCCTGCCC 3′), respectively, this combination of primers specifically amplifying a 326-bp amplicon on a *M. anisopliae* genomic DNA template, while on a cDNA template the amplicon comprised 265-bp. *M. anisopliae* genomic DNA was used as a control to distinguish the amplicon from cDNA. *L. migratoria* genomic DNA was use as a negative control. All samples containing the same quantity of first strand cDNA (0.5 μg) were subjected to different numbers of cycles of amplification (25 and 30 cycles). Amplification was performed in a PCR thermal cycler (Bio-Rad) with one cycle at 94°C for 5 min, and then 25 or 30 cycles as follows: 30 s of denaturation at 94°C, 30 s of annealing at 61°C, and 45 s of primer extension at 72°C. Amplicons were run on a 2% agarose gel.

RESULTS

Purification of Acid Trehalase from M. anisopliae—After 3 d culture the filtrate was dialyzed and concentrated, and then the crude enzyme was applied to 5% vertical isoelectric focusing gels (IEF) (pH 3.0–9.5), which were then stained for enzyme activity using the overlay method. With trehalose as the substrate, two brown trehalase activity bands appeared on the gel (Fig. 1), which exhibited pI values of about 4.7 and 8.4, respectively. The former isozyme was purified first because it exhibited the stronger activity and the same pI as the trehalase that appeared in the haemolymph of mycosed locusts (see later). Purification was achieved by two steps of anion exchange chromatography (High Q Sepharose and 25Q Sepharose) followed by gel filtration and narrow Precast IEF (pH 4.0–5.0). The purified protein gave a single band on a 12% SDS-PAGE gel stained with silver, with a molecular mass being *ca.* 170 kDa (Fig. 2A, lane 1). Five percent IEF also generated only one silver stained band with a pI of about 4.7 (Fig. 2B, lane 2). Table 1 summarizes the purification process; a 23.5% yield with approximately 98-fold purification was achieved.

Biochemical Characterization of the Pure Acid Trehalase—The acid trehalase is a glycoprotein with affinity for conA resin. On deglycosylation, the molecular weight of the acid trehalase decreased to 130 kDa as

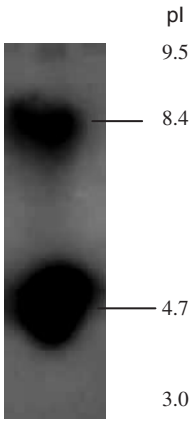

Fig. 1. **Isozymes of trehalase produced by 3 d cultures of *M.*** anisopliae. 20 μl Crude enzyme (about 20 × concentration from the culture filtrate of *M. anisopliae*) was subjected to 5% IEF and then stained for acid trehalase activity using the overlay gel method.

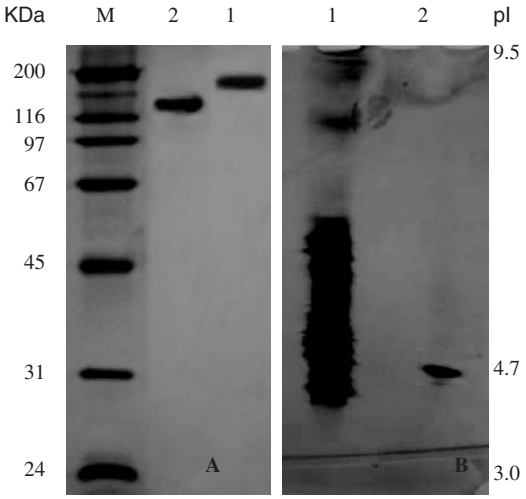

Fig. 2. **Panel A: SDS-PAGE.** Lane 1, purified acid trehalase (2 μg); lane 2, deglycosylated acid trehalase (2 μg); lane M, molecular markers **Panel B: IEF.** Lane 1, dialyzed culture filtrate (crude enzyme, 20 μg); lane 2, purified acid trehalase (2 μg). Stained with silver.

seen on 12% SDS-PAGE (Fig. 2A, lane 2), suggesting that carbohydrate comprises 23.5%.

A specific inhibitor of trehalases, trehazolin (*21*), inhibited the acid trehalase, the IC_{50} being about 1.3×10^{-8} M. The purified acid trehalase was highly specific for trehalose as a substrate and did not hydrolyze cellobiose (glucose β1-4 glucose), maltose (glucose α1-4 glucose), sucrose (glucose α1-2 β glucose), lactose (galactose 1-4 glucose), pnp galactopyranoside, pnp α-glucopyranoside, or pnp β-gluco-pyranoside. The enzyme exhibited strong activity across the pH range of 4.5 to 7.0, with an optimum of 5.5, which is close to the pH of locust haemolymph (about 6.0). The enzyme exhibited good activity from 20°C to 45°C, with an optimum at 30°C. The activity decreased rapidly at ⩾55°C.

The thermal stability of the purified acid trehalase essentially paralleled the effect of temperature on the

Table 1. **Purification of *M. anisopliae* acid trehalase.**

Purification step	Total protein (mg)	Total activity (U)	Specific activity (U/mg protein)	Purification factor	Yield (%)
Culture filtrate	25.0	30.6	1.224	–	100
High Q Sepharose	1.73	19.1	11.041	9.02	62.41
25Q Sepharose	0.94	15.3	16.277	13.30	50.00
Sepharcyl-200s HR	0.58	12.0	20.690	16.90	39.21
Precast IEF gel (pH 4.0–5.0)	0.06	7.2	120.0	98.04	23.53

activity in that preincubation at 40°C for 4 h had little impact on the activity while preincubation at 50°C caused the activity to decreased in proportion to the time, and as little as 10 min at ⩾60°C causing a complete loss of activity. The Michaelis constant, K_m, for trehalose is 2.3 mM and the maximum velocity V_{max} is 0.412 mmol min^{-1} mg $protein^{-1}$.

Isolation of the ATM1 *Gene and Structural Analysis of the Acid Trehalase Amino Acid Sequence*—A oligonucleotide probe designed from the amino acid sequence of peptide fragments was used to screen a *Metarhizium* Fosmid genomic DNA library, and the full length DNA and cDNA sequence encoding the acid trehalase were isolated (GenBank accession nos. DQ237957 and DQ237958). The conceptually translated ORF protein was confirmed by four other peptide sequences (N-terminal amino acid sequence: RDRVAKCLAR YSGSG, peptide: YSGSGLDSG, NAASSALSQGFY K and YATLLITGNQGLGQR) and exhibited homology (62%, 59%, 57%, 25% and 25%, respectively) with acid trehalases in three filamentous fungi (*Aspergillus fumigatus*, *Aspergillus nidalans* and *Talaromyces emrsonii*), and two other fungi (*Saccharomyces cerevisiae* and *Candida albicans*). Figure 3 shows alignment of the full length deduced sequences of the putative *Metarhizium* acid trehalase (Atm1p) and the five fungal/yeast acid trehalases. The result suggested that the gene we isolated is really the objective gene encoding the *Metarhizium* acid trehalase, and we named it *ATM1* (for acid trehalase of *Metarhizium anisopliae*).

The ORF encodes a putative polypeptide of 1,073 amino acids with a calculated molecular weight of 116,306. Analysis of the predicted amino acid revealed a 20 aa signal peptide, and a predicted cleavage site between positions 20 and 21 (CSA↓RD), which was confirmed by our N-terminal amino acid sequence. Hydropathy analysis (*20*) of the Atm1p amino acid sequence showed that the hydrophobic signal sequence is followed by a neutral region representing the mature protein (Fig. 4A). The mature protein comprises 1,053 amino acid residues with a calculated molecular weight of 114.3 kDa and a pI of 5.2. Thirty potential *N*-glycosylation sites (Asn-Xaa-Ser/Thr) were identified at amino acid positions 41, 132, 173, 203, 235, 273, 281, 305, 384, 491, 511, 568, 576, 616, 633, 642, 664, 733, 802, 826, 838, 847, 861, 904, 935, 942, 961, 993, 996 and 1028 (Fig. 4C).

A search of protein motifs in Atm1p revealed a match to the conserved sequence of glycosyl hydrolase family 65. N-terminal domain (amino acids 59–337), and the glycosyl hydrolase family 65 central catalytic domain (amino acids 403–769) (Fig. 4B). This family of glycosyl hydrolases includes vacuolar acid trehalase and maltose phosphorylase (*22*). The central domain is believed to be essential for catalytic activity, although its precise function remains unknown.

The Role of M. anisopliae Acid Trehalase in Fungal Pathogenesis—Ten nanograms pure *Metarhizium* acid trehalase was incubated *in vitro* with 10 μl locust haemolymph at 30°C for 0, 30 and 60 min, respectively. On incubation, the glucose concentration in the haemolymph had increased from 2.925 ± 0.25mM to 25.26 ± 1.10 mM (n = 3) at 30 min and 45.023 ± 0.69 mM at 60 min, while the denaturized acid trehalase (control) had no effect on the glucose concentration in locust haemolymph (Fig. 5), suggesting the acid trehalase can efficiently hydrolyze insect blood trehalose into available glucose.

Isoelectric focusing of haemolymph from mycosed locusts, in conjunction with the enzyme overlay detection method, using trehalose as a substrate (but not other disaccharide substrates, data not shown), revealed a single isoform of trehalase with a pI of *ca.* 4.7 (Fig. 6, lanes 2 and 4) that corresponded to the enzyme purified from the fungal culture (see above). The absence of this isoform in the other treatment groups also suggests a fungal origin. The appearance of the fungal acid trehalase in the haemolymph of mycosed locusts was accompanied by a decrease in the trehalose concentration (Fig. 7).

Evidence that the new acid trehalase in haemolymph from infected locusts corresponds to Atm1p was obtained from RT-PCR results. mRNA extracted from 2- and 4-day infected haemolymph was used to detect the *ATM1* gene's expression *in vivo*. *ATM1*-specific primers were employed that amplified a 326 bp fragment from genomic DNA (including a 61 bp intron) and a 265 bp amplicon from a cDNA template. A single 265 bp band was found on agarose gels in lanes corresponding to extracts of 2 d– and 4 d–infected haemolymph (Fig. 8, lanes 2 and 3); while a single 326 bp band appeared in lane 4 for amplification from *Metarhizium* genomic DNA. A band of neither size was found in lanes corresponding to control haemolymph cDNA or locust DNA (lanes 1 and 5). The presence of one intron in the corresponding region of the *ATM1* genomic DNA allowed the differentiation of bands of materials amplified from cDNA and any potentially contaminating genomic DNA. The expected band of 265 bp was obtained for the samples of infected haemolymph (lanes 2 and 3), while with cDNA from health locust haemolymph as a template the same band was not obtained (lane 1). Thus we have demonstrated that the *ATM1* gene was expressed in haemolymph of locusts during mycosis.

DISCUSSION

This is the first time that an acid trehalase has been isolated from *Metarhizium anisopliae*. Acid trehalases may

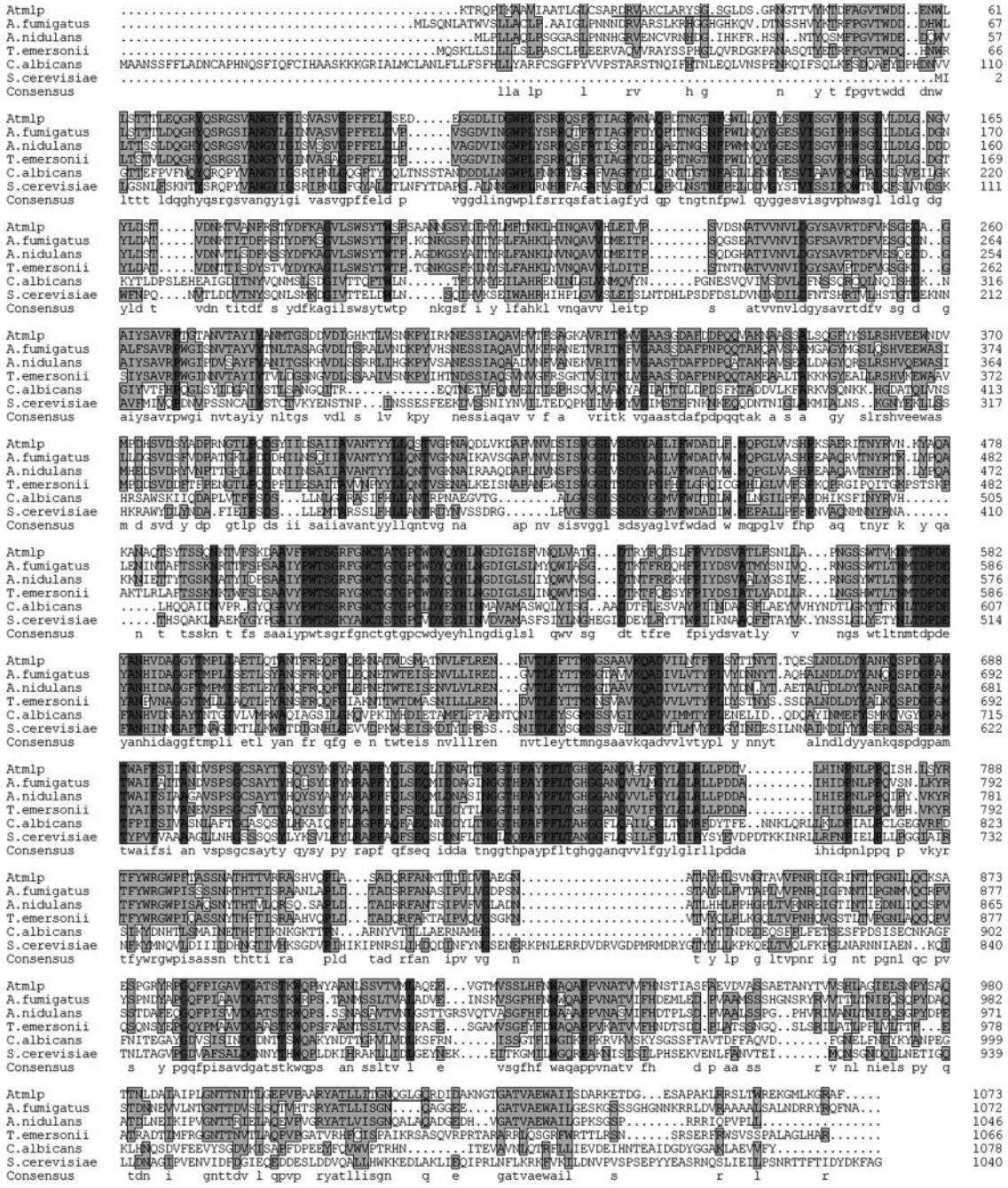

Fig. 3. **Predicted amino acid sequence of the *M. anisopliae ATM1* gene product.** Sequences were aligned by multiple sequence alignment with hierarchical clustering (Corpet, 1988). Atm1p is aligned with the sequences of α,α-trehalose glucohydrolase in *Aspergillus fumigatus* Af293, acid trehalase precursor in *Aspergillus nidulans* FGSC A4, acid trehalase in *Talaromyces emersonii*, vacuolar acid trehalase in *Candida albicans* SC5314, and Ath1p in *Saccharomyces cerevisiae*. The underlined amino acid regions indicated the sequences confirmed by peptide sequencing.

associate with other proteins during purification, e.g. a highly glycosylated protein invertase and gp37 (*23–25*), making them hard to purify. However, such problems were not encountered here. This enzyme is a glycoprotein with a molecular weight of 170 kDa, while after deglycosylation its molecular mass decreased to about 130 kDa, as seen on SDS-PAGE. The acid optimum pH of 5.5 is very similar to those of comparable enzymes from *Saccharomyces cerevisiae* (*8*, *24*) and *Aspergillus nidulans* (*26*). In common with most other trehalases examined the *Metarhizium* enzyme is strongly inhibited by trehazolin with an IC_{50} of 1.3×10^{-8} M, the values being 5.5×10^{-9} M, 3.7×10^{-9} M and 1.9×10^{-8} M for the porcine, silkworm and pig kidney trehalases, respectively (*27*). All well characterized trehalases are specific for trehalose and this enzyme is no exception (*28*, *29*). The properties of the acid trehalase distinguish it from the α-glucosidase which is the dominant trehalose-hydrolyzing enzyme

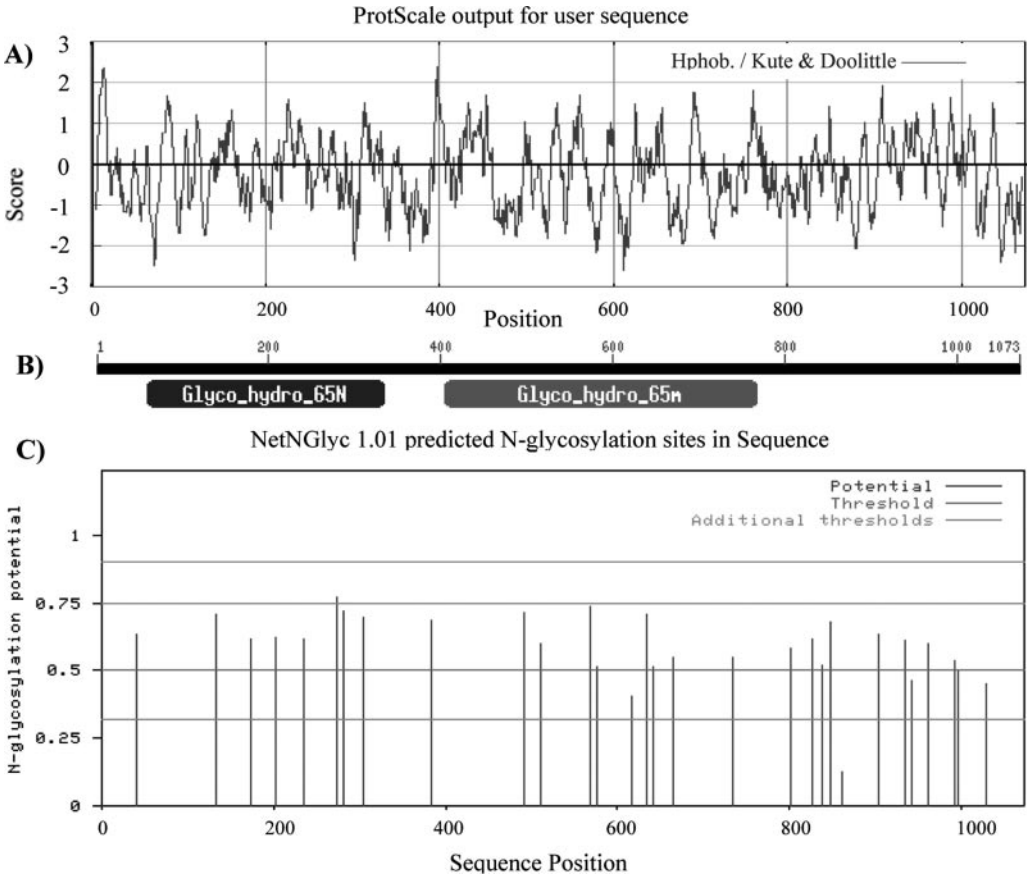

Fig. 4. **A: Hydropathic plot for the amino acid sequence of Atm1p**. Values above and below the horizontal line indicate hydrophobic and hydrophilic regions, respectively. The hydrophobic N-terminal domain corresponding to signal peptide (amino acids 1–20). **B: Features of Atm1p.** The regions exhibiting homology with the glycosyl hydrolase family 65 N-terminal domain (Glycohydro 65 N) and the glycosyl hydrolase family 65 central catalytic domain (Glycohydro 65 M) are indicated; **C: Potential *N*-glucosylation sites (Asn-Xaa-Ser/Thr).**

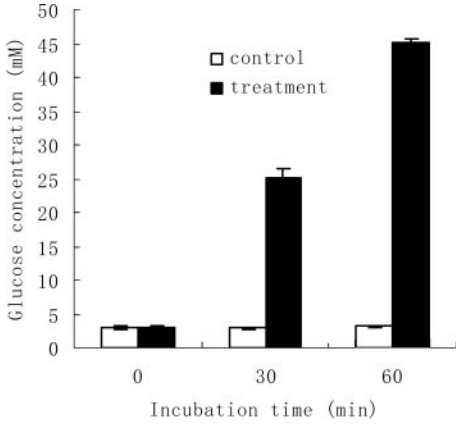

Fig. 5. **Glucose concentrations in the haemolymph of *Locusta migratoria in vitro* following incubation with pure *Metarhizium* acid trehalase.** Treatment, 10 ng pure acid trehalase was incubated *in vitro* with haemolymph for different times; Control, 10 ng denaturized pure acid trehalase was incubated *in vitro* with haemolymph for different times (n = 3).

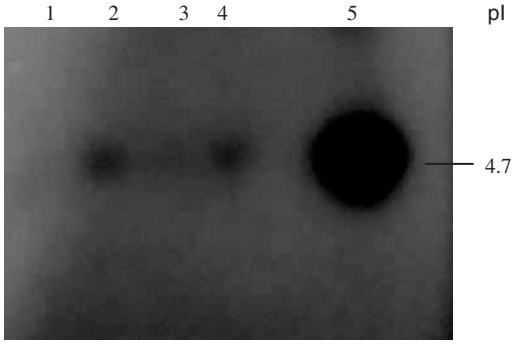

Fig. 6. **Isozymes of trehalose-hydrolyzing enzymes from haemolymph of *Locusta migratoria* and the acid trehalase purified from cultures (narrow range IEF, pH 4.0–6.0).** For each treatment, 20 μl haemolymph plasma was applied (lanes 1 to 4). Lane 1, day 4 haemolymph from locusts dipped in cotton oil; lane 2, day 4 haemolymph of locusts dipped in living spores; lane 3, day 2 haemolymph from locusts dipped in cotton oil; lane 4, day 2 haemolymph from locusts dipped in living spores; lane 5, the pure *Metarhizium* acid trehalase (10 ng).

secreted *in vitro* and *in vivo* by the generalist isolate *Metarhizium anisopliae* var. *anisopliae* ARSEF 3275 (*9*).

The temperature optimum (30°C) of the enzyme is the same as the optimum temperature for pathogenesis. Interestingly the temperature stability at *ca.* 45°C would help the fungus maintain integrity during the expression of behavioural fever in the host. The K_m of 2.3 mM is considerably less than the haemolymph trehalose concentration in the host, which is generally in the range of 4–20 mg/ml (*30*), and can be up to 100 mM in *M. sexta* (*31*). Thus it is not surprising that the pure acid trehalase efficiently hydrolyzed haemolymph trehalose into glucose *in vitro* (Fig. 5).

Acid trehalases are required for growth on trehalose as a sole carbon source and knockout mutants of several filmentous fungi are unable to grow on trehalose (*26*, *32–33*).

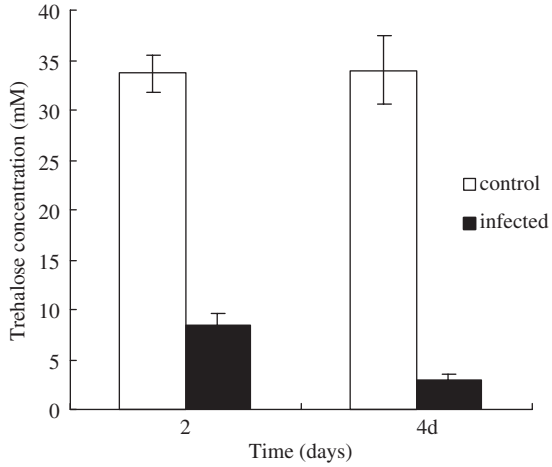

Fig. 7. **Trehalose concentration in the haemolymph of *Locusta migratoria* infected with *Metarhizium anisopliae*.** The locust haemolymph trehalose concentration was determined 2 and 4 days after inoculation.

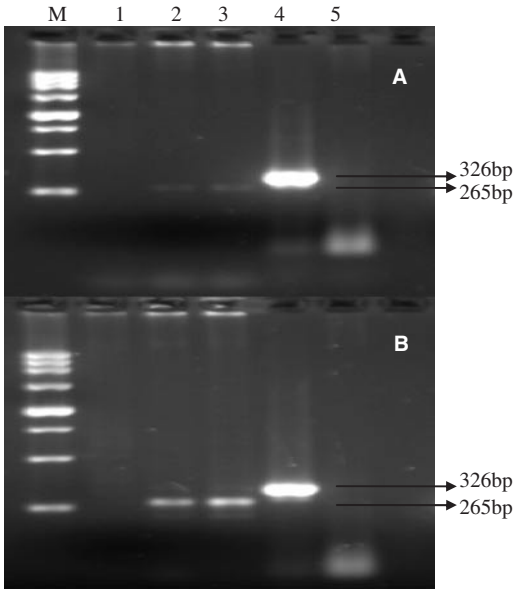

Fig. 8. **Expression of *ATM1* mRNA in the haemolymph of *Locusta migratoria* mycosed with *Metarhizium anisopliae*, as measured by RT-PCR.** Samples containing 0.5 μg of cDNA were subjected to 25 (A) and 30 (B) cycles of amplification (lanes 1–3), 10 ng DNA from *Metarhizium anisopliae* (lane 4) and *Locusta migratoria* (lane 5) being used as controls (35 cycles). The PCR samples were run on a 2% agarose. Lane 1, mRNA extracted from the haemolymph of control locusts; lane 2, mRNA extracted from the hemolymph of infected locusts at day 2; lane 3, mRNA extracted from the haemolymph of infected locusts at day 4; lane 4, *Metarhizium anisopliae* DNA; lane 5, *Locusta migratoria* DNA; lane M, molecular ladder.

These enzymes reported in fungi are generally present on the surface of spores and mycelium, or in vacuoles (*33*), and less frequently secreted into the medium (*28*, *34–35*). In contrast *Metarizium anisopliae* var. *acridum CQMa102* secretes a large amount of acid trehalase into the medium; indeed there is little bound or intracellular activity (*36*). This suggests a possible adaption to parasitism, as the secreted form would enable the scavenging of trehalose from the host haemolymph. Selection for trehalose utilization may also be suggested by the fact that the fungus can use trehalose as a sole carbon source (unpublished data).

Acid trehalase genes have been well identified in a limited number of species (two yeast species and one filamentous fungus) so far and amino sequence analysis shows they exhibit relatively low similarity (*37*). After sequencing the pure enzyme, and obtaining the N-terminal amino acid sequence and several peptide sequences, the gene encoding the acid trehalase was firstly isolated from *M. anisopliae* through DNA library and subclone screening combined with the 3′ RACE method. The deduced amino acid sequence reveals the presence of a signal peptide at the N terminal of the protein, which is a characteristic of proteins that transit through the secretory pathway. Atm1p exhibits relatively low amino acid similarity (25–62%) with the acid trehalases from 5 other fungi/yeasts, but domain prediction using NCBI Blastn revealed the presence of the glycosyl hydrolase family 65. N-terminal domain (amino acids 59–337), and glycosyl hydrolase family 65 central catalytic domain (amino acids 403–769) that characterize the classification of these proteins in glycosyl hydrolase family 65 (GH65) of the carbohydrate-active enzymes (GH65CAZy) (*37*). These results further confirmed that *ATM1* is the gene that encodes the purified acid trehalase. The theoretical molecular mass of mature Atm1p is 114.3 kDa, but on SDS-PAGE it shows an apparent mass of 170 kDa, which is due to *N*-glycosidic modification, as occurs for *Candida albicans* (*38*). Atm1p has 30 potential *N*-glycosylation sites. The difference between the deglycosylated enzyme (130 kDa) and the theoretical molecular mass of mature Atm1p (114.3 kDa) suggests there are other *N*-glycosidic modifications. This may because anhydrous trifluoromethanesulfonic acid (TFMS) is a mild chemical reagent for deglycosylation and can not remove all carbohydrates (*e.g. N*-linked GlcNAc) from proteins (*15*).

Trehalose is the major sugar in the haemolymph of most insects including *Schistocerca gregaria* and *Manduca sexta* (constituting 80–90% total sugar) and plays important roles in the insects' physiology (*4*). The depletion of blood trehalose may be detrimental to the insects. Fungal acid trehalase may be involved in the hydrolysis of haemolymph trehalose into available glucose for the invading fungus to grow. We have shown previously *in vitro* that external hydrolysis of trehalose and uptake of glucose is the primary means used by *M. anisopliae* var *anisopliae* to exploit an exogenous trehalose source (*9*). It seems likely that the isolate, CQMa102, investigated here does the same.

The results show that the pure *Metarhizium* acid trehalase can efficiently hydrolyze haemolymph trehalose into available glucose *in vitro*, and that a single isoform of acid trehalase appeared in the haemolymph of mycosed locusts at 2 and 4 d after inoculation. It is probably a fungal enzyme because the pI and substrate specificity are the same as these of *in vitro Metarhizium* enzyme. The decrease in haemolymph trehalose is consistent with the ability of this enzyme to release glucose for nutrition of the fungus. Contrary to the results of Xia *et al.* (*9*), the glucose concentration did not increase (unpublished data) although trehalose decreased sharply after the locust was mycosed with *M. anisopliae* var *anisopliae*, which may

suggest that the glucose released from the trehalose could be taken up rapidly by the fungus. Isolates of *M. anisopliae* invade their host using either the "growth strategy" or the "toxin strategy" (*39*). In common with other var *acridum* isolates *M. anisopliae* MaCQ102 probably employs the former, growing extensively in the haemolymph without recourse to toxins, which may explain why the haemolymph glucose did not increase after the locust was infected with this specific strain. All these findings may imply the important role of the acid trehalase in the pathogenesis of this fungus.

Further evidence that the decrease of trehalose in the haemolymph of infected locust was due to the fungal acid trehalase was obtained by RT-PCR. The results demonstrated that the *ATM1* gene was expressed effectively once *M. anisopliae* invaded the haemolymph of the locust, so we have reason to believe that the new acid trehalase appearing in the haemolymph of mycosed locusts is the product of *ATM1*.

M. anisopliae is a facultative saprophyte with both soil dwelling and insect pathogenic life stages, and when it traverses the cuticle and enters the haemolymph it must adapt to several different host environments. Wang *et al.* (*40*) have investigated the differential gene expression in isolated haemolymph and insect cuticle, and found that the genes concerned with the carbohydrate metabolism exhibited increased expression when grown *in vitro* on extracted insect haemolymph. Consistent with this, our research indicated that the *ATM1* gene is expressed when the fungus infects the insect's blood. This is the first time a fungal extracellular enzyme has been found to be secreted *in vivo* in the host and to have an impact on haemolymph biochemistry.

In conclusion this is the first time that fundamental knowledge about an acid trehalase and its gene from an entomopathogenic fungus has been obtained, and our results clearly show that this *Metarhizium* acid trehalase accounts for the decrease in haemolymph sugar in mycosed locusts. The contribution of this enzyme to pathogenesis and host death now need to be determined.

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