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Effects of efrapeptin and destruxin, metabolites of entomogenous fungi, on the hydrolytic activity of a vacuolar type ATPase identified on the brush border membrane vesicles of *Galleria mellonella* midgut and on plant membrane bound hydrolytic enzymes

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Abstract

The brush border membrane of the insect midgut is an initial site for interaction of insecticidal proteins. We have investigated the possibility that it may contain a target site for two insecticidal fungal toxins, destruxin and efrapeptin, both of which are ATPase inhibitors. We have studied the effects of the toxins on the hydrolytic activity of a vacuolar type ATPase (V-ATPase) that we have identified from *Galleria mellonella* midgut columnar cell brush border membrane vesicles (BBMV) by its cation and pH dependence, sensitivity to proton pump inhibitors and K_m (0.49 mM ATP). Efrapeptin strongly inhibited the BBMV V-ATPase but destruxin had little effect. We compared the effects of the inhibitors on known plant membrane hydrolytic enzymes, and although the vacuolar pyrophosphatase and plasma membrane ATPase were not inhibited by the toxins, the V-ATPase from mung bean, but not barley, was inhibited (50%) by 10 μM concentrations of both compounds. Different forms of the toxins were tested on the ATPases and destruxin B and efrapeptin F were the most effective. Kinetic analysis showed that the purified forms of both compounds inhibited the V-ATPases uncompetitively and modelling of data for inhibition of the BBMV V-ATPase by efrapeptin at concentrations of 0.06–12 μM yielded a K_i of 0.125 μM.

Keywords: Brush border membrane; Efrapeptin; Destruxin; ATPase; Vacuolar pyrophosphatase; Uncompetitive inhibition

1. Introduction

The insect midgut consists of two types of epithelial cells, columnar and goblet cells. The latter possess a unique apical cavity, with a connection to the gut lumen which is guarded by a constriction. Unlike the classical situation in animal cells, where the plasma membrane is energised by a sodium motive force, the insect midgut is energised by a proton gradient and a vacuolar (V-type) ATPase has been identified on the apical membrane surface of the goblet cell cavity [1]. A function of this enzyme is to support

Abbreviations: BBMV, brush border membrane vesicles; BTP/MES, (1,3-bis[tris (hydroxymethyl)-methylamino] propane)/(2-[*N*-morpholino]ethanesulphonic acid; DCCD, *N*,*N*′-dicyclohexylcarbodiimide; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide; NEM, *N*-ethylmaleimide; P-ATPase, plasma membrane ATPase; PPase, pyrophosphatase; V-ATPase, vacuolar ATPase

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a K^+/H^+ antiporter [2] that secretes K^+ ions into the apical cavity and maintains a high pH within the gut lumen [3,4]. Potassium ion channels on the haemolymph side of the epithelial cells allow the movement of ions into these cells from the blood [5]. Potassium ions in the gut lumen maintain the activity of a K^+/amino acid symporter on the brush border membrane of the columnar cells, which is also sensitive to electrical potential difference across the lumenal membrane [6]. The high concentration of K^+ in the insect's diet requires that the ion needs to be carefully regulated in the midgut [7].

The midgut columnar cell brush border membrane of the lepidopteran larva is the target site for *Bacillus thuringiensis* δ-endotoxins [8,9]. These toxins are non-competitive inhibitors of K^+/amino acid symport activity [10] either by interacting directly with the midgut symporter itself or with a functionally associated membrane bound aminopeptidase N.

Entomopathic fungi also produce mycotoxins that are believed to target the Malpighian tubules and the midgut. One such group of toxins, the destruxins [11], are of particular interest because, uniquely, they are present in sufficient quantities to cause insect death [12]. They are hexadepsipeptides produced by a range of fungi, including *Metarhizium anisopliae* [13], *Aschersonia aleyrodis* [14] and *Alternaria brassica* [15] and they possess insecticidal [16], phytotoxic [17] and antiviral [18] activity. Although the mechanism of action of the destruxins at the cellular and subcellular level remains poorly understood, in vitro experiments performed on isolated Malpighian tubules and midgut show that the toxins induce the formation of vesicles on the microvilli [19]. The effects of these toxins have been compared to those of the efrapeptins, which are a family of linear 15 residue petaibols produced by the filamentous fungus *Tolypocladium inflatum* [20]. The efrapeptins have also been shown to possess insecticidal properties [21] but the effects of the two inhibitor classes on isolated insect organs, including Malpighian tubules and midgut, have been found to differ significantly [19]. However, both families of toxins have been show to be potent inhibitors of ATPase activity – the destruxins inhibit V-type ATPases from *Saccharomyces cerevisiae* [22] and the efrapeptins inhibit photophosphorylation in plants and bacteria [23] and oxidative phosphorylation in bacteria and mammals [24] by interacting with the mitochondrial F_1F_0 ATPase [25].

An ATPase has been identified in columnar cell brush border membrane vesicles (BBMV) from the silk worm *Bombyx mori* which, unlike the V-ATPase identified in the goblet cells of tobacco horn worm [26] and blow fly [27], is not stimulated by K^+ [28] but resembles a V-type ATPase in other respects. The function of this enzyme may be to maintain the voltage dependent K^+/amino acid symport activity by regulating the membrane potential. In this paper we have identified an ATPase activity, present in *Galleria mellonella* larval BBMV, with similar properties to the *B. mori* enzyme. We have explored the possibility that the ATPase might be the site of action of the two insect toxins by studying the effects of destruxin and efrapeptin on the enzyme activity in the BBMV. Because of the phytotoxicity of the toxins [17,23] we have also studied the effects of the inhibitors on different membrane bound hydrolases of plant origin. These studies reveal that the two toxins differ in their relative inhibitory effects towards these enzymes but both display uncompetitive kinetics in their mode of action against V-type ATPases.

2. Materials and methods

2.1. Preparation of BBMV

Midguts were dissected from fifth instar *G. mellonella* larvae that had been fed an artificial diet at 30°C. The insects were immobilised by chilling on ice for 15 min before the midguts were removed, opened longitudinally and rinsed with 0.125 M NaCl. The isolated midguts were then blotted, weighed and either used immediately or placed in a vial with a small amount of 300 mM mannitol, 5 mM EGTA, 17 mM tris-(hydroxymethyl)aminomethane HCl, pH 7.5 (buffer A), snap frozen in liquid nitrogen and stored at −80°C.

BBMV were prepared from the tissue essentially as described in [29]. The midguts were placed in a 10-fold volume of an ice cold solution of buffer A and homogenised. The homogenate was mixed with an equal volume of 24 mM $MgCl_2$ and equilibrated on ice for 15 min (H). After centrifugation of the

preparation at 2500×*g*, the pellet (P1) was retained and the supernatant (S1) centrifuged at 30 000×*g*. The resulting pellet (P2) was homogenised in buffer A and mixed with an equal volume of 24 mM $MgCl_2$ and centrifuged at 2500×*g*. Both pellets from the 2500×*g* spins (P1 and P3) were retained for marker enzyme analysis and the supernatant from the second 2500×*g* spin (S3) was centrifuged at 30 000×*g*. The pellet, which was the BBMV preparation, was resuspended with a homogeniser in half strength buffer A, frozen in liquid nitrogen and stored at −80°C.

2.2. Plant material and vesicle preparation

Seeds of mung bean (*Vigna radiata*) were germinated and grown for 5 days in the dark at 25°C on water saturated vermiculite. Barley seeds (*Hordeum vulgare* cv. Alexis) were surface sterilised for 5 min with 70% ethanol followed by 20% sodium hypochlorite solution and imbibed for 6–8 h in tap water. The seeds were spread between two layers of cheese cloth and germinated in the dark at 25°C over an aerated solution of 0.5 mM $CaSO_4$. Roots were harvested 7 days after imbibition.

Tonoplast vesicles from mung bean were prepared according to the method of Rea et al. [30] and from barley as described in [31]. Plasma membrane vesicles were isolated from barley roots using reverse phase partitioning essentially as described by Larson et al. [32] except that sorbitol (500 mM) was used in the homogenisation and resuspension buffers instead of sucrose.

2.3. Production and purification of toxins

Crude preparations of the destruxins were isolated from the insect pathogenic fungus *M. anisopliae* [33]. The fungal strain was maintained on Sabouraud dextrose agar (Oxoid) at 25°C in the dark for 14 days. Conidial suspensions were prepared by scraping conidia from the culture into 0.03% (v/v) aqueous Tween 80 (BDH). 100 ml of Czapek–Dox medium containing 0.5% (w/v) Bactopeptone (CDB) adjusted to pH 7 was inoculated with 1×10^9 conidia and the mixture was incubated at 25±3°C for 12 days. The mixture was filtered and dried at 80°C for 24 h and the filtrate was extracted with AR dichloromethane (Fisons) (2×50 ml) and filtered through Whatman No. 1PS filter paper and concentrated by rotary evaporation. The residue (crude extract) was weighed and the destruxin content determined by chromatography and injection assay against *G. mellonella* [33]. The extracts were subjected to flash chromatography and the different forms of the toxins [34] were isolated on a reverse phase HPLC C18 column (Hichrom, 25×0.46 cm, 5 μm particle size) eluted with a 1:1 acetonitrile–water mixture with UV detection of 215 nm. The structure and purity of the compounds was verified by nuclear magnetic resonance and mass spectrometry [33] and the amount quantified by comparison of peak size to standard samples.

A similar procedure to that described above for the preparation of crude extracts of the destruxins was adopted for the in vitro production of crude extracts of the efrapeptins from *T. inflatum* [20,35]. Individual efrapeptins (D, E, and F) [20] were isolated from the crude preparations by HPLC on a reverse phase C8 column (Hichrom, 25×0.46 cm, 5 μm particle size) eluted with an acetonitrile–ammonium sulphate (65:35) gradient with UV detection of 225 nm. Each compound was identified by fast atom bombardment mass spectrometry [21] and quantified by comparison of peak size with standard samples.

2.4. Measurement of enzyme activity

ATPase assays were performed at 25°C in sealed tubes. The total volume of the assay mixture was 0.5 ml and it contained 250 mM glycerol, 50 mM KCl, 1 or 1.5 mM $MgCl_2$, 0.25 μg/μl gramicidin, 20 mM BTP (bis tris propane)/MES. The assays were performed at pH 6.5 for measurement of activity from plasma membrane, pH 8.0 for tonoplast, and pH 7.4 for the BBMV. Under standard conditions ATP concentration was 3 mM and the assays were allowed to proceed for 1 h, but where low ATP concentrations were required the assay time was reduced to 15 min to ensure that the ATP was not depleted. The linearity of the rate of increase of absorbance over these time periods was confirmed. Hydrolysis reactions were started by the addition of 5 μl of membrane preparation containing 5–10 μg of protein. The reactions were stopped by the addition of 700 μl of Ames reagent [36] containing 80 mM ascorbic acid, 3 mM ammonium molybdate, 0.85 M H_2SO_4 and 1% (w/v) sodium dodecyl sulphate. The

observed changes in absorbance were converted to specific activity (μM Pi released/mg protein/h) by reference to a standard curve. The specific activity of the barley plasma membrane and tonoplast preparations varied between 14 and 25 μM Pi released/mg protein/h, and the tonoplast preparations from mung bean between 3 and 6.5 μM Pi released/mg protein/h.

Pyrophosphatase (PPase) hydrolytic activity was measured using the method reported in [37] with imidazole as the pH buffer [38]. The specific activity of the tonoplast preparations ranged between 16 and 32 μM Pi released/mg protein/h.

Alkaline phosphatase and aminopeptidase activities were measured as described [28].

2.5. *Effect of proton pump inhibitors on the hydrolytic activity of BBMV ATPase*

The sensitivity of the BBMV ATPase to inhibitors specific for different classes of ATPases was examined [39]. These included ouabain, sodium azide, vanadate and potassium nitrate, which were added to the assay media at the concentrations indicated. BBMV preparations were preincubated with covalently binding inhibitors, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), *N*-ethylmaleimide (NEM), and *N*,*N*′-dicyclohexylcarbodiimide (DCCD) before assaying for ATPase activity as described [40].

Crude extracts and purified forms of the destruxins and efrapeptins were dissolved in the minimal volume of dimethyl sulphoxide and further diluted using distilled water. Their inhibitory effects on the different hydrolytic activities were tested by addition directly to the assay media.

2.6. *Kinetic analysis of the effects of destruxin B and efrapeptin F on the V-ATPases from mung bean and BBMV*

To investigate the kinetic mechanism involved in the inhibition of plant and insect V-ATPases the dependence of inhibition of hydrolytic activity on ATP concentration at different concentrations of toxin was measured. Enzyme concentrations were kept constant. Controls were run in parallel in which deionised water replaced the plant/insect membrane vesicles at each substrate/inhibitor concentration. Curve fitting was done with Sigmaplot 4.01 (Jandel Scientific, Ekrath, Germany).

2.7. *Protein measurement*

Protein was assayed by the method of Appelroth and Angsten [41] using bovine serum albumin as a standard.

3. Results

3.1. *Localisation of enzymic activity*

During the BBMV isolation procedure aliquots from each supernatant and precipitate were retained and assayed for ATPase, aminopeptidase and alkaline phosphatase activity. Alkaline phosphatase activity was enriched 10.2 times in the BBMV fraction

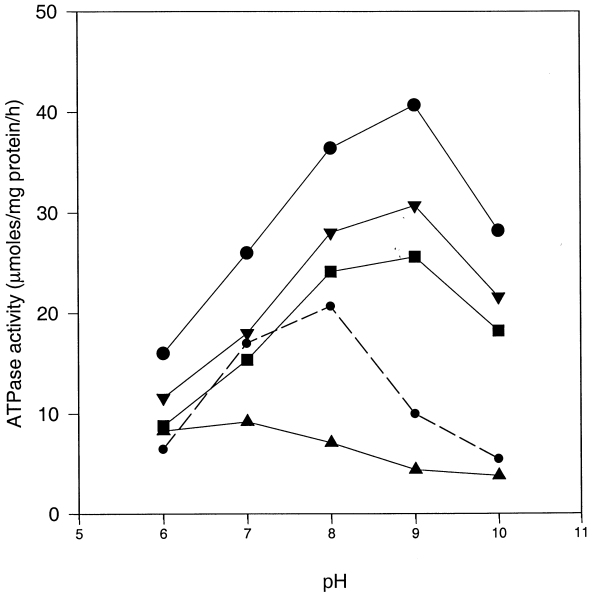

Fig. 1. The dependence of the V-ATPase activity from *G. mellonella* BBMV on pH. ATPase assays were performed as described in Section 2 and the pH was adjusted by altering the concentration of BTP/MES in the incubation medium. The figure shows the mean of three experiments. Error bars are omitted for clarity but the S.E.M. was less than 3% for each measurement. Assays were performed in the absence of KCl or NaCl with 1 mM $MgCl_2$ alone (●, solid line), or 2.5 mM $CaCl_2$ alone (●, dashed line) or both (▲) and in the presence of 50 mM NaCl (■, with 1 mM $MgCl_2$) or 50 mM KCl (▼, with 1 mM $MgCl_2$).

Table 1
Localisation of marker enzymes in subcellular fractions of midgut tissue from *G. mellonella*

Enzyme (specific activity)	Fraction					
	H	S1	S2	P1	P3	BBMV
ATPase	4.9 ± 0.2	4.51 ± 0.2	6.01 ± 0.2	7.7 ± 0.3	4.92 ± 0.3	30.0 ± 1.6
Alkaline phosphatase	208 ± 2.2	120.0 ± 9.0	143 ± 3.0	344 ± 5.0	228 ± 5.0	2115 ± 61
Aminopeptidase	72.6 ± 0.8	15.5 ± 1.7	35.0 ± 3.1	16.7 ± 1.5	85.4 ± 4.9	701 ± 5.7

Values for specific activity are expressed as μmol Pi released/mg protein/h (ATPase), μmol nitroalanine released/mg protein/min (aminopeptidase) and μmol *p*-nitrophenol released/mg protein/min (alkaline phosphatase).

compared to the original homogenate, aminopeptidase 9.6 times and ATPase 6.1 times (Table 1). The final ATPase specific activity of the BBMV fraction was 30.0 ± 1.6 μmol/mg protein/h for freshly prepared samples but the activity declined after freezing.

3.2. Characterisation of ATPase activity in BBMV

3.2.1. pH dependence

The dependence of the hydrolytic activity of the ATPase from BBMV on pH between the values of 6 and 10 in the presence of different cations was tested. In the presence of 2 mM $MgCl_2$ the activity increased up to pH 9 then declined (Fig. 1). When either 50 mM KCl or NaCl was added to the medium the activity was suppressed but the profile remained the same. When $CaCl_2$ replaced $MgCl_2$ in the medium the activity was lower with maximum activity at pH 8 and the presence of both cations supported very little activity.

In the absence of cations BBMV isolated from *B. mori* contain substantial amounts of ATPase activity above pH 9.5 [28], but this activity was almost negligible between pH 6 and 8. Consequently ATPase assays on *G. mellonella* BBMV were performed at pH 7.4.

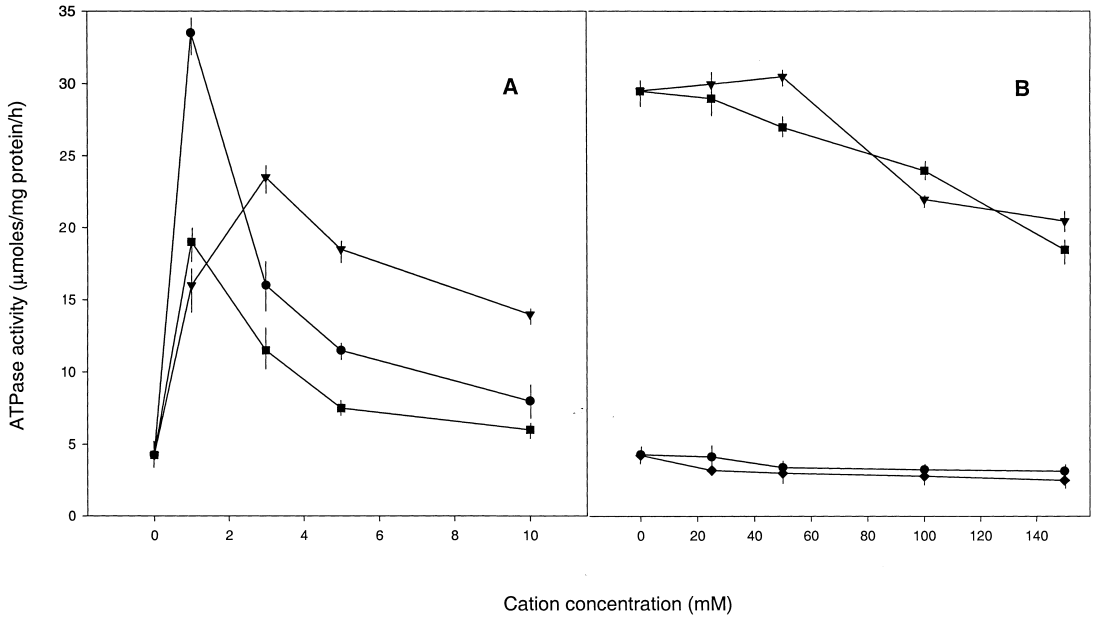

Fig. 2. The dependence of the V-ATPase activity from *G. mellonella* BBMV on cation concentration. ATPase assays were performed in triplicate as described in Section 2 at pH 7.4. (A) Assays in the absence of KCl or NaCl with varying concentrations of $MgCl_2$ alone (●) or $CaCl_2$ alone (▼) or varying concentrations of $MgCl_2$ with 2.5 mM $CaCl_2$ (■). (B) Assays in the presence of varying concentrations of NaCl (■, with 1 mM $MgCl_2$, or ●, without) or varying concentrations of KCl (▼, with 1 mM $MgCl_2$, or ♦, without).

3.2.2. The effect of various cations

The dependence of the ATPase activity from BBMV on the concentration of $MgCl_2$, $CaCl_2$, KCl and NaCl in the assay medium was investigated. Both $MgCl_2$ and $CaCl_2$ supported activity. In the presence of $MgCl_2$, without monovalent cations, activity was greater than in the presence of $CaCl_2$ at low concentrations, but both ions were inhibitory above 2.5 mM, with $MgCl_2$ having the greater inhibitory effect (Fig. 2A). When both salts were present the activity was suppressed, possibly due to the total combined concentration of the two ions providing an inhibitory effect. Activity in the presence of $MgCl_2$ was greatest at 1 mM (the concentration used for subsequent assays) and in the presence of $CaCl_2$ at 3 mM. In the absence of $MgCl_2$, neither NaCl nor KCl supported ATPase activity (Fig. 2B) but the addition of 1 mM $MgCl_2$ to the media restored activity although at concentrations above 50 mM both ions were inhibitory (50% at 150 mM).

3.2.3. The effect of ATPase inhibitors

The sensitivity of the ATPase activity in BBMV to inhibitors specific for different classes of ATPases was examined. As shown in Table 2, the enzyme was less sensitive to vanadate than nitrate, it was not inhibited by ouabain and only weakly by sodium azide. EDAC, NEM and DCCD were inhibitory.

Table 2
The effect of inhibitors on the hydrolytic activity of the V-ATPase from *G. mellonella*

Inhibitor	Concentration (mM)	Activity remaining (%)
Ouabain	0.1	103 ± 5.1
DCCD	0.02	55.4 ± 6.3
NEM	0.1	46.7 ± 2.8
EDAC	20	32.1 ± 1.4
Vanadate	1	72.2 ± 3.1
Nitrate	100	43.6 ± 9.0
Sodium azide	0.05	81.2 ± 2.7

Values are mean ± S.E.M. of three separate experiments.

3.3. The effects of destruxin and efrapeptin on the BBMV V-ATPase and on the activity of plant membrane bound hydrolytic enzymes

The ability of both the destruxins and the efrapeptins to inhibit ATPases has already been established [22,25] and we wished to study their effects on the ATPase activity from BBMV and on plant derived hydrolytic enzymes. To test the effects of the destruxins (100 μM) and the efrapeptins (60 μM), crude extracts (see Section 2) of the inhibitors were added to assay media containing the different enzyme preparations. Efrapeptin was a strong inhibitor of the ATPase activity from BBMV, reducing its activity

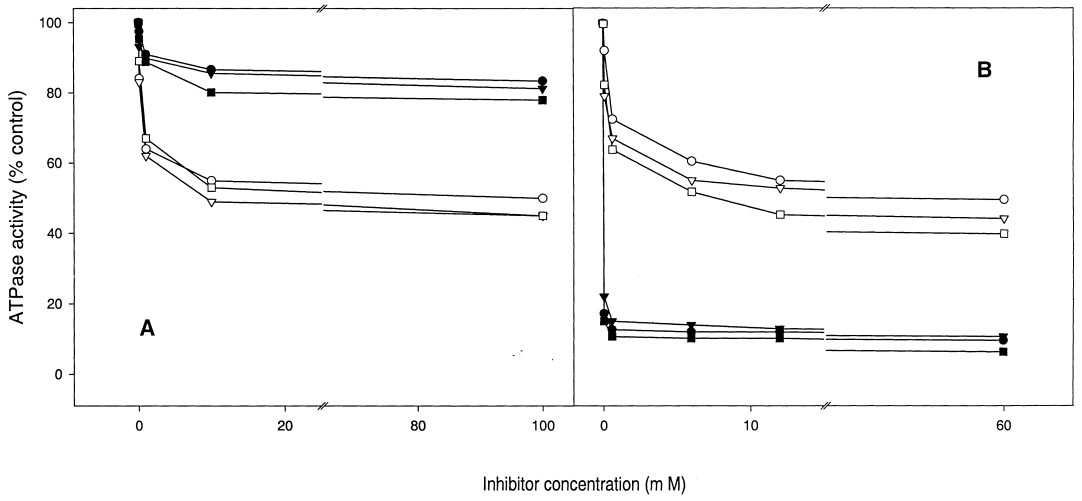

Fig. 3. Effect of (A) destruxins and (B) efrapeptins on the ATP hydrolytic activity from mung bean tonoplast vesicles (open symbols) and BBMV (filled symbols). Purified forms of destruxin (○ ●, A; □ ■, B; ▽ ▼, E) and efrapeptin (▽ ▼, D; ○ ●, E; □ ■, F) were added to the assay media which contained 3 mM ATP. Each experiment was performed three times and the figure shows a typical result.

Table 3
Effects of efrapeptin and destruxin on the hydrolytic activity of different membrane bound hydrolases

Inhibitor	Relative activity (% of control)					
	BBMV	Barley		Mung bean		
	V-ATPase	P-ATPase	V-ATPase	P-ATPase	V-ATPase	V-PPase
Efrapeptin (60 μM)	17.2 ± 1.5	97.1 ± 5.0	109 ± 4.0	103 ± 1.6	45.3 ± 3.1	87.2 ± 2.1
Destruxin (100 μM)	81.1 ± 1.8	101 ± 3.5	103 ± 6.5	100 ± 2.1	45.6 ± 1.4	89.4 ± 6.2

Values are mean ± S.E.M. of three separate experiments.

to 17%, whereas destruxin only inhibited the activity by 20% (Table 3). No effect on specific activity was detected when the inhibitors were tested against the plasma membrane ATPases (P-ATPases) from mung bean or barley, or the V-ATPase from barley. The vacuolar pyrophosphatase (V-PPase) from mung bean was weakly inhibited by both toxins and the V-ATPase was inhibited by about 55% by both destruxin and efrapeptin.

Different forms of the efrapeptins and destruxins were purified from the crude extracts using HPLC [20,34] and we examined the concentration dependence of the effects of the different forms of the inhibitors on the ATPase activity from BBMV and the V-ATPase from mung bean (Fig. 3). Maximum inhibition by all three forms of destruxin (Fig. 3A) was achieved with approximately 10 μM concentrations for both ATPases. Although there was little difference between the effects of the different forms of the toxin, B had a slightly greater effect on the BBMV enzyme and this compound was used in subsequent experiments. The concentration dependence of inhibition of the BBMV enzyme by the efrapeptins required much lower concentrations (< 0.6 μM) for

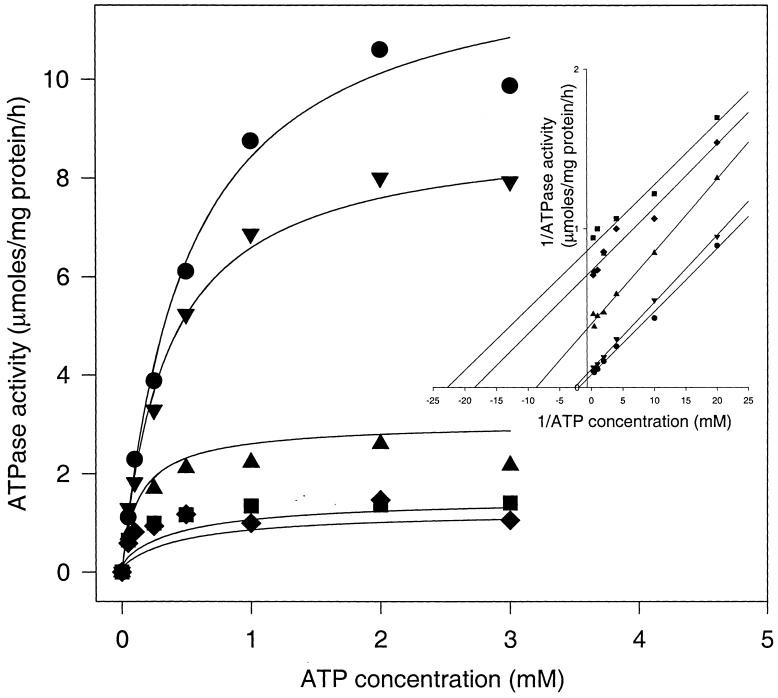

Fig. 4. (Inset) Double reciprocal plot of a typical experiment to show the dependence of the specific activity of the BBMV V-ATPase from *G. mellonella* on ATP concentration in the absence of inhibitors (●) and in the presence of 0.06 μM (▼), 0.6 μM (▲), 6.0 μM (♦) and 12.0 μM (■) efrapeptin F. (Main figure) ATP dependence of the specific activity of the BBMV ATPase from *G. mellonella* on ATP concentration (symbols represent experimental data as inset) modelled using Eq. 1 for uncompetitive inhibition (lines) with the following parameters: $V_{max} = 11.8$, $K_m = 0.49$ mM, $K_i = 0.125$ μM and maximum inhibition = 87%.

maximum inhibition (Fig. 3B) but their effect on the mung bean V-ATPase was similar to that observed for the destruxins. In both cases efrapeptin F was the most effective and was used for kinetic studies (see below).

3.4. Kinetic analysis of the effects of destruxin B and efrapeptin F on the ATPases from BBMV and mung bean tonoplast

To investigate the kinetic mechanism involved in the inhibition of the ATPases by the toxins the effect of varying the concentration of ATP on inhibition was studied. Double reciprocal plots of the data revealed that inhibition of the BBMV enzyme by efrapeptin F and the plant enzyme by both efrapeptin F and destruxin B followed uncompetitive kinetics, and both the V_{max} and the K_m were reduced (Figs. 4 and 5, insets). Increasing the concentration of efrapeptin F from 0 to 12 μM reduced the V_{max} of the hydrolytic activity of the BBMV ATPase from 11.79 ± 1.20 (mean ± S.E.M.) to 1.11 ± 0.02 nmol Pi/mg protein/h and the K_m from 0.49 ± 0.03 to 0.04 ± 0.002 mM ATP. 100 μM concentration of destruxin B had only a very slight effect on the BBMV enzyme (data not shown). The K_m for ATP concentration for the plant enzyme was reduced from 0.13 ± 0.002 to 0.11 ± 0.001 mM and the V_{max} from 3.27 ± 0.07 to 2.57 ± 0.08 nmol Pi/mg protein/h by 12 μM efrapeptin F. The effect of 100 μM destruxin B on these parameters was to reduce V_{max} of the plant enzyme to 1.75 ± 0.05 nmol Pi/mg protein/h and the K_m to 0.09 ± 0.001 mM.

The data from a typical experiment measuring the ATP concentration dependence of the ATPase activity from BBMV in the presence of different concentrations of efrapeptin F were modelled using the equation for uncompetitive inhibition, modified to take into account a reaction with less than 100% maximal inhibition:

$$V = \frac{\dfrac{a.V_{max}}{1+\dfrac{[I]}{K_i}} \times S}{s + \dfrac{K_m}{1+\dfrac{[I]}{K_i}}} + \frac{(1-a).V_{max} + s}{S + K_m} \quad (1)$$

assuming a value of 87% for a, maximum inhibition, 11.79 nmol Pi/mg protein/h for the V_{max} and 0.49 mM ATP for the K_m, a value for the K_i for inhibition of the ATPase from BBMV of 0.125 μM provided the best fit to the data (Fig. 4).

Efrapeptin F and destruxin B only achieved a maximum inhibition of 55% when tested with the plant V-ATPase and it was not possible to accurately model their effect on ATP concentration dependence using Eq. 1 to obtain values for K_is. IC_{50} values for the inhibition of the plant V-ATPase by both destruxin B and efrapeptin F of less than 1.0 μM were estimated from the dose–response curves (Fig. 3). However, Michaelis–Menten kinetics were obeyed in the presence of the inhibitors and the data for a typical experiment could be successfully modelled with this equation using the actual values for the

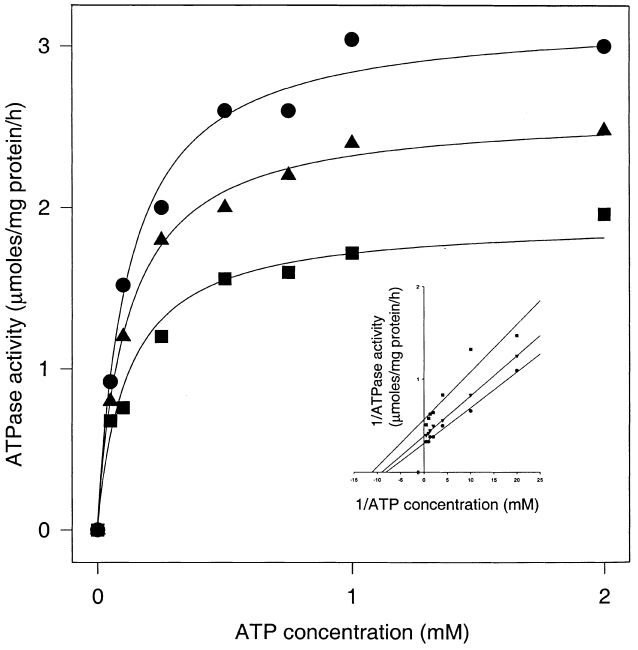

Fig. 5. (Inset) Double reciprocal plot of a typical experiment to show the dependence of the specific activity of the V-ATPase from mung bean on ATP concentration in the absence of inhibitors (●) and in the presence of 12 μM efrapeptin F (▼) and 100 μM destruxin B (■). The experiment was performed three times and the figure shows a typical result. (Main figure) ATP dependence of the specific activity of the V-ATPase from mung bean on ATP concentration (symbols represent experimental data as inset) modelled using the Michaelis–Menten equation (lines) with the following parameters: in the absence on inhibitor, V_{max} = 3.21, K_m = 0.12 mM, in the presence of 12 μM efrapeptin F, V_{max} = 2.56, K_m = 0.11 mM, in the presence of 100 μM destruxin B, V_{max} = 1.75, K_m = 0.09 mM.

K_m and V_{max} obtained from double reciprocal plots (Fig. 5).

4. Discussion

The presence of a V-ATPase in the BBMV from *B. mori* has been reported and this enzyme, unlike the V-ATPase from the goblet cell plasma membrane, is insensitive to K^+ [28,42]. Our results suggest that we have identified a similar enzyme in the BBMV from *G. mellonella*. Firstly, marker enzyme analysis indicates that we have successfully purified the BBMV fraction from the tissue homogenate. The activities of alkaline phosphatase and amino peptidase, two important BBMV marker enzymes [29], were enriched (10.2 and 9.6 times respectively) in the purified vesicle fraction compared to the starting material and the ATPase specific activity was enriched six-fold. These values are comparable with those published for the relative enrichment of enzyme activity in BBMV isolated from *B. mori* [28] and other insect species ([29] and references therein). In addition, the ATPase that we have detected resembles the BBMV ATPase from *B. mori* in its cation dependence. It is not stimulated by K^+ and the ion is inhibitory at high concentrations and its dependence on $MgCl_2$, $CaCl_2$ and pH resembles that of the BBMV V-ATPase from *B. mori* [28].

The effects of several inhibitors routinely used to characterise ATPases [39] on the activity associated with the *G. mellonella* BBMV fraction suggest that a major proportion of the activity is due to a V-type enzyme. The activity was more sensitive to nitrate, an inhibitor of the V-ATPase, than to vanadate, an inhibitor of the P-ATPase. However, the difference was less marked than the effects of the two inhibitors on either the plant V-ATPase [39] or the V-ATPase from *B. mori* [28]. Ouabain, an inhibitor of Na/K-ATPases, was ineffective against the activity, but azide, an inhibitor of the mitochondrial ATPase, had a slight effect, possibly indicating contamination of the preparation by mitochondria. Published values for the K_m of V-ATPases with respect to substrate concentration vary between 0.16 mM for the enzyme from locust Malpighian tubules [43], 0.26 mM for the enzyme from *B. mori* BBMV [28] and 0.65 mM for the red beet tonoplast enzyme [39]. All these are in the same order of magnitude as the value of 0.49 mM obtained here for the ATPase identified in *G. mellonella* BBMV.

Comparison of the effects of crude extracts of the fungal toxins on the ATPase from BBMV reveals that only the efrapeptins inhibit the activity significantly and only up to 20% of this effect could have been due to inhibition of the mitochondrial ATPase. The remaining inhibitory activity must be due to its effect on the V-ATPase. Although both compounds reduced the activity of the V-ATPase from mung bean to a similar degree, neither provided complete inhibition. The toxins had no effect on the activity of the plant P-ATPase or the tonoplast V-ATPase from barley. The ability of monocotyledons to demonstrate enhanced resistance to certain toxic chemicals is a property potentially exploitable in the development of herbicides. Fungal extracts containing both toxins have been shown to be phytotoxic [21] which may be due, in part, to their effects on the V-ATPases, although their size would prevent them from moving passively across the plasma membrane [44]. The effects of the destruxins on the yeast vacuolar enzyme have been well characterised [22]. The IC_{50} value obtained for the yeast V-ATPase (5.4 μM) is similar to that which we have found for the plant V-ATPase (see Fig. 3A) but the destruxins have no effect against the yeast mitochondrial ATPase [22]. Their high degree of specificity suggests that they could be used as powerful tools to study the localisation and function of ATPases. However, because they do not have significant inhibitory activity against the BBMV ATPase their entomopathic activity does not appear to be due to a direct effect on the enzyme. Comparative studies between the effects of the destruxins and the efrapeptins on insect tissues have confirmed that the destruxins do not have strong lytic effects on mitochondria or act as ionophores although they may have other destructive effects on these organelle. Unique cytological changes in the morphology of the BBMV that may relate to their toxicity [19] are induced by these compounds, but the biochemical mechanisms responsible for these alterations have yet to be elucidated.

Lineweaver–Burk analysis of the effects of destruxin B and efrapeptin F on the plant V-ATPase and the BBMV associated activity suggests that, similar to the effects of the efrapeptins on the mitochondrial

ATPase [45], inhibition obeys uncompetitive kinetics. Modelling of the experimental data enabled a K_i for the uncompetitive inhibition of the BBMV enzyme by efrapeptin F of 0.125 μM to be calculated but it was not possible to accurately model the effects of the inhibitors on the plant enzyme because only 55% maximal inhibition was achieved. The effect of efrapeptin on mitochondrial enzymes from a number of sources has been studied and IC_{50} values of 0.025 μM and 0.075 μM respectively for inhibition of fungal and house fly mitochondrial enzymes [21] have been measured. The K_d for inhibition of oxidative phosphorylation by the beef heart mitochondrial ATPase was found to be 10^{-8} M [45]. The compound has also been shown to inhibit the ATPase from spinach chloroplasts (IC_{50}, approximately 1 μM) [46], bacteria (≤ 84 μM) [47] and both the Mg-ATPase and the soluble Ca-ATPase of the coupling factor from *Rhodospirillum rubrum* chromatophore (0.15 μM) [23]. Hence, although they are inactive against the P-ATPase, efrapeptins can be considered to be inhibitors with broad specificity and it is not surprising that they also inhibit a V-ATPase from the BBMV. Presumably, by targeting different components in the insect, the pathogen is able to debilitate its host and facilitate colonisation. It is also possible that the toxins are host specific and by producing a wide range the pathogen can increase its host range. Indeed, Amri et al. [33] found a crude correlation between the quantity and type of toxin produced by *M. anisopliae* and host range: strains producing a wide range often had a wide host range. However, a V-ATPase located on the brush border plasma membrane is likely to be the initial site of interaction of the toxin. Inhibition of the mitochondrial ATPase will only contribute to the insecticidal effect if the compound is internalised.

When the purified forms of the efrapeptins were tested we found that efrapeptin F was the most effective against both ATPases. It has been found that F is more potent than D in in vivo insect assays [35], however, in vitro studies show the latter to be more inhibitory to mitochondrial ATPases than efrapeptin F [21]. This observation further supports the suggestion that the principal target for the inhibitor is the BBMV V-ATPase or the plant V-ATPase, not the mitochondrial ATPase.

Analysis of the effects of efrapeptin on the mitochondrial ATPase from beef heart has enabled some of the kinetic steps involved in ATP hydrolysis and proton transport to be elucidated [45,48]. X-ray crystallography of the bovine F_1 ATPase–efrapeptin complex reveals that efrapeptin binds to a site in the core of the enzyme which does not overlap with the substrate binding sites [49]. The potentially crucial role played by the BBMV V-ATPase in maintaining amino acid transport in the insect midgut indicates that clarification of its physiological function and its purification to enable characterisation of its structure and catalytic mechanism are warranted. Our evidence, that it is inhibited by efrapeptin and may be the target for its toxic effects, indicates that the compound is an appropriate tool for such investigations to achieve this. The kinetics of inhibition of the plant V-ATPase by efrapeptin and destruxin are similar and it would be of interest to establish whether similar binding sites are involved.

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