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ISOLATION AND INHIBITION OF A TRYPSIN-LIKE ACTIVITY FROM LARVAE OF CORN BORER *(Ostrinia nubilalis)* USING REVERSE MICELLES

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SUMMARY

Endoproteinase(s) was isolated from a freeze-dried powder of larvae of *Ostrinia nubilalis* using reverse micellar solutions. The inhibition of proteinase was studied in reverse micelles with commercial Bowman-Birk soybean trypsin inhibitor and three trypsin inhibitors recently isolated from ripe cruciferous seeds.

INTRODUCTION

The properties of some enzymes which interact with hydrophobic substrates, viz. lipases, lipoxygenases, but also bovine β-trypsin and α-chymotrypsin have been often studied in reverse micellar solution both for technological and analytical purposes (Luisi and Laane, 1986; Hochkoeppler and Palmieri, 1990). A reverse micellar solution is a micro-dispersion of water droplets in an organic solvent stabilized by a surfactant. This dynamic system is regulated by W_0, which is the ratio of water molarity to surfactant molarity ($W_0 = [H_2O]/[surfactant]$). In particular, micelle size is strongly affected by W_0: as W_0 increases, the size of micelles increases. Reverse micelles offer an interesting opportunity to study membrane-associated enzymes in an aqueous micro-environment, separated from a bulk of organic solvent by a thin layer of surfactant, simulating a biological membrane (Martinek *et al.*, 1989). In addition, it has been demonstrated that this surfactant/hydrocarbon water ternary system makes it possible to efficiently isolate water-insoluble proteins which are bound on biological membranes surface or inside them (Leser *et al.*, 1986).

We isolated a trypsin-like enzyme(s) from larvae of corn borer (*Ostrinia nubilalis*) because this insect causes heavy damage to numerous important crops and maize in particular. This endoproteinase(s) was previously described by Houseman *et al.* (1989) who, however, reported data obtained using an aqueous crude extract.

The aim of this work was twofold: (i) to isolate the proteinase(s) contained in the digestive system of *O. nubilalis* larvae, and (ii) to study its activity in a background mimicking the situation *in vivo*. The main objective of this second aim was to study the efficacy of a new

family of proteinase inhibitors recently isolated from cruciferous seeds and characterized (Menegatti *et al.*, 1992; Ceciliani *et al.*, 1994; Iori *et al.*, 1995).

MATERIALS AND METHODS

Insects

Larvae of *O. nubilalis* were obtained from the Research Institute for Cereal Crops, Bergamo, Italy. The larvae were maintained in the laboratory under constant light at 28°C and 80% relative humidity. They were reared according to the method of Guthrie *et al.* (1985). The late instar larvae were collected and frozen at -20 °C.

Materials

Reverse micelles constituents included bis (2-ethylhexyl) sodium sulfosuccinate (AOT; Sigma 99% purity), isooctane (Fluka, for ultraviolet spectroscopy), double distilled water. Nα-Benzoyl-L-Arginine ethyl ester (BzArgOEt), Bowman-Birk soybean trypsin inhibitor (SBTI) were from Sigma; glycine (electrophoresis purity reagent) was from Bio-Rad. All other chemicals used in this study were of analytical grade or equivalent.

Reverse micellar solutions preparation and enzyme isolation

Different amounts of a 50 mM acetate buffer pH 4.2 were injected in a solution of 20, 50, 100, 200, 300 mM AOT dissolved in isooctane to reach the desired W_0. The cloudy mixture was shaken until it was clear. The larvae freeze-dried powder (80 mg) was added to 20 ml of micellar solutions with different W_0 or AOT concentrations and stirred at room temperature for about two hours. The solution was then centrifuged and the precipitate was discarded or used for other extraction using solutions with increasing W_0.

Enzyme Assays

The enzymatic activity was assayed at 30 °C, W_0 = 40; 0.2 ml of isolated proteinase in micellar solution was added to 0.145 ml of 0.1 M glycine buffer pH 10 and to 0.655 ml of 300 mM AOT solution in isooctane. The transparent micellar solution was thermostated for 10 min at 30° C and then 3 µl of substrate (BzArgOEt) aqueous solution (0.233 M) were added and shaken until clear again. The reaction rate was then determined following the absorbance increase at 265 nm, using a Kontron 940 spectrophotometer equipped with a thermostated cell compartment. The spontaneous hydrolysis of BzArgOEt was noticeable, although its rate was negligible in comparison to the enzymatic-catalyzed reaction rate. The same procedure was used when inhibitors were added to the assay. One unit of trypsin-like activity was defined as the amount that catalyzes the cleavage of 1 µmol of substrate per minute, using a $\Delta\varepsilon$ = 850 $M^{-1}cm^{-1}$ calculated at 265 nm in micellar solution.

Protein determination

The protein concentration was evaluated using an ultraviolet absorption method (Stoscheck, 1990) and the formula: mg/ml = 1.55 A_{280} - 0.76 A_{260}. The absorbances were determined with extraction micellar solutions in the reference quartz cell.

Proteinase Inhibitor isolation

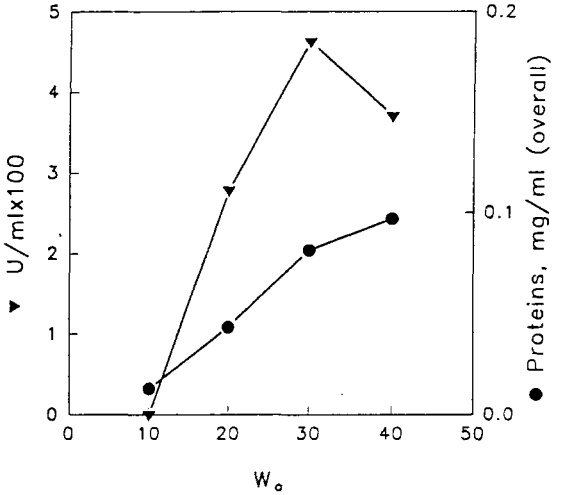
The non commercial proteinase inhibitors used in this work were isolated from ripe seeds of some cruciferous plants such as rapeseed (*Brassica napus)*, *Brassica carinata* and *Cheirantus cheiri*, following the method previously described (Ceciliani *et al.* 1994; Iori *et al.* 1995).

RESULTS AND DISCUSSION

Reverse micelles appear to be one of the most promising non conventional systems, which has been reported in the literature for the extraction and purification of proteins (Leser *et al.*, 1986; Leser *et al.*, 1989). We applied this procedure to isolate a trypsin-like enzyme(s),

difficult to isolate by traditional chromatographic techniques. In our opinion, the failure of conventional methods was due to the relevant high hydrophobic nature of this protein, which *in vivo* is presumably dispersed in a fatty environment, or more likely is associated with the intestinal membrane.

The procedure that we applied essentially consists in a phase-transfer from the freeze-dried larvae. Micellar solutions were used with a fixed AOT concentration (100 mM) at varying W_0 (W_0= 10, 20, 30, 40) (see Figure) (Leser *et al.*, 1986), or with a constant W_0 and increasing AOT concentrations (25, 50, 100, 200 and 300 mM) (data not shown). As the figure shows, the best enzyme isolation conditions were W_0 = 30, 100 mM AOT, which made it possible to isolate a trypsin-like enzyme(s) with the highest specific activity (0.22 U/mg). However, when the freeze-dried powder was previously extracted by a micellar solution with W_0 = 10, 100 mM AOT, the final specific activity improved notably (0.78 U/mg), without affecting the isolated enzyme yield. Although the latter datum appears to be much better than the previous one, this activity difference must be considered with a certain caution. In fact, the preliminary treatment with micellar solution W_0 = 10, as well as removing part of the non active soluble proteins, also eliminates many other water and isooctane soluble compounds. Many of these compounds absorb around 280 nm, which is the wavelength used for protein content determination.



Solubilization of *O. nubilalis* endoproteinase(s) from freeze-dried larvae powder in 100mM AOT/ isooctane/ water reverse micelles as a function of the water content (W_0).

These results also suggest that proteinase is associated with the midgut biological membrane; subcellular structure of the latter and the enzyme compartimentalization certainly play an important role in enzyme stability and the regulation mechanism. Although biological membranes are mostly structured as a flat bilayer of lipid molecules, certain proteins can induce the development of "non-bilayer" structures that incorporate hydrophobic enzymes for their protection and, in general, for a better control of their activity (Martinek *et al.*, 1989). These considerations strongly suggest that reverse micelles offer a possibility of reaching a better understanding of enzyme functioning in natural lipid media and, in our case, in the midgut of *O. nubilalis* larvae. Therefore, apart from easily isolating this trypsin-like activity directly from crude material, the enzyme(s) confined in reverse micelles make it easier to assess the inhibition

potential of a new family of proteinase inhibitors, recently isolated, in a state closer to that found *in vivo*. As the Table shows both cruciferous inhibitors and SBTI strongly inhibit endoproteinase(s) of *O. nubilalis* in micellar solution.

TABLE. Effect of a commercial (SBTI) and some other proteinase inhibitors isolated from cruciferous seeds on endoproteinase(s) isolated from larvae of *O. nubilalis* in micellar solution.

Inhibitors from ripe seed of	IC_{50} (μM) overall[a]
Soybean (SBTI)	0.12
Rapeseed (*Brassica napus*)	0.50
Brassica carinata	0.24
Cheirantus cheiri	0.30

[a] Inhibitory concentration 50%: Compound concentration required for 50% inhibition

Although it was interesting to determine the inhibition constants for each inhibitor in water solution, in reverse micelles we consider more useful to evaluate the IC_{50} which is, however, directly related to the Ki. These values demonstrated that the new inhibitors also strongly inhibit *O. nubilalis* proteinase, with an activity comparable to the well known SBTI. These results suggest similar activities *in vivo*, thus indicating the possibility, after further studies, to use these inhibitors in plant protection.

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