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Identification and recombinant expression of a novel chymotrypsin from *Spodoptera exigua*

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Abstract

A novel chymotrypsin which is expressed in the midgut of the lepidopteran insect *Spodoptera exigua* is described. This enzyme, referred to as SeCT34, represents a novel class of chymotrypsins. Its amino-acid sequence shares common features of gut chymotrpysins, but can be clearly distinguished from other serine proteinases that are expressed in the insect gut. Most notable, SeCT34 contains a chymotrypsin activation site and the highly conserved motive DSGGP in the catalytic domain around the active-site serine is changed to DSGSA. Recombinant expression of SeCT34 was achieved in Sf21 insect cells using a special baculovirus vector, which has been engineered for optimized protein production. This is the first example of recombinant expression of an active serine proteinase which functions in the lepidopteran digestive tract. Purified recombinant SeCT34 enzyme was characterized by its ability to hydrolyze various synthetic substrates and its susceptibility to proteinase inhibitors. It appeared to be highly selective for substrates carrying a phenylalanine residue at the cleavage site. SeCT34 showed a pH-dependence and sensitivity to inhibitors, which is characteristic for semi-purified lepidopteran gut proteinases. Expression analysis revealed that SeCT34 was only expressed in the midgut of larvae at the end of their last instar, just before the onset of pupation. This suggests a possible role of this protein in the proteolytic remodelling that occurs in the gut during the larval to pupal molt.

Keywords: Serine proteinase; Chymotrypsin; Trypsin; Baculovirus; Proteinase inhibitor; Lepidoptera

1. Introduction

Serine proteinases (SP) belong to one of the largest gene families in the animal kingdom. Within the human genome, for instance, around 500 proteinase-encoding genes have been identified, of which around 30% are SP or SP homologues (SPH) (Southan, 2001). A similar complexity exists in the *Drosophila melanogaster* genome, where around 200 SP- and SPH-encoding genes have been identified (Ross et al., 2003). SPs are involved in a wide range of physiological functions, including digestion of dietary proteins, blood coagulation, immune responses, signal transduction, hormone activation and development (Barrett et al., 2003). In insects, the most abundant and best studied group of SPs contains those expressed in the larval midgut, and these are supposed to be involved in the digestion of dietary protein.

Usually, the architecture of such proteinases is comparatively simple. While most regulatory SPs, for

Abbreviations: SeCT34, *Spodoptera exigua* chymotrypsin 34; BApNA, *N*α-benzoyl-L-arginine *p*-nitroanilide; SAAPFpNA, *N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide; SAAPLpNA, *N*-succinyl-alanine-alanine-proline-leucine *p*-nitroanilide; SAAApNA, *N*-succinyl-alanine-alanine-alanine *p*-nitroanilide; EFLpNA, pyroglutamyl-phenylalanine-leucine *p*-nitroanilide; BBI, Bowman–Birk trypsin inhibitor; PMSF, phenylmethylsulfonyl fluoride; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; EDTA, ethylenediamine tetraacetic acid.

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instance those involved in polyphenol-oxidase activation (Cerenius and Soderhall, 2004; Ji et al., 2004), or those involved in dorsoventral patterning (Rose et al., 2003), have a number of non-proteolytic protein modules attached to their *N*-terminus, the SP genes isolated from lepidopteran midgut do not contain such modules, and have a relatively small size (i.e. less than 300 amino-acids) (Bown et al., 1997). Generally, the immature protein (also called zymogen) contains a signal for its secretion into the gut lumen and a pro-protein part which keeps the protein in an inactive form until it is cleaved off (Barrett et al., 2003).

The study of digestive proteinases in lepidoptera is generally motivated by the fact that many lepidoptera are severe agricultural pests and that their digestive system is a suitable target for crop-protection strategies. For instance, herbivory of *Manduca sexta* on tobacco plants can be reduced by expressing a recombinant potato proteinase inhibitor in the leaves (Johnson et al., 1989). Proteinase inhibitors are also employed by the natural defence of plants against insects (Zavala et al., 2004). The inhibitors function by blocking the digestive proteinases in the larval gut, thereby limiting the release of amino acids from food protein. As a consequence, the larvae are arrested in development and eventually die. However, this strategy has not worked in all cases. Polyphagous insects like *Helicoverpa zea* and *Spodoptera exigua* have been shown to adapt to the presence of proteinase inhibitors in their diet, by switching to the production of proteinases that are resistant to plant proteinase inhibitors (Jongsma et al., 1995; Mazumdar-Leighton and Broadway, 2001b). Lepidopteran midgut SPs have also been studied in relation to their interaction with the Cry toxins from the entomopathogenic bacterium *Bacillus thuringiensis* (Oppert, 1999). Cry toxins accumulate in the bacteria in a protoxin form which, upon ingestion by the insect, is converted into an active form by action of the insect's SP. In addition, SP are also involved in the inactivation of such toxins by degradation. Resistance to Cry toxins has been described to be mediated both by down-regulation of proteinase expression thereby decreasing the activation of the protoxin (Oppert et al., 1997; Herrero et al., 2001) as well as by up-regulation of SPs increasing toxin inactivation (Forcada et al, 1996).

Despite their importance, not much is known about the catalytic properties of individual midgut SPs from lepidopteran insects. They have been studied following two different approaches. In a biochemical approach, the SPs have been purified from the midgut of the insects, which allowed characterization of their activity (Volpicella et al., 2003). By this approach, only the most abundant proteins in the mixture have been identified and characterized. In a genomic approach, sequences from different proteinases have been obtained from cDNA libraries (Bown et al., 1997) or by RT-PCR techniques using conserved primers (Mazumdar-Leighton and Broadway, 2001a). This approach does consider low abundant proteins, but no information on the catalytic characteristics of these proteins has so far been obtained due to the absence of a suitable expression system.

In the current work, we studied a novel and low abundant midgut proteinase from the beet armyworm, *S. exigua*. The proteinase is characterized by sequence comparison with related proteinases and detailed analysis of recombinant expressed protein. A recombinant baculovirus (*Autographa californica* multicapsid nucleopolyhedrovirus, AcMNPV) containing a deletion of the *chitinase* and *cathepsin* genes was employed for the expression of a functional proteinase in insect cells. Purified recombinant enzyme was characterized by its ability to hydrolyze synthetic substrates, its kinetic parameters and its susceptibility to different proteinase inhibitors.

2. Material and methods

2.1. Proteinase substrates and inhibitors

Synthetic substrates *N*α-benzoyl-L-arginine *p*-nitroanilide (BApNA), *N*-succinyl-alanine-alanine-proline-phenylalanine *p*-nitroanilide (SAAPFpNA), *N*-succinyl-alanine-alanine-proline-leucine *p*-nitroanilide (SAAPLρNA), *N*-succinyl-alanine-alanine-alanine *p*-nitroanilide (SAA ApNA) and pyroglutamyl-phenylalanine-leucine *p*-nitroanilide (EFLpNA) were purchased from Bachem AG (Bubendorf) and Sigma-Aldrich Chemie BV (Zwijndrecht). Proteinase inhibitors aprotinin, Bowman–Birk trypsin inhibitor (BBI), phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-phenylalanine chloromethyl ketone (TPCK), ethylenediamine tetra-acetic acid (EDTA) and antipain were purchased from Sigma-Aldrich Chemie BV (Zwijndrecht). Stock solutions were prepared according the supplier's specifications.

2.2. Insect RNA isolation

S. exigua larvae were continuously reared on artificial diet at 28 °C as described before (Smits and Vlak, 1988). RNA was isolated at different instars from whole larvae, from the larval midgut, the adult gut, hemocytes, and eggs. Larval midguts were pulled from the larvae after cutting off the hindbody between the last two pseudoleg pairs. Next, midguts were cut longitudinally with scissors and washed in phosphate-buffered physiological saline to remove the gut contents. Adult guts were obtained by longitudinally cutting of the abdomen. Although attention was given to remove all non-gut tissues during dissections, minor contamination could not be ruled out. For hemocyte isolation hemolymph