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Biochemical comparison of two proteolytic enzymes from *Carica candamarcensis*: Structural motifs underlying resistance to cystatin inhibition

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ABSTRACT

The lattices of *Carica candamarcensis* and *Carica papaya*, members of the Caricaceae family, contain isoforms of cysteine proteinases that help protect these plants against injury. In a prior study, we fractionated 14 discrete proteinaceous components from *C. candamarcensis*, two of them displaying mitogenic activity in mammalian cells. In this study, we compared the kinetic parameters of one of the mitogenic proteinases (CMS2MS2) with one of the isoforms displaying the highest enzyme activity of this group (CMS1MS2). Both enzymes display a similar Km value with either BAPNA (Benzoyl-Arg-pNA) or PFLPNA (Pyr-Phe-Leu-pNA), but the *kcat* of CMS1MS2 is about 14-fold higher for BAPNA and 129-fold higher with PFLPNA. While both enzymes are inhibited by E-64 and iodoacetamide, chicken cystatin fully inhibits CMS1MS2, but scarcely affects activity of CMS2MS2. Based on the structure of these proteins and other enzymes from the Caricaceae family whose structures have been resolved, it is proposed that Arg^{180} located in the cleft at the active site in CMS2MS2 is responsible for its resistance to cystatin.

1. Introduction

Plants store a variety of fluids, including latexes, resins, gums and mucilages within secretory cell cavities and canals. Latex is typically contained within laticifer cells, which may be interconnected by ramified structures creating a network throughout most of the plant (Dussourd and Eisner, 1987; Joel, 1980). Latex contains several compounds, whose biological properties confer protection against environmental damage. In *Hevea brasiliensis* L. (rubber tree), latex contains isoprene monomers that are released following tree-tapping until spontaneous polymerization leads to rubber formation (Gidrol et al., 1994).

Latex of *Carica papaya* contains a mixture of cysteine endopeptidases, such as papain (EC 3.4.22.2) (Mitchel et al., 1970), chymopapain (EC 3.4.22.6) (Watson et al., 1990), papaya endopeptidase III (Barrett and Buttle, 1985), papaya endopeptidase IV (also named glycyl endopeptidase or chymopapain M) (Ritonja et al., 1989a) and endopeptidase Ω (caricain) (Dubois et al., 1988). When fruits are injured, latex exudates transiently until a protein clot forms around the wounded area. The coagulation process is vital in creating a physical barrier against predator attack. Earlier, we provided evidence that during latex coagulation a number of peptides are proteolytically processed in a non-random manner (Moutim et al., 1999; Silva et al., 1997). Many of these latex changes take place concomitant with drastic variations in proteolytic activity, suggesting the involvement of proteolytic enzymes during clot formation.

Latex of *Carica candamarcensis* (also known as *Vasconcellea cundinamarcensis*), another member of the Caricaceae family common to many areas of South America, contains cysteine proteinases participating in clot formation *C. papaya*. Interestingly, *C. candamarcensis* proteinases display higher (five- to sevenfold) proteolytic activity than their homologues from *C. papaya* (Baeza et al., 1990; Bravo et al., 1994), and at least two of these proteases exert proliferative effects when incubated with mammalian cells (Gomes et al., 2005).

Although the latex components of *C. candamarcensis* are less studied than those from *C. papaya*, some reports describing their proteolytic activities and structure are available. Early studies described the most basic proteinase CC28 (Gravina de Moraes et al., 1994), the most acid proteinase CC23 (Pereira et al., 2001), the primary structure of three cysteine proteinases (CC-III, CC-Ia and CC-Ib) (Jaziri et al., 1994; Walraevens et al., 1999) and the primary structure of one of the proliferative proteinases (Gomes et al., 2007).

In a recent report, we described the proteinase composition of latex purified from this plant by a non-denaturing chromatographic procedure (Teixeira et al., 2008). Essentially, two main

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Table 1
Kinetic parameters of CMS1MS2 and CMS2MS2.

	CMS1MS2 BAPNA	CMS2MS2 BAPNA	CMS1MS2 Pyr-Phe-Leu-pNa	CMS2MS2 Pyr-Phe-Leu-pNa
kcat (s^{-1})	2.09 ± 0.14	0.15 ± 0.01	15.5 ± 2.10	0.12 ± 0.03
Km (mM)	7.24 ± 0.70	9.27 ± 1.28	0.27 ± 0.05	0.32 ± 0.08
kcat/Km ($M^{-1} \times s^{-1}$)	288.7 ± 17.7	16.2 ± 1.36	57,407 ± 5,330	375.0 ± 68.1

CMS1MS2 (1 μg) or CMS2MS2 (16 μg) was incubated with variable amounts of the substrates BAPNA or PFLPNA, in Tris–HCl activation buffer (pH 8) containing 2 mM EDTA and 5 mM cysteine as detailed in Section 4. The initial velocities obtained between 3 and 5 min were fitted into the Michaelis function by non-linear least-square method using the conjugate gradient algorithm (Solver).

proteolytic fractions were found plus a third fraction containing smaller amounts of 6 proteolytic enzymes. The major fractions CMS1 and CMS2 contain 3 and 5 isoforms, respectively, which were resolved by Mono S FPLC chromatography. Interestingly, the isoforms derived from CMS1 (CMS1MS1-3) display on average 18-fold higher amidase activity than isoforms from CMS2 (CMS2MS1-5), and two of the isoforms derived from CMS2 (CMS2MS2 and CMS2MS3) display mitogenic action on cultured fibroblasts (Gomes et al., 2005), while no effect is seen with the isoforms from CMS1. The isolation of two cysteine isoforms displaying proliferative effects on heterologous systems prompted us to study in more detail the differences between the proteolytic components of *C. candamarcensis*. Furthermore, in this study we present evidence that the mitogenic proteinase CMS2MS2 is barely inhibited by 3.5 μM egg white cystatin, while CMS1MS2 is sensitive like most papain-like enzymes. Thus, a search for structural evidence to explain the differential inhibitory effect of cystatin was conducted.

Table 2
Kinetic parameters of cysteine proteinases from *C. papaya* and *C. candamarcensis*.

Enzyme	pH	Substrate	*kcat*/Km ($M^{-1} \times s^{-1}$)
CMS1MS2	8.0	L-D-BAPNA	288.7
CMS2MS2	8.0	L-D-BAPNA	16.2
Chymopapain[a]	7.4	L-BAPNA	6.6
	9.2	L-BAPNA	4.1
Papain[a]	6.9	L-BAPNA	145.0
	6.2	L-BAPNA	145.0

[a] Baines et al. (1986).

2. Results and discussion

The purity of the isolated enzymes CMS1MS2 and CMS2MS2 was confirmed by SDS–PAGE and mass spectrometry (not shown). The kinetic parameters for each enzyme determined with L-D-BAPNA (Benzoyl-Arg-pNA) and Pyr-Phe-Leu-pNa (PFLPNA) substrates confirm that each enzyme has similar Km values, while *kcat* of CMS1MS2 is 14-fold higher for BAPNA and 129-fold higher with PFLPNA (Table 1). A comparison of the catalytic efficiencies of CMS1MS2 and CMS2MS2 with those described for chymopapain and papain from *C. papaya* is shown in Table 2. The data establish that the *kcat*/Km for CMS1MS2 is closer to papain, while the value for CMS2MS2 ranks close to chymopapain. The higher *kcat*/Km of papain compared to chymopapain has been explained by the papain preference for an aromatic (Phe or Tyr) residue at P2. The Val^{133} and Val^{157} residues at the S2 subsite from papain are substituted by L^{133} and L^{157} in chymopapain (Watson et al., 1990). These substitutions were predicted to restrict the accessibility of the F residue from PFLPNA to the S2 pocket, decreasing the reactivity of chymopapain (Maes et al., 1996). Interestingly, CMS2MS2 like chymopapain has L^{133} and L^{157} in these positions, while CMS1MS2 contains V^{133} and I^{157}. These differences may account for the discrepant enzymatic activity observed with PFLPNA substrate. Furthermore, the kinetic similarities between chymopapain and CMS2MS2 and also between papain and CMS1MS2 observed here are supported by the structural relationship previously observed by the phylogenetic tree clustering of the enzymes from Caricaceae (Gomes et al., 2007).

A comparison between these two enzymes in their ability to cleave the β-chain of insulin is illustrated in Fig. 1. The results show

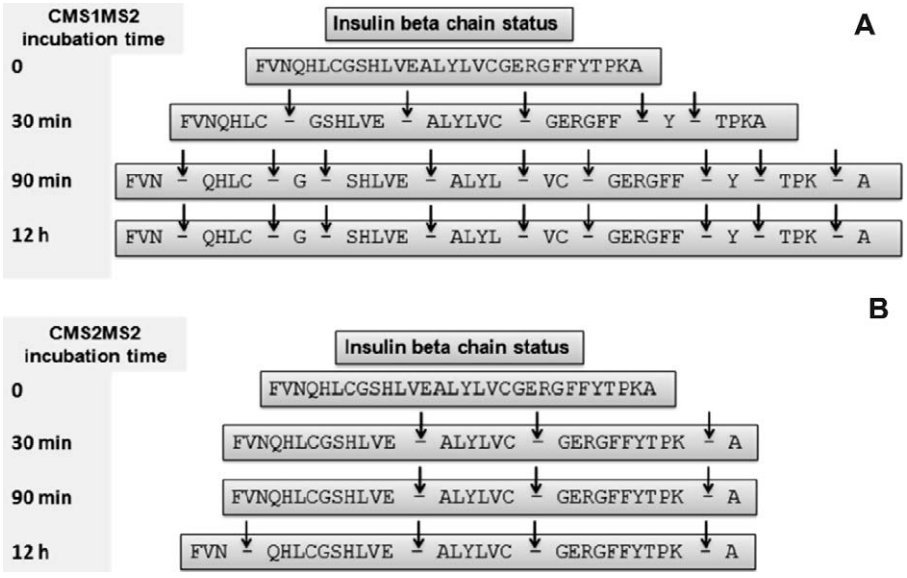

Fig. 1. Insulin β-chain cleavage by CMS1MS2 and CMS2MS2. β-chain insulin and the proteases CMS1MS2 (A) or CMS2MS2 (B) were incubated as described in Section 4. The arrows indicate the hydrolyzed peptide bond in each incubation time.