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A novel form of ficin from *Ficus carica* latex: Purification and characterization

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ABSTRACT

A novel ficin form, named ficin E, was purified from fig tree latex by a combination of cation-exchange chromatography on SP-Sepharose Fast Flow, Thiopropyl Sepharose 4B and fplc-gel filtration chromatography. The new ficin appeared not to be sensitive to thiol derivatization by a polyethylene glycol derivative, allowing its purification. The protease is homogeneous according to PAGE, SDS–PAGE, mass spectrometry, N-terminal micro-sequencing analyses and E-64 active site titration. N-terminal sequencing of the first ten residues has shown high identity with the other known ficin (iso)forms. The molecular weight was found to be (24,294 ± 10) Da by mass spectrometry, a lower value than the apparent molecular weight observed on SDS–PAGE, around 27 kDa. Far-UV CD data revealed a secondary structure content of 22% α-helix and 26% β-sheet. The protein is not glycosylated as shown by carbohydrate analysis. pH and temperature measurements indicated maxima activity at pH 6.0 and 50 °C, respectively. Preliminary pH stability analyses have shown that the protease conserved its compact structure in slightly acidic, neutral and alkaline media but at acidic pH (<3), the formation of some relaxed or molten state was evidenced by 8-anilino-1-naphtalenesulfonic acid binding characteristics. Comparison with the known ficins A, B, C, D1 and D2 (iso)forms revealed that ficin E showed activity profile that looked like ficin A against two chromogenic substrates while it resembled ficins D1 and D2 against three fluorogenic substrates. Enzymatic activity of ficin E was not affected by Mg^{2+}, Ca^{2+} and Mn^{2+} at a concentration up to 10 mM. However, the activity was completely suppressed by Zn^{2+} at a concentration of 1 mM. Inhibitory activity measurements clearly identified the enzyme as a cysteine protease, being unaffected by synthetic (Pefabloc SC, benzamidine) and by natural proteinaceous (aprotinin) serine proteases inhibitors, by aspartic proteases inhibitors (pepstatin A) and by metallo-proteases inhibitors (EDTA, EGTA). Surprisingly, it was well affected by the metallo-protease inhibitor o-phenanthroline. The enzymatic activity was however completely blocked by cysteine proteases inhibitors (E-64, iodoacetamide), by thiol-blocking compounds ($HgCl_2$) and by cysteine/serine proteases inhibitors (TLCK and TPCK). This is a novel ficin form according to peptide mass fingerprint analysis, specific amidase activity, SDS–PAGE and PAGE electrophoretic mobility, N-terminal sequencing and unproneness to thiol pegylation.

1. Introduction

Proteases are important biomolecules that regulate the turnover, localization and activity of a variety of proteins, control protein–protein interactions, fashion new bioactive molecules, contribute to the processing of cellular information, and also generate, transduce and amplify molecular signals. As a consequence, proteases influence DNA replication and transcription, cell proliferation and differentiation, tissue morphogenesis and remodeling, angiogenesis, fertilization, wound repair, inflammation, immunity, autophagy, senescence, necrosis and apoptosis. Consistent with these essential roles of proteases in cell behavior, survival and death of all organisms, alterations in proteolytic systems underlie multiple pathological conditions such as cancer, neurodegenerative disorders, inflammatory and cardiovascular diseases. Accordingly, many proteases are potential drug targets or diagnostic (Turk, 2006), and hence constitutes a major focus of attention for the pharmaceutical industry. In plants, proteases have been shown to be involved in xylem formation, seeds maturation,

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nutrient supply mobilization, removal of signal peptides from several nucleus-encoded organellar proteins, degradation of damaged proteins, responses to environmental stimuli, virulence factors and senescence of programmed cell death (Huffaker, 1990; van der Hoorn, 2008). They have been isolated from various parts of the plant, including fruits, stems, seeds and latex (Boller, 1986). The properties of latex in plant defense and in alleviating various diseases have been attributed to the presence of several hydrolytic enzymes from which proteases play a key role. Most of the proteases found in latex of different plants belong to cysteine and serine proteases families (Singh et al., 2008; El Moussaoui et al., 2001; Azarkan et al., 2004, 2011; Torres et al., 2012; Ramos et al., 2013; Yariswamy et al., 2013). Why latex contains high amounts of such proteases still remain enigmatic. It was reported, however, that they participate to defense mechanisms by protecting ripening fruits against plant pathogens like fungi and insects (Baker and Drenth, 1987), which they can attack directly (Konno et al., 2004). *In vitro* experiments clearly demonstrated that cysteine proteases act by degrading the protective cuticule (Stepek et al., 2004, 2005) or the peritrophic matrix (Pechan et al., 2002; Mohan et al., 2006). It was also demonstrated that artificial diets containing mixtures of clan CA1 cysteine proteases, like papain and ficin from papaya and common fig tree latex, respectively, both at concentrations observed in latex, are toxic for silkworm larvae. It was observed that the larvae died when fed on latex-containing leaves but did not on latex-free leaves or when the cysteine proteases were selectively inactivated by 4-[(2S,3S)-3-carboxyoxiran-2-yl-carbonyl-L-leucylamido]-butylguanidine (E-64) (Konno et al., 2004). Cysteine proteases are also required for latex coagulation upon biotic or abiotic injuries (Azarkan et al., 2006). This wound-healing property is probably the most efficient plant protection from pathogens entry and further spread (El Moussaoui et al., 2001). All these findings are in favor of a common defensive role of latex proteases, suggesting that these enzymes possess other functions than the simple digestive role they are often assumed to play.

The latex of *Ficus carica* constitutes an important source of many proteolytic components, known under the general term ficin (EC 3.4.22.3), which belong to the cysteine proteases of the papain family (family C1, clan CA). Fractionation of several ficin forms from *F. carica* latex was reported as early as 1964 (Kramer and Whitaker, 1964; Sgarbieri et al., 1964) and since then, the existence of ficin forms complexity was confirmed by other researchers (Kramer and Whitaker, 1969, 1969b; Devaraj et al., 2008). On the basis of activity measurements, Kramer and Whitaker (1969, 1969b) could identify at least seven ficin molecular species. Moreover, Sgarbieri et al. (1964) obtained ten proteolytic components from *F. carica* latex.

We recently isolated and characterized five ficin (iso)forms (Azarkan et al., 2011). In the present work, we report the purification and characterization of an additional cysteine peptidase, termed ficin E. Purification of this novel protease was possible after depletion, by thiol-derivatization, of ficin A which co-eluted with ficin E on all the chromatographic media tested. The novel ficin is clearly distinguishable from the other known ficin (iso)forms on the basis of its N-terminal sequence, its electrophoretic behavior on both native and SDS–PAGE, and its resistance to chemical modification by a thiol-specific polyethylene glycol derivative.

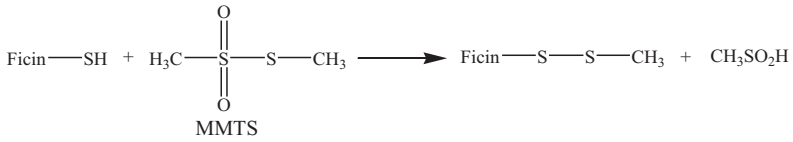
2. Results and discussion

2.1. Fractionation of the Ficus carica latex proteases

Fresh *F. carica* latex was collected in the presence of methylmethanethiol sulfonate (MMTS), leading to *in situ* direct chemical modification of the catalytic cysteine of papain-like proteases. This procedure was used to inhibit these enzymes from irreversible oxidation and also to prevent both, their autolysis and possible proteolytic degradation of other latex proteins. Both, colorimetric (DL-BAPNA and Boc-Ala-Ala-Gly-pNA) and fluorimetric (Z-Phe-Arg-AMC) assays clearly demonstrated that no enzymatic activity could be detected after this chemical treatment. However, full activity could be recovered, upon addition of DTT in the reaction mixture, showing the reversible character of the inhibition process. This result also indicated that the proteases found in *F. carica* latex all contain an essential active site cysteine. A schematic representation of the corresponding reaction is presented in Scheme 1.

According to Fig. 1, the fractionation of the whole *F. carica* latex soluble fraction, on a SP-Sepharose Fast Flow column, showed five major peaks designated as unretained fraction (UF) and pools I, II, III and IV. The most apparent heterogeneity was observed for pool I. Consistent with this are the SDS–PAGE experiments where a protein band with an apparent molecular weight of 27 kDa (ficin E) (Inset Fig. 1, lane 2) can be detected in addition to the protein bands migrating with an apparent molecular weight of 24 kDa (ficin A) and other low molecular weight species. The presence of such low molecular weight protein material indicated that some proteolysis/autolysis occurs even when the proteases were immediately inhibited upon latex collection. It is known that unprotected cysteine proteases can form intermolecular linkages and/or catalyze their autolysis during storage. It is important to note that ficin forms with molecular masses comprised between 14 kDa and 18 kDa, i.e. far below the 24 kDa value characterizing native ficin, have been reported, which could reasonably be explained by some autolysis/proteolysis processes (Kramer and Whitaker, 1969). More recently, Zare et al. (2013) investigated ficins' autolysis, in absence of inhibitors and after being fractionated on a cation-exchange support, by using HPLC chromatogram changes and ultrafiltrations at different temperatures and storage times. These authors concluded that ficin's autolysis process directly and inversely correlated with surface charges and hydrophobic patches, respectively. They thus demonstrated that some ficin forms were more prone to proteolysis/autolysis than others.

The band of ficin E is not detected in the other pools upon SDS–PAGE analyses (inset Fig. 1, lines 3, 4 and 5). Pools II, III and IV contained almost exclusively molecular species with an apparent molecular weight of ~24 kDa, corresponding to different ficin forms (B, C and D1/D2). After rechromatography of pool I, on the SP-Sepharose Fast Flow column (Fig. 2), SDS–PAGE analyses, indeed (inset Fig. 2), clearly indicated that ficin E was mainly eluted in peak b, with however some presence in peak a. The first



Scheme 1.