PURIFICATION AND SOME MOLECULAR PROPERTIES OF A SERINE PROTEINASE INHIBITOR FROM *Brassica carinata* SEED

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ABSTRACT

A serine proteinase inhibitor, *Brassica carinata* inhibitor (BCTI), has been isolated from ethiopian mustard (*B. carinata*) seeds by ion exchange and gel filtration chromatography. BCTI besides inhibiting the catalytic activity of bovine β-trypsin and α-chymotrypsin at pH 8.0 and 21°C, strongly inhibits a serine proteinase isolated from larvae of European corn borer (*Ostrinia nubilalis).* The inhibitor shows a molecular weight of about 7 kD and the stoichiometry presumably is 1:1 in the complexes with both the bovine proteinases. No structural similarity of BCTI with other serine proteinase inhibitors has been revealed, apart from the other low molecular weight proteinase inhibitors from cruciferous seeds such as MTI-2 from *Sinapis alba* and RTI from oil seed rape (*Brassica napus*) previously studied. The possibility that BCTI, MTI-2 and RTI are members of a new class of plant serine proteinase inhibitors is discussed.

INTRODUCTION

In previous studies two new serine proteinase inhibitors from ripe seeds of *S. alba* (MTI-2) and *B. napus* (RTI) were purified and characterized (Menegatti *et al.* 1992; Ceciliani *et al.* 1994). The results of these studies strongly indicate the existence of a new family of serine proteinase inhibitors. In fact, their amino acid sequence is considerably different from plant inhibitors of other families. Nevertheless, MTI-2 and RTI show a high degree of identity in their primary structure, including the reactive site position, the molecular weight, and the dissociation constants.

The aim of this work is two fold. Firstly, to propose a new family of serine proteinase inhibitors belonging to the Cruciferae on a wider basis of observations. Secondly, to promote the isolation and introduction of genes encoding those proteins into the plants for their protection against insect attack. To this end, we describe the purification, a part of the primary structure and the inhibitory properties of inhibitor from *B. carinata* (BCTI). These data are discussed in comparison to those of MTI-2 and RTI.

EXPERIMENTAL

Purification

The seeds of *B. carinata,* cv BRK29 were supplied from Agra S.p.A. Italy. The defatted meal was homogenized in distilled water with a Ultra Turrax (IKA-Werke), centrifuged and then titred to pH 3.8. After centrifugation, which removes more than 80% of inactive proteins, the clear solution was loaded onto a SP-Sepharose F.F.

(Pharmacia) previously conditioned with 50 mM citrate buffer, pH 3.8 (starting buffer). The inhibitor was eluted with 1M NaCl in the same buffer. After dialysis against starting buffer (pH 3.8), the active solution was loaded by FPLC apparatus into a SP-Sepharose HP HR 16/10 column (Pharmacia) and eluted with a gradient of NaCl from 0.0 to 0.5 M in starting buffer. The active fractions were pooled, dialyzed, concentrated and loaded by the FPLC in a column Superose 12 (Pharmacia). Molecular weight was determined by SDS-PAGE electrophoresis (Laemmly, 1970).

Sequence determination, Amino acid analysis and Computer sequence analysis

The methodology for determining the primary structure of BCTI is based on S-pyridylethylation. N-terminal amino acid sequencing was performed as reported by Menegatti *et al.*, (1992). The amino acid analysis was carried out after gas-phase hydrolysis by pre-column derivatization with 6-aminoquinoyl-N-hydroxysuccinimidyl carbamate using a JASCO HPLC equipped with a monitor mod. 820-FP (Cohen and Michaud., 1993). Similarities between the structures of inhibitors isolated from cruciferous seeds and other proteins were searched using the Swiss Protein Data Bank. As regard the BCTI structure, although still not complete, it appears to be the same of RTI and very similar to the MTI-2, which are not assignable to any of the families in the current inhibitor classification.

RTI	Asp	Ser	Glu	Cys	Leu	Lys	Glu	Tyr	Gly	Gly	Asp	Val	Gly	Phe	Gly
MTI-2	Asp	Ser	Glu	Cys	Leu	Lys	Glu	Tyr	Gly	Gly	Asp	Val	Gly	Phe	Pro
BCTI	....	Ser	Glu	Cys	Leu	Lys	Glu	Tyr	Gly	Gly	Asp	Val	Gly	Phe	Gly
RTI	Phe	Cys	Ala	Pro	**Arg**	**Ile**	Tyr	Pro	Ser	Phe	Cys	Val	Gln	Arg	Cys
MTI-2	Phe	Cys	Ala	Pro	**Arg**	**Ile**	Phe	Pro	Thr	Phe	Cys	Tyr	Thr	Arg	Cys
BCTI	Phe	Cys	Ala	Pro	**Arg**	**Ile**	Tyr	Pro	....	....	....	....	....	....	....
RTI	Arg	Ala	Asp	Lys	Gly	Ala	Leu	Ser	Gly	Lys	Cys	Ile	Trp	Gly	Gln
MTI-2	Arg	Glu	Asn	Lys	Gly	Ala	Lys	Gly	Gly	Lys	Cys	Ile	Trp	Gly	Glu
BCTI	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
RTI	Gly	Ser	Asn	Val	Lys	Cys	Leu	Cys	Asn	Phe	Cys	Arg	His	Glu	Pro
MTI-2	Gly	Thr	Asn	Val	Lys	Cys	Leu	Cys	Asn	Tyr	Cys	Asn	Asp	Ser	Pro
BCTI	....	....	....	....	....	....	....	....	....	....	....	....	....	....	....
MTI-2	Phe	Asp	Gln												

Fig. 1 The primary structures of RTI, MTI-2 and the N-terminus of BCTI. The Arg-Ile (bold face) bond appears to be the reactive sites of the inhibitors. This peptide bond was cleaved by immobilized trypsin which can be used as ligand for the affinity chromatography purification of the inhibitors.

Inhibitory properties

The crude extract of *B. carinata* seeds shows both anti-trypsin and anti-chymotrypsin activities, similarly to those obtained from *S. alba* and *B. napus* seeds. (Iori *et al.* 1991). In addition, BCTI, but also RTI and MTI-2, strongly inhibits a serine proteinase isolated by affinity chromatography from eight instars larvae of European corn borer (*Ostrinia nubilalis*) both in water solution and in reverse micelles dispersed in

organic solvent (Bernardi *et al.* 1994). These similar results obtained for all three inhibitors are presumably due to their impressive homology, both of the N-terminal sequences and the position of reactive site bond at Arg^{20}-Ile^{21}(Fig.1). Taking into account these findings, one can reasonably assume that the apparent dissociation constant (*Kd)* of pure BCTI, although its accurate determination is still in progress, should not be so different from the *Kd* values determined for MTI-2 and RTI, which are respectively 1.6×10^{-10}M and 3.0×10^{-10}M for β-trypsin; 5.0×10^{-7}M and 4.1×10^{-7}M for α-chymotrypsin. Likewise, one can also assume that the apparent stoichiometry for the enzyme-inhibitor complex is 1:1, with an apparent Hill coefficient nearly to 1.00, and that the *Kd* value, as those of MTI-2 and RTI, is independent from the enzyme and substrate concentrations. In conclusion, although part of this work is still in progress and some experiments remain to be done and confirmed, we believe that BCTI, but also RTI and MTI-2, are members of a new class of serine proteinase inhibitors. In fact, beside to display both new primary structures and chemicophysical properties, they show interesting biological activities which appear to be of potential practical use.

As regard the possible utilization of these molecules, we want to emphasize the strong inhibition of these new inhibitors on a pure proteinase isolated from an important noxious insect, as well as the great potentiality of these molecules in plant biotechnology as reported by Hilder *et al.*, (1987) and McManus *et al.*, (1993). In fact, these authors indicate that similar genes encoding proteinase inhibitors, beside being correctly processed in transgenic plants, are also expressed and accumulated as a fully functional protein, which confer field resistance to several phytophagous insects.

Finally, as regard the nutritional aspect of defatted proteinic seed meals for animal feeding, as already reported for oil seed rape (Visentin *et al.*, 1992), the presence of this inhibitor in the seed of *B. carinata* has little significance from a nutritional viewpoint, given that the activity values are lower than the toasted soybean protein concentrate.

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