Characterisation of Serine Proteinase Inhibitors in Dry Seeds of Cultivated Pasture Grass Species

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Abstract

A chymotrypsin inhibitor has been partially purified from *Lolium perenne* cv. Grasslands Ruanui that is stable at pH 3 and after heating at 80°C for 10 minutes (acid/heat stable). The inhibitor had a native molecular weight of *ca.* 20-22 kDa determined using a 2.5 cm x 100 cm Sephadex G-75 column and gel electrophoresis indicated that it may be comprised of two subunits that had molecular weights of *ca.* 11 kDa and 12 kDa. To determine the occurrence of such inhibitors in other grass species, a survey of seeds from several cultivars of pasture grass species was conducted. Seeds of two cultivars of *Festuca arundinaceae*, cv. Grasslands Garland and cv. Grasslands Roa have been found to contain the most potent chymotrypsin inhibitory activity of the species surveyed. Seeds of two cultivars of an economically-important genus, *Lolium*, *L. perenne* cv Grasslands Ruanui and *L. x boucheanum* cv Grasslands Greenstone also exhibited significant chymotrypsin inhibitory activity, and so these four species were studied further. Using a 2.5 cm x 100 cm gel filtration column, chymotrypsin inhibitory activity eluted as two peaks in all four cultivars examined which had native molecular weights of *ca.* 20-22 kDa for peak I and *ca.* 10-12 kDa for peak II. A *ca.* 12 kDa chymotrypsin inhibitor was observed in peak 1 of *F. arundinaceae* cv. Grasslands Garland during gel electrophoresis, and was further purified by ion exchange chromatography. Twelve amino acid residues were sequenced from the N-terminal and the protein was found to be homologous with members of the cereal α-amylase inhibitor family. To purify this activity further, a 10 cm x 100 cm gel filtration column was used. This time trypsin inhibitory activity was also assayed and for all four cultivars, both chymotrypsin and trypsin inhibitory activities co-eluted within two peaks, both with similar molecular weights (peak I, 20-22 kDa and peak II, 10-12 kDa) to that observed previously using the smaller gel filtration column. Gel electrophoresis of peak I from all the four cultivars revealed at least six comparatively lower molecular weight chymotrypsin inhibitors ranging from *ca.* 12 kDa to 3 kDa for the *Festuca* cultivars, and at least two ranging from *ca.* 8 kDa and 12 kDa for the *Lolium* cultivars. However, higher molecular weight trypsin inhibitors were present in Peak I with 5 inhibitors

ranging from *ca.* 12-18 kDa for *Festuca*, and three ranging from *ca.* 12-16 kDa for *Lolium*. Peak II from all the four cultivars contained only one 12 kDa inhibitor band. The 12 kDa inhibitor is active against both trypsin and chymotrypsin. The 12 kDa inhibitor from peak II of *F. arundinaceae* cv. Grasslands Garland was purified further using an anhydro-trypsin affinity chromatography and then to homogeneity by reverse-phase HPLC. Two inhibitory peaks were separated that had dual trypsin/chymotrypsin inhibitory activity and had an identical 20 amino acid residues at the N-terminal. The two inhibitor polypeptides were found to be homologous to members of the barley trypsin inhibitor family, but did not share homology with the *ca.* 12 kDa inhibitor protein purified by ion exchange from gel filtration peak I.

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LIST OF ABBREVIATIONS

BApNA	N-benzoyl-DL-arginine-p-nitroanalide
BBI	Soybean (Bowman-Birk) trypsin inhibitor
BTI	Barley trypsin inhibitor
BTpNA	N-benzoyl-L-tyrosine-p-nitroanalide
Ca.	Circa
CI-1	Barley chymotrypsin inhibitor 1
CI-2	Barley chymotrypsin inhibitor 2
CMe	Chloroform methanol soluble
CPTI	Cow pea trypsin inhibitor
ELISA	Enzyme-linked immunosorbent assay
FMTI	Foxtail millet trypsin inhibitor
h	Hour
HPLC	High pressure liquid chromatography
kDa	Kilo dalton
Ki	Enzyme inhibition constant
LPC	Leaf protein concentrate
min	Minute
OD	Optical density
P1	Proteinase inhibitor reactive site
PIIF	Proteinase inhibitor inducing factor
PVDF	Polyvinylidene difluoride membrane
RBTI	Rice bran trypsin inhibitor
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STI	Soybean (Kunitz) trypsin inhibitor
TFA	Trifluoroacetic acid
Tris	Tris (hydroxymethyl) amino-methane
cv.	Cultivar