CLONING, EXPRESSION AND SITE-DIRECTED MUTAGENESIS OF THE BOWMAN-BIRK INHIBITOR OF HORSEGRAM (*Dolichos biflorus*).

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by

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DECLARATION

I hereby declare that this thesis entitled **CLONING, EXPRESSION AND SITE-DIRECTED MUTAGENESIS OF THE BOWMAN-BIRK INHIBITOR OF HORSEGRAM (*Dolichos biflorus*),** submitted herewith, for the degree of Doctor of Philosophy in Biochemistry of the University of Mysore, Mysore, is the result of work done by me in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, India, under the guidance and supervision of Dr. Lalitha R. Gowda, during the period of May, 2004 - September, 2011.

I further declare that the results of this work have not been previously submitted for any other degree or fellowship.

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CERTIFICATE

I hereby certify that this thesis `Cloning, expression and site-directed mutagenesis of the Bowman-Birk inhibitor of horsegram (*Dolichos biflorus)'*, submitted by **Mrs.Deepa G. Muricken** to the University of Mysore, Mysore, for the degree of **Doctor of Philosophy in Biochemistry** is the result of research work carried out by her in the Department of Protein Chemistry and Technology, Central Food Technological Research Institute (CFTRI), Mysore, under my guidance and supervision. This work has not been submitted either partially or fully for any other degree or fellowship.

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ABSTRACT

Bowman Birk inhibitors (BBI) are small protease inhibitors found in the seeds of legumes in particular. Their molecular masses are in the range of 6-9 kDa. They comprise of a binary arrangement of two sub domains with a conserved array of seven disulphide bridges, which play a pivotal role in the stability of the inhibitors. These inhibitors interact simultaneously and independently with two molecules of proteinases. In addition to the protease inhibitor activity, BBI is reported to have anticarcinogenic and radio protective activity and immune stimulating properties. BBIs have also been implicated to play a vital role in plant defense mechanism. Horse gram (*Dolichos biflorus)* is a pulse crop native to South East Asia and Tropical Africa. Four isoforms of BBIs, from horsegram seeds have been isolated. The inhibitors of horsegram (HGIs) are single polypeptides with a molecular mass of 8.5 kDa. However SDS-PAGE and analytical gel filtration indicate the molecular mass to be 16 kDa, suggesting that they exist as dimers in solution. In contrast, inhibitors of germinated horse gram seeds, HGGIs exist as monomers. The role of active site residue Lys^{24} and C-terminal end in the dimeric status of the major inhibitor (HGI-III) was previously established by in vitro and homology modeling. To delineate their role in vivo the HGI-III gene was cloned in *E. coli* and expressed.

HGI-III specific gene was isolated from the genomic DNA of horsegram by PCR based method. The gene was cloned in pRSET C vector such that the extra residues from the vector are avoided. pRSET-rHGI was functionally expressed in *E.coli* cells and was purified to homogeneity. The characterization of rHGI was carried out and was comparable to the HGI-III already reported. rHGI also existed as a dimer and the kinetic constants of the inhibitor towards trypsin and chymotrypsin were comparable to the HGI-III.

To evaluate the role of active site residue Lys^{24} and the C-terminal end in dimerisation, site directed mutagenesis was performed. Characterization of the inhibitory activities of the mutants revealed that the K24A mutant inhibited elastase instead of trypsin. D75A mutant and Δ76 mutant retained the trypsin inhibitory activity. All the mutants and rHGI exhibited similar chymotrypsin inhibitory activity. The oligomerisation status of the mutants was studied using SDS-PAGE and size exclusion chromatography. The studies pointed that K24A mutant existed as a monomer. The C-terminal mutants, D75A and Δ 76 also existed as monomers. Thermal stability studies revealed that the monomers were less stable than the dimers. Thus the cloning and heterologous expression of a functional rHGI provides a platform to unveil the fine specificity of the interactions involved in the dimeric status of HGI-III.

The BBIs have been extensively studied and known to prevent malignant transformation in cancerous cells. The large size of BBIs hinders the bioavailability of orally administered the BBIs. Smaller peptides comprising the inhibitor domain are an attractive alternative. The trypsin inhibitory (TID) domain of horsegram BBI was genetically engineered. The expressed rTID was purified to homogeneity and was evaluated. The apparent molecular mass of the expressed protein was ~4000 Da. The purified inhibitor was stable thermally and also to proteases like pepsin and pancreatin. Kinetic studies indicated that the expressed peptide (rTID) is a non-competitive inhibitor of trypsin and also inhibited tryptase. Preliminary studies revealed that rTID inhibits other trypsin like proteases. This smaller peptide is a better prospect for drug design targeted at tryptase, the enzyme implicated in inflammatory, allergic disorders and multiple sclerosis.

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LIST OF ABBREVIATIONS

%	Percentage
λ	Wavelength
°C	Degree centigrade
λ_{max}	Absorption maximum (wavelength)
ε	Molar extinction coefficient
µg	Micro gram
[E]	Enzyme concentration
[S]	Substrate concentration
[I]	Inhibitor concentration
A	Absorbance
A.U.	Absorbance unit
APNE	N-acetyl-DL-phenylalanine-β-napthyl ester
Amp	Ampicillin
APS	Ammonium persulfate
BBI	Bowman-Birk inhibitor
BBIC	Bowman-Birk inhibitor concentrate
BAPNA	∝-N-Benzoyl-DL-arginine-*p*-nitroanilide
BCIP	5-Bromo-4-chloro-3-indolylphosphate
BSA	Bovine serum albumin
BTPNA	N-Benzoyl-L-tyrosine-*p*-nitroanilide
$CaCl_2$	Calcium chloride
CAPS	3-[Cyclohexylamino]-1-propanesulfonic acid
CBB	Coomassie brilliant blue
CCl_4	Carbon tetrachloride
cDNA	Complementary DNA
CIU	Chymotrypsin inhibitory units

cm	Centimeter
CNBr	Cyanogen bromide
CU	Chymotrypsin unit
DEAE	Diethylaminoethyl
DMSO	Dimethylsulfoxide
DTT	DL-Dithiothreitol
dNTPs	Deoxynuleotide mixture
DNA	Deoxyribonucleic acid
EDTA	Ethylene diamine tetraacetic acid
E. coli	*Escherichia coli*
EtBr	Ethidium bromide
EIU	Elastase inhibitory unit
g	Grams
GuHCl	Guanidine hydrochloride
h	Hour
HRP	Horseradish peroxidase
HCl	Hydrochloric acid
HGGI	Horsegram germinated inhibitor
HGI	Horsegram inhibitor
HPLC	High performance liquid chromatography
IPTG	Isopropyl thiogalactopyranoside
IU	Inhibitory units
i.d.	Internal diameter
kDa/Da	Kilo Daltons/Daltons
K_i	Inhibitory constant
K_m	Michaelis-Menten constant
L	Liter
LB	Luria Broth
$MgCl_2$	Magnesium chloride

M	Molar concentration
MALDI-MS	Matrix assisted laser desorption ionization - Mass spectrometry
$MgCl_2$	Magnesium chloride
MgSO4	Magnesium sulphate
$MnCl_2$	Manganese chloride
min	Minute
mL	Milliliter
M_r	Molecular weight
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NAPNA	N-Succinyl-Ala-Ala-Ala-*p*-nitroanilide.
NBT	Nitroblue tetrazolium
NTSB	2-Nitro-5-thiosulfobenzoate
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
pmole	Picomole
PCR	Polymerase chain reaction
PMSF	Phenylmethyl-sulfonyl fluoride
PTC	Phenylthiocarbamyl
PVDF	Polyvinilidine diflouride membrane
rHGI	Recombinant horsegram inhibitor
rTID	Recombinant trypsin inhibitory domain
RP	Reverse phase
RNA	Ribonucleic acid
R_t	Retention time
RP-HPLC	Reverse phase-High performance liquid chromatography
SDS	Sodium dodecyl sulfate
Sec	Second

TCA	Trichloroacetic acid
TAE	Tris acetate ethylene diamine tetraacetic acid
TE	Tris ethylene diamine tetraacetic acid
TBS	Tris-buffer saline
TEMED	N-N, N'-N'-Tetramethyl 1, 2-diaminoethane
TFA	Trifluoroacetic acid
TIU	Trypsin inhibitory unit
TPCK	Tosyl-phenyalanine chloromethylketone
Tris	Tris (hydroxymethyl) amino methane
TU	Trypsin unit
TLCK	Tosyl-lysine chloromethylketone
T_m	Melting temperature
UV	Ultraviolet
v/v	Volume by volume
vs	Versus
w/v	Weight by volume
V_{max}	Maximum velocity
V_e	Elution volume

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