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Complete Amino Acid Sequence of the Lentil Trypsin–Chymotrypsin Inhibitor LCI-1.7 and a Discussion of Atypical Binding Sites of Bowman–Birk Inhibitors

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The complete primary structure of the lentil (*Lens culinaris*) trypsin−chymotrypsin inhibitor LCI-1.7 was determined by conventional methods in order to find relationships between partial sequences and the difference in action against human and bovine chymotrypsin. As other Bowman−Birk type inhibitors, LCI-1.7 contained 68 amino acid residues, seven disulfide bridges, and two reactive sites, Arg16−Ser17 for trypsin and Tyr42−Ser43 for chymotrypsin. Evaluation of sequence homologies showed that it belonged to the group III Bowman−Birk inhibitors. The atypical additional binding site of LCI-1.7 for human chymotrypsin was discussed and compared with such binding sites of two other Bowman−Birk inhibitors, the Bowman−Birk soybean proteinase inhibitor BBI, and the lima bean proteinase inhibitor LBI I, for human and bovine trypsin and chymotrypsin. A concept to reduce the action of these inhibitors against human enzymes by genetic engineering was proposed.

KEYWORDS: Amino acid sequence; atypical binding sites; Bowman−Birk inhibitor; chymotrypsin inhibitor; disulfide bridges; *Glycine max*; inhibition of bovine proteinases; inhibition of human proteinases; *Lens culinaris*; lentil; lima bean; *Phaseolus lunatus*; primary structure; reactive sites; soybean; trypsin inhibitor

INTRODUCTION

Four Bowman−Birk type trypsin−chymotrypsin inhibitors, *Lens culinaris* inhibitors (LCI) LCI-1.7, -2.2, -3.3, and -4.6, have been isolated from Syrian local small lentils (*Lens culinaris* Medik. ssp. *microsperma*) and characterized (*1*). The four inhibitors show characteristic differences in their action against human and bovine proteinases: human trypsin (HT) is inhibited somewhat less (∼75%) than bovine trypsin (BT), and human chymotrypsin (HCT) is inhibited much more (∼250%) than bovine chymotrypsin (BCT). Their molecular mass, high cystine content, and ability to inhibit trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) indicate that they are members of the Bowman−Birk inhibitor family. Chemical modification experiments, enzymatic modifications followed by carboxypeptidase degradation, and characterization of the enzyme/inhibitor complexes formed have been performed to clarify the reasons for their different actions against the same enzyme from different species (*2*). All four inhibitors contain arginine at the trypsin-reactive site and tyrosine (LCI-1.7 and LCI-2.2), phenylalanine (LCI-3.3), or leucine (LCI-4.6) at the chymotrypsin-reactive site. On a molar basis, the inhibition of HT, BT, and BCT is within the usual range: <1 mol of HT and BCT and ∼1 mol of BT are inhibited per mole of inhibitor. The differences in action against HT and BT are caused by different affinities of the inhibitors toward the two trypsins. The inhibition of >1 mol of HCT per mole of inhibitor is caused by the additional and atypical binding at the trypsin-reactive site of all four inhibitors. The so much higher inhibition of HCT compared to BCT results from a combination of two effects: the additional binding of HCT at the trypsin-reactive site and the weak inhibition of BCT. To further clarify the reasons for the differences in action against HCT and BCT, the most prominent lentil isoinhibitor that also shows those differences most pronounced, LCI-1.7, has been selected to determine its primary structure in order to find relationships between sections of the amino acid sequence and the differences in inhibition properties. The understanding of these relationships offers the possibility to produce varieties by breeding or genetic engineering that show minimal inhibition of proteinases in the species for whose nutrition they are produced without totally reducing their biological activity, which is thought to function in plant protection (*3*). The results of these studies are presented in this paper.

MATERIALS AND METHODS

Trypsin−Chymotrypsin Inhibitor LCI-1.7 from Lentils. Extraction of the inhibitors from seed meal of Syrian local small lentils (*Lens culinaris* Medik. ssp. *microsperma*, ICARDA ILL 4401), fractionation with ammonium sulfate, affinity chromatography on anhydrotrypsin−Sepharose, and anion exchange chromatography on Whatman DE-52 were performed as previously described (*1*). The pooled fractions

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containing the inhibitors of group LCI-1, after ultrafiltration (Amicon membrane YM 3) and lyophilization, were applied to reversed-phase high-pressure liquid chromatography (RP-HPLC) on Nucleosil 300-5 C_8 (column 4.6 × 250 mm) eluted with a gradient of acetonitrile in 0.01 M triethylammonium formate (TEAF) buffer, pH 6.0, at 58 °C and 1.5 mL/min (isocratic 13.75% acetonitrile in TEAF buffer for 2 min, linear gradient of 13.75− 20.75% acetonitrile in 38 min). The resulting main peak, crude LCI-1.7, was rechromatographed using the same system and lyophilized.

Materials. Ultrafiltration membranes YM 3, 43 mm ∅, were purchased from Amicon (Witten, Germany), carboxypeptidase Y from bakers' yeast and thermolysin from *Bacillus thermoproteolyticus* from Boehringer (Mannheim, Germany), and trifluoroacetic acid (TFA), for protein sequential analysis, and 4-vinylpyridine, ∼96%, practical grade, from Fluka (Neu-Ulm, Germany). Nucleosil 300-5 C_8, 5 μm, 300 Å, was from Macherey-Nagel (Düren, Germany), and hydroperoxide, 30%, Selectipur, and tetrahydrofuran, Lichrosolv, were from Merck (Darmstadt, Germany), Sephadex G-25 fine was obtained from Pharmacia (Freiburg, Germany), acetonitrile, Chromosolv, from Riedel de Haen (Seelze, Germany), ribonuclease from bovine pancreas from Serva (Heidelberg, Germany), and ODS-Hypersil, 5 μm, 100 Å, Shandon, from Bischoff (Leonberg, Germany). Triethylamine, ≥99%, and trypsin type XIII, *N-p*-tosyl-L-phenylalanine chloromethyl ketone(TPCK)-treated, from bovine pancreas, were from Sigma (St. Louis, MO). Biobrene Plus TM, PTH-C18 cartridge (2.1 × 220 mm), TFA-treated cartridge filters, and the sequencing grade reagents were from Applied Biosystems (Weiterstadt, Germany). All other chemicals were of reagent grade purchased from Merck, Serva, or Sigma.

Tryptic Peptides. Tryptic peptides of performic acid-oxidized LCI-1.7 (LCI-1.7/PA), prepared as previously described (*2*), were obtained by incubation with TPCK-trypsin. LCI-1.7/PA (∼400 μg) was dissolved in 100 μL of 0.1 M tris(hydroxymethyl)aminomethane (Tris)-HCl buffer, pH 8.0, 10 mM in $CaCl_2$, and mixed with 10 μL of enzyme solution (7.2 μg of TPCK-trypsin dissolved in Tris-HCl buffer, enzyme/inhibitor protein ratio 1:56, w/w). After 8 h at 37 °C, the reaction was stopped by adding 30 μL of 30% acetic acid. After drying under flowing nitrogen, the peptides were separated by RP-HPLC on ODS-Hypersil (column 4.6 × 250 mm) eluted with a gradient of acetonitrile in 0.1% TFA at 60 °C and 1.5 mL/min (isocratic 0.1% TFA for 5 min, linear gradient of 0−50% acetonitrile in 60 min). The resulting protein-containing peaks (Tr′1−Tr′8) were dried under flowing nitrogen and rechromatographed using the same system, but with an isocratic part of the start acetonitrile concentration in 0.1% TFA for 5 min (all samples) followed by a linear gradient of acetonitrile 0−10% in 20 min (Tr′5), 0−30% in 25 min (Tr′6b), 10−40% in 30 min (Tr′4 and Tr′7), 15−35% in 20 min (Tr′3), 15−40% in 25 min (Tr′1+2 and Tr′6a), and 20−40% in 20 min (Tr′4+8).

Tryptic peptides of reduced and *S*-β-(4-pyridyl)ethylated LCI-1.7 (LCI-1.7/PE) were prepared accordingly. Reduction and derivatization were performed according to a modification of the method of Friedman et al. (*4*). Portions of LCI-1.7 (100−500 μg) were mixed with 1.5 mL of a freshly prepared solution of 1 mg of dithiothreitol in 100 mL of 0.2 M Tris-HCl buffer, pH 7.5, 2 M in urea, and stirred for 2 h at ambient temperature under nitrogen. A solution of freshly distilled 4-vinylpyridine (80 μL; 50 μL of 4-vinylpyridine in 1 mL of 2-propanol) was added. The mixture was stirred for 5 min, kept in the dark for 2 h, and dried under flowing nitrogen. LCI-1.7/PE was isolated by chromatography on Sephadex G-25 fine (column 10 × 570 mm) eluted with 0.1 M acetic acid at a flow rate of 14 mL/h, dried under flowing nitrogen, and then lyophilized. The product (∼400 μg) was dissolved in 950 μL of 0.1 M Tris-HCl buffer, pH 8.0, 10 mM in $CaCl_2$, and mixed with 51 μL of enzyme solution (16 μg of TPCK-trypsin dissolved in Tris-HCl buffer, enzyme/inhibitor protein ratio 1:25, w/w). After 8 h at 37 °C, the reaction was stopped by adding 100 μL of glacial acetic acid. After drying under flowing nitrogen, the peptides were separated by RP-HPLC on ODS-Hypersil (column 4.6 × 250 mm) eluted with a gradient of acetonitrile in 0.01 M TEAF buffer, pH 6.0, at 60 °C and 1.5 mL/min (isocratic 5% acetonitrile in TEAF buffer for 5 min, linear gradient of 5−40% acetonitrile in 60 min). The resulting protein-containing peaks (Tr1−Tr8) were dried under flowing nitrogen and rechromatographed using the same system, but with an isocratic part of the start acetonitrile concentration in TEAF buffer for 5 min (all samples) followed by a linear gradient of acetonitrile 5−25% in 20 min (Tr3 and Tr5), 15−40% in 25 min (Tr4 and Tr8), 25−55% in 25 min (Tr6a and Tr7), and 35−65% in 25 min (Tr2).

Thermolytic Peptides. Thermolytic peptides of LCI-1.7/PE were prepared by dissolving the derivatized product (∼1 mg) in 950 μL of 0.1 M Tris-HCl buffer, pH 8.0, 2 mM in $CaCl_2$, and mixing with 50 μL of enzyme solution [40 μg of thermolysin (EC 3.4.24.4) dissolved in Tris-HCl buffer, enzyme/inhibitor protein ratio 1:25, w/w]. After 4 h at 50 °C, the reaction was stopped by adding 100 μL of glacial acetic acid. After drying under flowing nitrogen, the peptides were separated by RP-HPLC on ODS-Hypersil (column 4.6 × 250 mm) eluted with a gradient of acetonitrile in 0.01 M TEAF buffer, pH 6.0, at 60 °C and 1.5 mL/min (isocratic 5% acetonitrile in TEAF buffer for 5 min, linear gradient of 5−40% acetonitrile in 60 min). Some of the resulting protein-containing peaks were dried under flowing nitrogen and rechromatographed using the same system, but with an isocratic part of the start acetonitrile concentration in TEAF buffer for 5 min (all samples) followed by a linear gradient of acetonitrile 0−16% in 20 min (Th2 and Th3), 10−30% in 25 min (Th5, Th6, and Th7), 15−30% in 30 min (Th13 and Th14), 20−35% in 25 min (Th18 and Th19), and 20−45% in 35 min (Th16).

Amino Acid Analysis. Samples were hydrolyzed (5.7 M HCl, 10 mM in phenol, 110 °C, 24 h, in vacuo; LCI-1.7/PA and peptides Tr′1−Tr′8 without phenol), dried over KOH pellets in vacuo, and analyzed (Biotronik LC 5001 amino acid analyzer, Maintal, Germany).

N-Terminal Sequencing. Automated Edman degradation was performed on a "pulsed liquid" protein sequencer 471A (Applied Biosystems, Foster City, CA) using the program provided by the company. Phenylthiohydantoin−amino acid derivatives were identified on-line by RP-HPLC using a 140 B solvent system from Applied Biosystems.

C-Terminal Sequencing. LCI-1.7/PA (250 μg in 500 μL of 0.1 M TEAE buffer, pH 6.0) was degraded with carboxypeptidase Y (EC 3.4.16.1; 2.7 μg of enzyme in 100 μL of distilled water) at 37 °C. Aliquots were taken after 60, 180, and 360 min, and the amino acids liberated were determined by amino acid analysis.

RESULTS AND DISCUSSION

Purified LCI-1.7 was performic acid-oxidized, yielding LCI-1.7/PA. After incubation of LCI-1.7/PA with TPCK-trypsin, separation by RP-HPLC on ODS-Hypersil resulted in nine peptide fractions (results not shown). The amino acid compositions of these fractions are compiled in **Table 1**. The final denotation of the fractions as tryptic fragments Tr′1−Tr′8 was made after finishing the sequencing studies. For these studies, the fractions were rechromatographed on ODS-Hypersil, whereby Tr′1+2 was separated into Tr′1 and Tr′2 and Tr′4+8 was separated into Tr′4 and Tr′8. Unexpectedly, two further fragments, Tr′6a and Tr′6b, were obtained, which resulted from Tr′6 by cleavage of the Tyr−Ser bond, although TPCK-trypsin with blocked chymotryptic activity was used.

In parallel, purified LCI-1.7 was reduced and *S*-β-(4-pyridyl)-ethylated, yielding LCI-1.7/PE. After incubation of LCI-1.7/PE with TPCK-trypsin and separation of the peptides by RP-HPLC on ODS-Hypersil, seven peptide fractions were isolated (results not shown). Comparison of their amino acid compositions (**Table 2**) with those of Tr′1−Tr′8 (**Table 1**) and sequencing of some of the fragments, after rechromatography, identified the fractions as the tryptic fragments Tr2−Tr8 with Tr6a instead of Tr6 (see above).

To obtain overlapping sequences, LCI-1.7/PE was also cleaved with thermolysin, and the resulting fragments were separated by RP-HPLC on ODS-Hypersil (**Figure 1**). Of the 20 fractions obtained, designated Th1−Th20, the amino acid compositions of 11 selected fractions were determined (**Table 3**). Rechromatography on ODS-Hypersil, done for further

Table 1. Amino Acid Composition of Performic Acid-Oxidized LCI-1.7 (LCI-1.7/PA) and Fragments Thereof Obtained with TPCK-Trypsin (Tr′1–Tr′8)

amino acid[b]	LCI-1.7/PA		Tr′1+2		Tr′3		Tr′4		Tr′5		Tr′6		Tr′6a		Tr′6b		Tr′7		Tr′8+4		Tr′8[a]	
	C[c]	S[c]	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
Asx	8.2	8	3.0	3	0.2	0	1.1	1	1.0	1	2.3	2	0.3	0	1.9	2	0.2	0	2.3	2	1.2	1
Thr	3.8	4	1.9	2	1.0	1	0	0	0.1	0	1.0	1	0.2	0	1.0	1	0.1	0	0.2	0	0.2	0
Ser	5.6	6	1.2	1	1.0	1	0.1	0	1.7	2	1.4	1	0.4	0	1.0	1	0.2	0	1.4	1	1.3	1
Glx	7.7	7	0.3	0	1.2	1	0.1	0	1.0	1	2.4	2	0.3	0	2.2	2	0.2	0	3.9	3	3.8	3
Pro	4.1	4	0	0	2.1	2	0	0	0.1	0	1.4	2	0.2	0	1.7	2	0.1	0	0.2	0	0.2	0
Gly	1.1	1	1.2	1	0.1	0	0.1	0	0.2	0	0.3	0	0.2	0	0.9	0	0.1	0	0.3	0	0.2	0
Ala	3.9	4	0.9	1	0.1	0	0.1	0	0.9	1	1.0	1	1.2	1	0.5	0	0.1	0	1.3	1	1.2	1
Cys	14.4	14	3.8	4	1.2	1	1.1	1	1.7	2	3.1	4	2.6	2	1.7	2	1.1	1	2.4	2	1.3	1
Val	3.9	4	1.1	1	0.1	0	2.0	2	0.1	0	1.3	1	1.2	1	0.3	0	0.1	0	1.9	2	0	0
Ile	1.3	1	0	0	0.1	0	0	0	0.1	0	0.3	0	0	0	0.2	0	0	0	1.1	1	1.1	1
Leu	1.0	1	0.9	1	0	0	0	0	0.1	0	0	0	0.1	0	0.2	0	0	0	0.1	0	0.1	0
Tyr	2.1	3	0	0	0	0	0	0	0	0	1.0	2	1.0	1	0.8	1	0.8	1	0.1	0	0.1	0
Phe	1.0	1	0	0	0	0	0	0	0	0	0	0	0	0	0.2	0	0.9	1	0	0	0	0
His	3.6	3	0	0	0.1	0	0	0	0.9	1	1.0	1	0.1	0	0.9	1	0.1	0	1.3	1	1.3	1
Lys	4.3	4	1.0	1	0.1	0	0	0	1.0	1	1.0	1	0.2	0	1.0	1	1.0	1	0.1	0	0.1	0
Arg	3.0	3	0.9	1	1.0	1	1.0	1	0.1	0	0	0	0.1	0	0.2	0	0	0	0.9	1	0	0
Σ	69.0	68	16.2	16	8.3	7	5.6	5	9.0	9	17.5	18	8.1	5	14.7	13	5.0	4	17.5	14	12.1	9

[a] Differences between Tr′8+4 and Tr′4. [b] LCI-1.7 does not contain methionine and tryptophan (*1*). [c] Columns C and S are residues per molecule from amino acid composition and sequence, respectively.

Table 2. Amino Acid Composition of *S*-*β*-(4-Pyridyl)ethylated LCI-1.7 (LCI-1.7/PE) and of Some Fragments Thereof Obtained with TPCK-Trypsin (Tr2–Tr8)

amino acid	LCI-1.7/PE		Tr2		Tr3		Tr4		Tr5		Tr6a		Tr7		Tr8	
	C[a]	S[a]	C	S	C	S	C	S	C	S	C	S	C	S	C	S
Asx	7.5	8	1.0	1	0.1	0	1.3	1	1.2	1	0.2	0	0.4	0	0.9	1
Thr	3.9	4	2.2	2	0.9	1	0	0	0.3	0	0.2	0	0.2	0	0	0
Ser	5.5	6	1.9	1	1.0	1	0.3	0	2.5	2	0.2	0	0.4	0	1.0	1
Glx	7.4	7	1.0	0	1.2	1	0.3	0	1.5	1	0.2	0	0.4	0	2.8	3
Pro	4.7	4	0.7	0	1.6	2	0	0	0	0	0	0	0	0	0	0
Gly	1.1	1	0.5	0	0.1	0	0.3	0	0.7	0	0.2	0	0.4	0	0.2	0
Ala	4.3	4	1.2	1	0.1	0	0	0	1.2	1	1.0	1	0.2	0	1.0	1
Cys	8.5	14	4.0[b]	4	1.0[b]	1	1.0[b]	1	2.0[b]	2	2.0[b]	2	1.0[b]	1	1.0[b]	1
Val	4.2	4	0.7	0	0.1	0	2.4	2	0.3	0	1.0	1	0.2	0	0.2	0
Ile	1.0	1	0.5	0	0.1	0	0	0	0.3	0	0.2	0	0.2	0	1.0	1
Leu	1.1	1	0.9	1	0.1	0	0	0	0	0	0	0	0.2	0	0	0
Tyr	3.1	3	0	0	0	0	0	0	0	0	1.0	1	1.1	1	0	0
Phe	1.2	1	0	0	0	0	0	0	0	0	0	0	1.0	1	0	0
His	3.1	3	0	0	0	0	0	0	1.0	1	0	0	0	0	1.0	1
Lys	4.2	4	0	0	0	0	0	0	1.0	1	0	0	0.9	1	0	0
Arg	3.3	3	1.0	1	1.0	1	1.0	1	0	0	0	0	0	0	0	0
Σ	64.1	68	15.6	11	7.3	7	6.6	5	12.0	9	6.2	5	6.6	4	9.1	9

[a] Columns C and S as in **Table 1**. [b] Cysteine residues taken from sequencing; *S*-*β*-(4-pyridylethyl)cysteine not determined.

Table 3. Amino Acid Composition of Some Fragments from *S*-*β*-(4-Pyridyl)ethylated LCI-1.7 Obtained with Thermolysin (Th2–Th19)

amino acid	Th2		Th3		Th5		Th6		$Th7_1$		Th8		Th13		Th14		Th16		Th18		Th19	
	C[a]	S[a]	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S	C	S
Asx	0.9	1	1.1	1	0.9	1	0.1	0	1.0	1	1.8	1	0	0	0	0	0.3	0	0.2	0	0.9	1
Thr	0.1	0	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	1.9	2	1.1	1	1.2	1
Ser	0.9	1	1.0	1	0	0	0	0	1.0	1	2.2	2	0.1	0	0	0	1.2	1	1.1	1	0.3	0
Glx	2.6	3	1.1	1	0	0	0	0	0.3	0	2.0	1	0	0	0	0	1.3	1	1.3	1	0.2	0
Pro	0.2	0	2.8	2	0	0	0	0	0.4	0	0.2	0	0.2	0	0	0	1.9	2	2.4	2	0.5	0
Gly	0.1	0	0.1	0	0	0	0	0	0	0	0.8	0	0	0	0	0	0.1	0	0.1	0	0	0
Ala	1.1	1	0	0	0.9	1	0.1	0	0.9	1	0.8	0	0	0	0	0	0.1	0	0.2	0	1.0	1
Cys	0.4	1	0.2	1	1.1	2	0.7	1	1.2	2	0.2	1	1.0	2	0.8	1	0.9	1	0.9	2	2.0	3
Val	0.4	0	0	0	0	0	0	0	0	0	1.0	2	0	0	0	0	0	0	0.1	0	0	0
Ile	0.8	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Leu	0.1	0	0	0	0	0	1.3	1	0.2	0	0	0	0	0	0	0	0.9	1	0.1	0	0	0
Tyr	0	0	0	0	0	0	0	0	0.2	0	0	0	0	0	0	0	0	0	0.1	0	0	0
Phe	0	0	0	0	0	0	0	0	0	0	0.5	0	0	0	1.2	1	0.1	0	0	0	0	0
His	0.8	1	0	0	0	0	0	0	0	0	0.8	1	0.2	0	0	0	0	0	0	0	0	0
Lys	0.1	0	0	0	0.9	1	0.1	0	0.9	1	0.5	0	0.1	0	0	0	0.3	0	0.1	0	0	0
Arg	0.1	0	0	0	0	0	0	0	0	0	0.8	1	1.2	1	0.1	0	1.2	1	1.0	1	0.4	0
Σ	8.6	9	6.3	6	3.8	5	2.3	2	6.1	6	11.8	9	2.8	3	2.1	2	10.2	9	8.7	8	6.5	6

[a] Columns C and S as in **Table 1**.

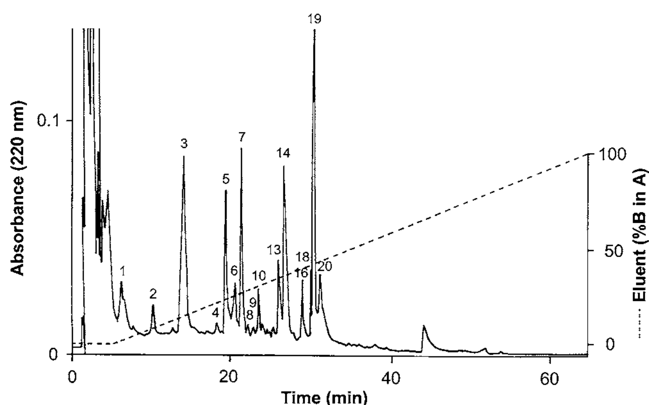

Figure 1. RP-HPLC of thermolytic peptides from LCI-1.7/PE: column (4.6 × 250 mm) of ODS-Hypersil, eluted with 5% acetonitrile in 0.01 M TEAF buffer, pH 6.0, (A) for 5 min, followed by a linear gradient of 5–40% acetonitrile in TEAF buffer (B) in 60 min (1.5 mL/min, 60 °C); 1–20, peptides Th1–Th20.

purification prior to sequencing, separated the fraction initially designated Th7 into one major peak ($Th7_1$) and one minor component ($Th7_2$).

Automated Edman degradation of LCI-1.7/PE resulted in the N-terminal amino acid sequence of LCI-1.7 up to position 40 (**Figure 2**). Sequencing of the tryptic fragments Tr′1, Tr′2, Tr3, Tr′4, and Tr5 allowed their arrangement in that order, starting at the N-terminus of LCI-1.7. Sequencing of Tr′6, Tr′6a, Tr′6b, and Tr6a resulted in their location with the aid of three characteristic overlapping amino acids (Cys38−Val39−Cys40), as shown in **Figure 2**. Carboxypeptidase Y degradation of LCI-1.7/PA, yielding the C-terminal sequence -Ile-Glu-Glu, indicated Tr′8 as the C-terminal fragment of the remaining two tryptic fragments. Sequencing of 12 of the thermolytic fragments confirmed most of the partial sequences and arrangements by overlapping sequences (**Figure 2**). The complete amino acid sequence of the lentil trypsin−chymotrypsin inhibitor LCI-1.7 was derived from these results. It is confirmed by at least two partial sequences, two residues (Tyr58−Lys59) excepted. Inhibitor LCI-1.7 contains 68 amino acid residues, and its relative molecular mass, calculated from the amino acid composition derived from the sequence, is 7576. This value is close to that determined with gel−liquid chromatography earlier (7700) and smaller than values determined with other methods (*1*).

The primary structure of LCI-1.7 shows the typical features of Bowman−Birk proteinase inhibitors (*5*−*7*): a molecular mass of ~8000, 14 half-cystine residues, and two repetitive domains (residues 9−34 and 35−60) with two reactive sites. The location of the two reactive sites of LCI-1.7, Arg16−Ser17 for trypsin and Tyr42−Ser43 for chymotrypsin, was deduced from homology with other Bowman−Birk inhibitors (**Figure 3**). Arginine has already been identified to occur in the trypsin-reactive site of LCI-1.7 recently (*2*). Whereas chemical modification experiments have also indicated tyrosine in the chymotrypsin-reactive site in the same study, enzymatic modification with catalytic amounts of chymotrypsin followed by carboxypeptidase degradation has resulted in leucine occurring there. Thus, this study confirmed the results of the chemical modification experiments; not even traces of leucine were found at that position by sequencing the four fragments Tr′6, Tr′6a, Tr6a, and $Th7_2$ (**Figure 2**). The discrepancy between the two experiments has been thought to result from a microheterogeneity at the chymotrypsin-reactive site (*2*); this isoform of LCI-1.7 was

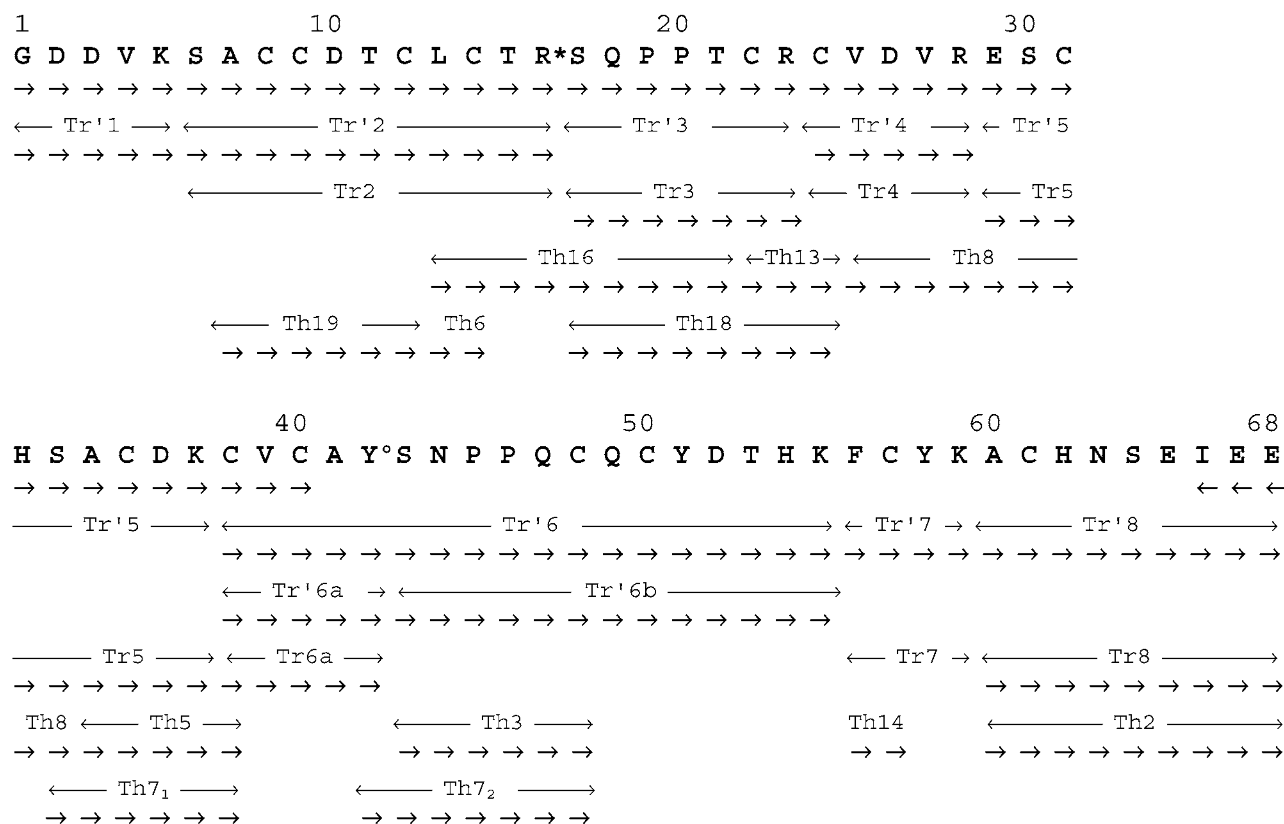

Figure 2. Results of sequencing studies of LCI-1.7: Tr′ and Tr, tryptic peptides from LCI-1.7/PA and LCI-1.7/PE, respectively; Th, thermolytic peptides from LCI-1.7/PE; ★, trypsin-reactive site; ○, chymotrypsin-reactive site; ⟶, automated Edman degradation; ⟵, carboxypeptidase Y degradation; amino acid composition determined for all peptides, $Th7_2$ excepted.

	1			10			20			30			40			50			60			68	
LCI-1.7	GDD	VKS	ACC	DTC	LCT	R[a]SQ	PPT	CRC	VDV	RES	CHS	ACD	KCV	CAY[b]	SNP	PQC	QCY	DTH	KFC	YKA	CHN	SEI	EE	
LCI-L1[F]	GDD	VKS	ACC	DTC	LCT	R[x]SQ	PPT	CRC	VDV	RES	CHS	ACD	KCV	CAY[x]	SNP	PQC	QCY	DTH	NFC	YKT	CH/			
PSTI IVA	GDD	VKS	ACC	DTC	LCT	K[a]SN	PPT	CRC	VDV	RET	CHS	ACD	SCI	CAY[b]	SNP	PKC	QCF	DTH	KFC	YKA	CHN	SEV	EEV	IKN
PSTI IVB	GDD	VKS	ACC	DTC	LCT	K[a]SN	PPT	CRC	VDV	GET	CHS	ACL	SCI	CAY[b]	SNP	PKC	QCF	DTQ	KFC	YKA	CHN	SEL	EEV	IKN
PSI TI-5-72	GDD	VKS	ACC	DTC	LCT	K[a]SD	PPT	CRC	VDV	GET	CHS	ACD	SCI	CAL[b]	SYP	PQC	QCF	DTH	KFC	YKA	CHN	SEV	EEV	IKN
PSI TI-F	GDD	VKS	ACC	DTC	LCT	K[x]SN	PPT	CRC	VDV	GET	CHS	ACN	SCI	CAL[x]	SYP	PKC	QCF	DTQ	KFC	YKA	CHN	SEL	EEV	IKN
PSI TI-7V	GDD	VKS	ACC	DTC	LCT	K[x]SN	PPT	CRC	VDV	GET	CHS	ACN	SCI	CAL[x]	SYP	PQC	QCF	DTQ	KFC	YKA	CHN	SEV	EEV	IKN
PSI TI-6V[F]	GDD	VKS	ACC	DTC	LCT	A[x]SN	PPT	CRC	VDV	GET	CHS	ACN	SCI	CAY[x]	SNP	PKC	QCF	DTH	KFC	YKA	CH/			
VAI	GDD	VKS	ACC	DTC	LCT	R[a]SQ	PPT	CRC	VDV	GER	CHS	ACN	HCV	CNY[b]	SNP	PQC	QCF	DTH	KFC	YKA	CHS	SEK	EEV	IKN
FBI	GDD	VKS	ACC	DTC	LCT	K[a]SE	PPT	CRC	VDV	GER	CHS	ACN	SCV	CRY[b]	SNP	PKC	QCF	DTH	KFC	YKS	CHN			

Figure 3. Comparison of the primary structures of group III Bowman–Birk inhibitors from legume seeds: LCI-1.7, trypsin–chymotrypsin inhibitor from lentils (this paper); LCI-L1[F], trypsin–chymotrypsin inhibitor fragment from L. culinaris leaves (10), included for comparison (sequence from nucleic acid; residues 1–42 not shown, probably propeptide and/or signal as in garden pea); PSTI IVA and IVB, trypsin inhibitors from winter pea (P. sativum cv. Frilene) (11, 12); PSI TI-5–72, trypsin inhibitor from garden pea (P. sativum cv. Birte; sequence from cDNA) (13); PSI TI-F, trypsin–chymotrypsin inhibitor from garden pea (P. sativum cv. Frisson; sequence from nucleic acid) (14); PSI TI-7V, trypsin–chymotrypsin inhibitor from garden pea (P. sativum cv. Victor; sequence from nucleic acid) (14); PSI TI-6V[F], trypsin–chymotrypsin inhibitor fragment from garden pea (P. sativum cv. Victor; sequence from nucleic acid) (14); VAI, proteinase inhibitor from common vetch (V. angustifolia var. segetalis) (15); FBI, proteinase inhibitor from faba bean (V. faba) (16); [a] and [b], demonstrated or putative reactive site for trypsin and chymotrypsin, respectively; [x], no information given in the reference; boldface letters, identical amino acid residue in all representatives.

probably separated by RP-HPLC rechromatography prior to sequencing in the present study.

The highest degrees of homology exist between the primary structure of LCI-1.7 and those of representatives of a group of Bowman-Birk inhibitors designated group III by Norioka and Ikenaka (*8*, *9*). The amino acid sequences of LCI-1.7 and of some representatives of that group are shown in **Figure 3**. As expected, a very high degree of homology exists between LCI-1.7 and LCI-L1[F], the trypsin−chymotrypsin inhibitor fragment from *Lens culinaris* leaves (*10*): the two sequences differ by only two amino acids (97% homology if amino acids 1−62 of LCI-1.7 are taken as the basis). The degrees of homology between LCI-1.7 and the other representatives of **Figure 3** range from 90% for VAI, the proteinase inhibitor from common vetch, *Vicia angustifolia* L. (*15*), to 81% for PSI TI-F, the trypsin−chymotrypsin inhibitor from garden pea, *Pisum sativum* L. cv. Frisson (*14*). The four species from which the inhibitors have been isolated, the sequences of which are shown in **Figure 3**, *L. culinaris*, *P. sativum*, *V. angustifolia*, and *Vicia faba* L., all belong to the tribe Vicieae of the Papilionoideae subfamily of the Leguminosae (Fabales). The high degrees of homology between the primary structures of Bowman−Birk inhibitors from this group (the lowest value was 77% between LCI-L1[F] and PSI TI-F) confirm the treatment of these inhibitors as a separate group by Norioka and Ikenaka (*8*, *9*), although the authors knew the sequence of only one inhibitor, VAI. The authors have constructed a phylogenetic tree of legume Bowman−Birk inhibitors on the basis of their amino acid sequence relationships. In this tree, group III inhibitors are located in the middle between group I and II inhibitors [from soybean, *Glycine max* (L.) Merr.; adzuki bean, *Vigna angularis* (Willd.) Ohwi & Ohashi; mung bean, *Vigna radiata* (L.) Wilczek; garden bean, *Phaseolus vulgaris* L.; lima bean, *Pisum lunatus* L.; and *Macrotyloma axillare* (E. Mey.) Verdc.] and those of group IV (peanut, *Arachis hypogaea* L.). This phylogenetic tree has been confirmed by studies of other authors (*17*−*19*).

The locations of the 14 half-cystine residues in Bowman−Birk inhibitors are highly conserved (*5*−*7*). The seven disulfide bridges between these residues play an important role in maintaining their active conformation and are responsible for their relative stability against heating and strong acids. The disulfide linking pattern has proven to be identical in all cases studied: in BBI, the Bowman−Birk soybean proteinase inhibitor, as established by chemical methods (*20*), as well as in ABI I, the adzuki bean inhibitor I (*21*), PI A-II, the peanut inhibitor A-II (*22*), PI-II from soybeans (*23*), and PSTI IVB, the *Pisum sativum* trypsin inhibitor IVB from winter pea (*24*), as derived from X-ray diffraction data at 2.5−3.3 Å resolution. From this disulfide pattern, the disulfide bridges of LCI-1.7 were deduced to be Cys8−Cys61, Cys9−Cys24, Cys12−Cys57, Cys14−Cys22, Cys31−Cys38, Cys35−Cys50, and Cys40−Cys48.

Figure 4 shows the covalent structures of three Bowman−Birk inhibitors in a representation that Odani and Ikenaka (*20*) have used for BBI. The three inhibitors were selected because the differences in their action against human and bovine proteinases have been studied in detail. Inhibitor LCI-1.7 (**Figure 4a**) binds HCT additionally at Arg16 of the trypsin-reactive site (see Introduction for more details) (*2*). Because one of the porcine chymotrypsin components, chymotrypsin C, cleaves peptide bonds also after glutamine and methionine (*26*), and a chymotrypsinogen similar to chymotrypsinogen C from porcine pancreas has been isolated from human pancreatic juice (*27*), binding of HCT at Gln18 close to that reactive site has

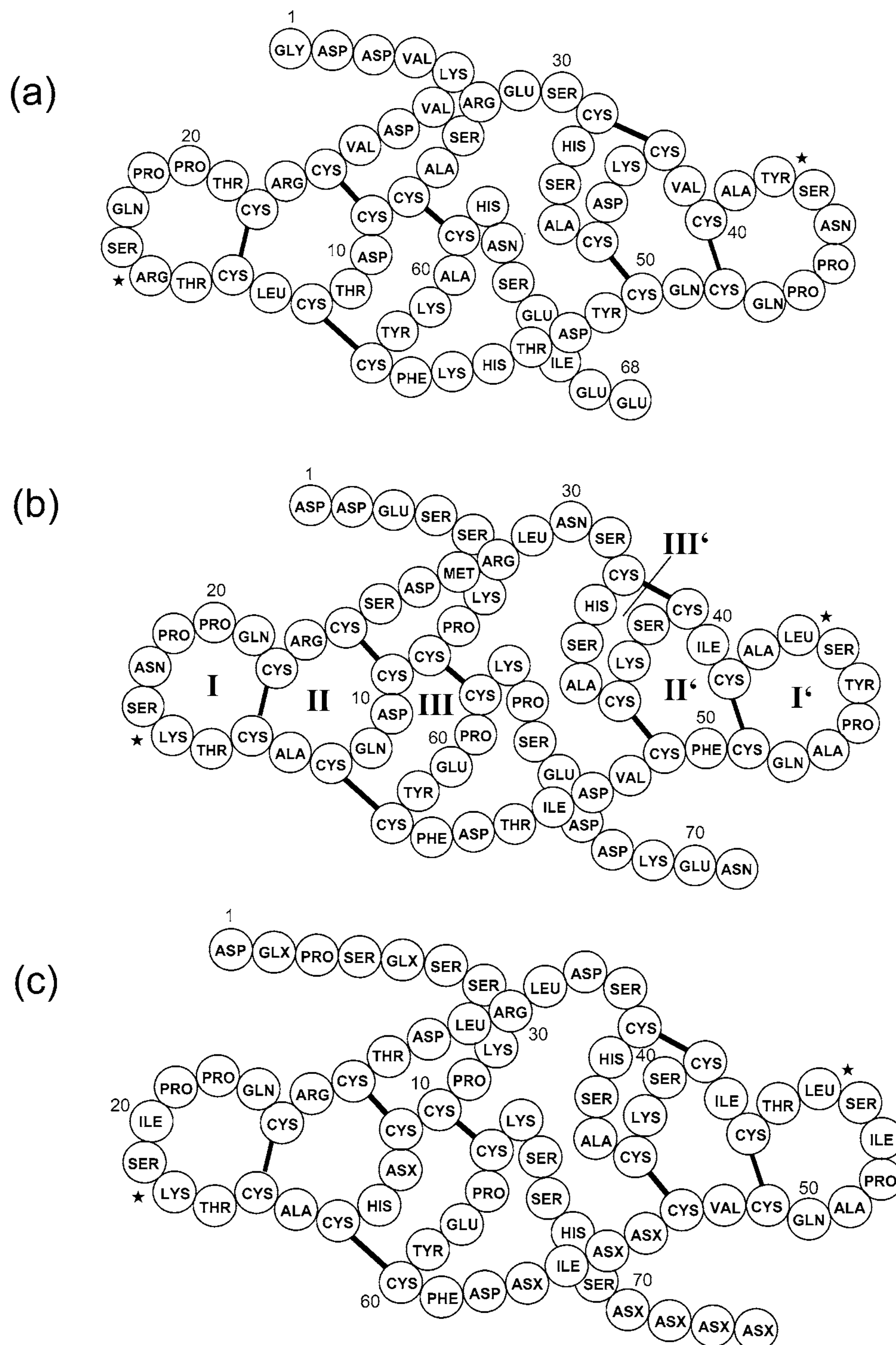

Figure 4. Covalent structures of Bowman–Birk inhibitors: (**a**) trypsin–chymotrypsin inhibitor from lentils LCI-1.7 (this paper, disulfide bridges by similarity); (**b**) Bowman–Birk soybean proteinase inhibitor BBI (*20*), I–III and I′–III′, corresponding loops in domains 1 and 2, respectively; (**c**) lima bean proteinase inhibitor LBI I (*25*); ★, reactive site.

also to be considered. This binding would explain the reduced activity of the LCI-1.7/BT complex against HCT, but not the influence of arginine modification with the 2,3-butanedione trimer on that activity (*2*). Thus, Gln18 as the additional binding site of LCI-1.7 for HCT is not very probable.

Inhibitor BBI (**Figure 4b**) maximally binds ∼2 mol of HT, BT, HCT, or BCT per mole inhibitor (*28*). The two trypsins are bound at Lys16 of the trypsin-reactive site and a further lysine and the two chymotrypsins at Leu43 of the chymotrypsin-reactive site and a further leucine or phenylalanine. In contrast to LCI-1.7, and also to the three fenugreek (*Trigonella foenum-graecum* L.) seed inhibitors (*29*), no atypical binding of enzymes at "wrong" reactive sites has been observed. As to lysine as the additional binding site for HT and BT, four further lysine residues occur in BBI. Two of them, Lys37 and Lys63, are not very probable because they are adjacent to disulfide bridges. The remaining two lysines, Lys6 and Lys69, are potential candidates for that binding, because they are located in the N- and C-terminal regions of BBI and not too close to the chymotrypsin-reactive site to be influenced by the BCT bound there (*28*). As to the additional binding site for HCT and BCT, the second leucine, Leu29, is thought to be that binding site,

because the two phenylalanines occurring in BBI, Phe50 and Phe57, are both also adjacent to disulfide bonds.

The lima bean proteinase inhibitor I, LBI I (**Figure 4c**), maximally inhibits ~2 mol of HT and BT, 1.5 mol of HCT, but only 1 mol of BCT per mole of inhibitor (*30*). Like BBI, LBI I binds HT and BT at its trypsin-reactive site, Lys18−Ser19, and at an additional binding site for trypsin containing lysine. The two chymotrypsins are bound at its chymotrypsin-reactive site, Leu45−Ser46, but only HCT is bound at an additional binding site containing either leucine or phenylalanine. Again, no binding at "wrong" reactive sites has been observed. As for BBI, two of the remaining three lysine residues, Lys39 and Lys65, are not very probable as binding sites for HT and BT, because they are adjacent to disulfide bridges. Thus, Lys8 remains for that binding, which is located in the N-terminal region of LBI I. For the additional binding of HCT, the two remaining leucines, Leu29 and Leu31, are the only potential candidates, because the one phenylalanine occurring in LBI I, Phe59, is adjacent to a disulfide bridge. Similar studies with LBI IV, another lima bean inhibitor that differs from LBI I mainly by an extended N-terminus and a somewhat longer C-terminus, have led to results that parallel those for LBI I (*30*).

Comparing the results discussed above, some common features become visible. Both BBI and LBI I contain in their N-terminal sequence Lys-Pro (Lys6−Pro7 and Lys8−Pro9, respectively) at the corresponding position, adjacent to the half-cystine that is involved in the formation of loop III (**Figure 4b**,**c**). Because Lys8 has been discussed as the only possible additional binding site for HT and BT in LBI I, Lys6 is proposed as that binding site in BBI by sequence similarity, excluding Lys69 for that binding. Stereographic drawings derived from X-ray data show that the N-terminal region is located in the middle of the soybean inhibitor PI-II and that the two reactive sites are located near the tips of the "bow tie"-shaped molecule (*23*). Backbone superposition of PI-II with BBI and PSTI IVB shows that this is true also for other Bowman−Birk inhibitors (*24*). Thus, Lys6 and Lys8 of BBI and LBI I, respectively, may be accessible to an additional binding of HT and BT. Inhibitor LCI-1.7 contains a different sequence, Ser6−Ala7, at that position (**Figure 4a**), which cannot bind trypsin. Why Lys5−Ser6 of LCI-1.7, located next but one to the half-cystine of loop III, cannot act as additional binding site for trypsin, cannot be answered for the moment.

Similarly, both BBI and LBI I contain the sequence Leu-Asx (Leu29−Asn30 and Leu31−Asp32, respectively) at the corresponding position in the connecting bridge between domain 1 (loop II) and domain 2 (loop III′) (**Figure 4b**,**c**). Because Leu29 has been discussed as the only possible additional binding site for HCT and BCT in BBI, Leu31 is proposed as the binding site for HCT in LBI I by similarity, excluding Leu29 for that binding. According to representations derived from X-ray data, these residues are located in a bend not too much distant from the loop containing the chymotrypsin-reactive site (*23*, *24*). Obviously, the flexibility of this region allows an additional binding of HCT and BCT by BBI, but only of HCT by LBI I. Inhibitor LCI-1.7 does not contain an amino acid residue in the bridge between domains 1 and 2 that is specific for chymotrypsin (**Figure 4a**). Furthermore, this bridge contains one residue less than those in BBI and LBI I. Why the trypsin-reactive site of LCI-1.7, Arg16−Ser17, can atypically bind HCT, and only HCT but not BCT, is still an open question. Such atypical binding of proteinases at "wrong" reactive sites has also been observed for other Bowman−Birk type inhibitors: the trypsin−chymotrypsin−elastase inhibitor C-II from soybeans (*31*), the five trypsin−chymotrypsin inhibitors from peanuts (*8*), and three trypsin−chymotrypsin inhibitors from fenugreek seeds (*29*). Initially we have thought that this is a general principle for legume Bowman−Birk inhibitors to explain the inhibition of >1 mol of enzyme per mole of inhibitor at higher enzyme concentrations; if not, two typical reactive sites for the same enzyme are present, as in the peanut inhibitors. However, studies with soybean and lima bean inhibitors show that they do not follow this principle. A prediction from the primary structure of whether an inhibitor exhibits additional binding and, if it does, based on which principle, is not possible for the moment and demands further studies.

To reduce the action of these inhibitors against human enzymes by genetic engineering, some possibilities might be derived from these results. The inhibition of HT and also BT by BBI and LBI I might be reduced by deletion or replacement of Lys6 and Lys8, respectively. Similarly, the inhibition of HCT, and also of BCT for BBI, by the two inhibitors might by reduced by deletion or replacement of Leu29 and Leu31, respectively. The inhibition of HCT by LCI-1.7 might be reduced by replacement of Arg16 by lysine, which seems to be not able to bind HCT at loop I, as deduced from homology with loop I of BBI and LBI I. These preliminary concepts may serve as a stimulation to adapt so-called antinutritional factors to human demands.

ABBREVIATIONS USED

ABI, adzuki bean inhibitor; BBI, Bowman−Birk soybean proteinase inhibitor; BCT, bovine chymotrypsin; BT, bovine trypsin; FBI, faba bean inhibitor; HCT, human chymotrypsin; HT, human trypsin; LBI, lima bean inhibitor; LCI, *Lens culinaris* inhibitor; LCI-1.7/PA, performic acid-oxidized LCI-1.7; LCI-1.7/PE, *S*-β-(4-pyridyl)ethylated LCI-1.7; PI, peanut inhibitor; PSI, *Pisum sativum* inhibitor; PSTI, *P. sativum* trypsin inhibitor; RP-HPLC, reversed-phase high-pressure liquid chromatography; TEAF, triethylammonium formate; TFA, trifluoroacetic acid; Th, thermolytic peptide from LCI-1.7/PE; TPCK, *N*-*p*-tosyl-L-phenylalanine chloromethyl ketone; Tr, tryptic peptide from LCI-1.7/PE; Tr′, tryptic peptide from LCI-1.7/PA; Tris, tris-(hydroxymethyl)aminomethane; VAI, *Vicia angustifolia* inhibitor.

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