JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Reaction of Lentil Trypsin−Chymotrypsin Inhibitors with Human and Bovine Proteinases

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Four trypsin−chymotrypsin inhibitors from Syrian local small lentils were selected to study the reasons for their different action against human and bovine proteinases. Chemical modification experiments, enzymatic modifications followed by carboxypeptidase degradation, and characterization of the inhibitor/enzyme complexes formed were performed. All four *Lens culinaris* inhibitors (LCI) contained 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. The inhibition of more than one molecule of human chymotrypsin per molecule of inhibitor was caused by the additional and atypical binding at the trypsin-reactive site of all four inhibitors. The ∼2.5-fold higher inhibition of human chymotrypsin compared to bovine chymotrypsin was the result of two effects, the additional binding of human chymotrypsin at the trypsin-reactive site and the low inhibition of bovine chymotrypsin. As a consequence, human enzyme preparations or suitable conversion factors should be used to evaluate the effect of such inhibitors in foods.

KEYWORDS: Bowman−Birk inhibitor; chymotrypsin inhibitor; inhibition of bovine proteinases; inhibition of human proteinases; inhibition of porcine trypsin; inhibitor constants; *Lens culinaris*; lentil; reactive sites; trypsin inhibitor

INTRODUCTION

Proteinase inhibitors are widely distributed in plants, and thus, occur in many plant foodstuffs, in particular in pulses (*1*−*3*). In legumes, mainly two types of proteinase inhibitors have been demonstrated: the larger Kunitz inhibitors (Kunitz or soybean trypsin inhibitor family), generally with two disulfide bonds and one reactive site for trypsin (EC 3.4.21.4) or chymotrypsin (EC 3.4.21.1), and the smaller, double-headed Bowman−Birk inhibitors (Bowman−Birk or soybean proteinase inhibitor family), generally with seven disulfide bridges and two reactive sites, one for trypsin and one for chymotrypsin. In a previous paper, the isolation of four trypsin−chymotrypsin inhibitors from Syrian local small lentils (*Lens culinaris* Medik., ssp. *microsperma*), the *Lens culinaris* inhibitors (LCI) LCI-1.7, −2.2, −3.3, and −4.6, and their characterization have been described (*4*). Their high cystine content, their molecular mass and their ability to inhibit trypsin and chymotrypsin indicate that they belong to the Bowman−Birk inhibitor family. The four inhibitors display characteristic differences in action against human and bovine proteinases: the amount of human trypsin (HT) inhibited is ∼75% of that of bovine trypsin (BT) and the amount of human chymotrypsin (HCT) inhibited is ∼250% of that of the bovine (BCT). From another lentil variety, Italian red lentils, two trypsin−chymotrypsin inhibitors have been isolated that display similar properties (*5*). The two inhibitors inhibit 1 mol of HT, more than 1 mol of BT and HCT, and less than 1 mol of BCT per mol of inhibitor (*6*). The amounts of enzyme exceeding 1 mol are atypically bound at the "wrong" reactive sites: BT at the chymotrypsin-reactive and HCT at the trypsin-reactive site. In contrast, the three proteinase inhibitors isolated from the seeds of another legume species, fenugreek (*Trigonella foenum-graecum* L.), show intra-species differences in this respect (*7*). The trypsin−chymotrypsin inhibitor TFI-B2 binds more than 1 mol of HT and BT and less than 1 mol of HCT and BCT per mol of inhibitor, made possible by an atypical binding of both HT and BT at the chymotrypsin-reactive site. The trypsin−chymotrypsin inhibitor TFI-N2 binds 1 mol of BT and BCT and more than 1 mol of HT and HCT per mol of inhibitor, through an atypical binding of the two human enzymes at the respective wrong reactive sites. The trypsin inhibitor TFI-A8 binds 2 mol of HT and BT per mol of inhibitor at its two trypsin-reactive sites and small amounts of HCT and BCT atypically at one of those sites. To further clarify the reasons for the differences in action against human and bovine proteinases, and to find out whether the reasons for those differences are restricted to a given species or variety, the four isoinhibitors from Syrian local small lentils have been selected to study those differences on a molecular level. Understanding the reasons for those differences, based on a broader range of data, would also help to evaluate the nutritional significance of proteinase

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10.1021/jf0305682 CCC: $25.00
Published on Web 12/02/2003

inhibitors for human beings. The results of these studies are described in the present paper.

MATERIALS AND METHODS

Samples. Four trypsin−chymotrypsin inhibitors from Syrian local small lentil seeds, LCI-1.7, −2.2, −3.3, and −4.6, were isolated and purified as previously described (*4*). This variety (ILL 4401 from ICARDA, Aleppo, Syria) was originally chosen from 38 varieties studied because of the predominance of inhibitor LCI-4, which should be isolated, and its high inhibitor activity (*8*).

Enzymes. Bovine trypsin (BT), trypsin from bovine pancreas, 40 units/mg, and porcine trypsin (PT), trypsin from porcine pancreas, 40 units/mg, both biochemical grade, were purchased from Merck (Darmstadt, Germany). Bovine chymotrypsin (BCT), α-chymotrypsin from bovine pancreas, ∼45 units/mg, research grade, was from Serva (Heidelberg, Germany). Carboxypeptidase Y from bakers yeast, ∼20% protein, ∼100 units/mg protein, and trypsin type XIII, *N*−*p*-tosyl-L-phenylalanine chloromethyl-ketone(TPCK)-treated, from bovine pancreas, were purchased from Sigma (St. Louis, MO). Human duodenal juice, taken from the duodenum by using a Dreiling tube after stimulation of pancreas secretion with dried cattle bile (*9*), was obtained from a local hospital. The samples were taken from 10 fasting healthy volunteers (age, 25 ± 0.8 years; weight, 69.1 ± 3.3 kg) with the consent of the individuals.

Other Materials. Fractogel TSK HW-50 (S), 25−40 μm, 2,3-butanedione (diacetyl), practical grade, and porcine thyroglobulin were purchased from Merck. L-Alanyl-L-alanyl-L-phenylalanine-4-methyl-coumarinyl-7-amide (Ala-Ala-Phe-MCA), carbobenzoxy-glycyl-glycyl-L-arginine-4-trifluoromethylcoumarinyl-7-amide (Z-Gly-Gly-Arg-TFM-CA), Coomassie Brilliant Blue G-250 (Serva Blue G), C. I. 42655, 1,2-cyclohexanedione (CHD), trypsin inhibitor from bovine lung (aprotinin), 140 units/mg, trypsin inhibitor from bovine pancreas, all of research grade, and porcine pepsin, ∼15 mAnson units/mg, were from Serva. Maleic anhydride (MA), 95%, myoglobin from sperm whale, and trypsin inhibitor type I-S from soybean (Kunitz) were purchased from Sigma. Acrylamide and *N*,*N*′-methylenebisacrylamide, both ultrograde, were from Pharmacia LKB (Uppsala, Sweden). Bovine ribonuclease, reagent grade, was obtained from Boehringer (Mannheim, Germany), limabean inhibitor from Worthington (Freehold, NJ), and tetranitromethane (TNM), reagent grade, from Aldrich (Milwaukee, WI). All other chemicals were of biochemical or analytical grade from Merck, Serva, or Sigma.

Inhibitor Determinations. Determination of inhibitor activities was performed with synthetic substrates, N^{α}-benzoyl-L-arginine *p*-nitroanilide and glutaryl-L-phenylalanine *p*-nitroanilide for the trypsins and chymotrypsins, respectively (*5*). Activities were expressed as mol of active enzyme (determined by active-site titration with 4-nitrophenyl 4′-guanidinobenzoate and 2-hydroxy-5-nitro-α-toluenesulfonic acid sultone for trypsin and chymotrypsin, respectively) inhibited by 1 mol of inhibitor. Human duodenal juice was used as HT and HCT preparation. Studies on purified inhibitors from fenugreek seeds had shown that this approach resulted in clear figures with regard to the inhibition of HT and HCT (*7*). Moreover, the experimental conditions more closely approximated the situation in vivo than those with purified human enzymes and are more meaningful nutritionally (*10*). The batches of commercial BT, PT, and BCT contained 55.5, 82.7, and 70.6% of active enzyme, respectively.

Chemical Modifications. Modification of arginine residues with 2,3-butanedione trimer (BDT) was performed following the procedure of Means and Feeney (*11*). The BDT reagent was prepared keeping 3 mL of 2,3-butanedione in 16 mL of 0.3 M sodium borate buffer, pH 8.2, adjusted to pH 8.9 with 5 M NaOH, at pH 8.9 for 48 h at 20 °C in an automatic titrator (Radiometer, Copenhagen, Denmark). Equal amounts of inhibitor solution (0.1−0.2 mg in 1 mL of borate buffer, pH 8.2) and BDT reagent were mixed and kept at ambient temperature for 48 h.

Modification of arginine residues with 1,2-cyclohexanedione (CHD) was done as described by Patthy and Smith (*12*). Inhibitor solutions (1.7 mL; 0.1−0.2 mg in 1 mL of 0.2 M sodium borate buffer, pH 8.9) and reagent (0.3 mL; 5 mg CHD in 30 mL of distilled water) were mixed and kept for 6 h at 37 °C in the dark.

Modification of lysine residues with maleic anhydride (MA) was performed by modifying the procedure of Butler et al. (*13*). Ice-cold inhibitor solutions (0.1−0.2 mg in 1 mL of 0.2 M sodium bicarbonate solution) were mixed with 20 mg of MA, kept on ice for 15 min and then at ambient temperature for 30 min with shaking from time to time.

Modification of tyrosine residues with tetranitromethane (TNM) was done as outlined by Lundblad and Noyes (*14*). Inhibitor solutions (0.1−0.2 mg in 1 mL of 75 mM tris(hydroxymethyl)aminomethane(TRIS)-HCl buffer, pH 8.0) were mixed with 50 μL of TNM and kept at ambient temperature for 2 h while vigorously shaking from time to time.

Inhibitor activities of the modified inhibitors were determined in triplicate in diluted aliquots of the incubation mixtures taking into consideration the influence of the reagent itself on the assay. Preliminary experiments on pure inhibitors with known reactive-site amino acids, the trypspin-chymotrypsin inhibitor LBI IV from limabeans (*2*) and PCI-2 from *Phaseolus coccineus* (*15*), had shown that these experimental conditions are suitable to indicate reactive-site amino acids.

Carboxypeptidase Y Degradation after Enzymatic Modification and Performic Acid Oxidation. Enzymatic modification with catalytic amounts of BT and BCT was performed as described by Krahn and Stevens (*16*), but using TPCK-treated BT instead of BT. Enzymatically modified and native (for comparison) inhibitors were oxidized with performic acid modifying the method of Hirs (*17*). The inhibitors (∼1 mg) were dissolved in 0.2 mL of formic acid and mixed with 5 μL of hydrogen peroxide (30%, w/v). After 30 min at ambient temperature, the reaction was stopped by the addition of 10 mL of ice-cold distilled water. The samples were frozen in liquid nitrogen and lyophilized three times. The performic acid-oxidized samples were degraded by carboxypeptidase Y (EC 3.4.16.1) in 0.1 M ammonium acetate buffer, pH 5.0, at an enzyme preparation/inhibitor protein ratio of 1.5:10 (w/w) (*18*, *19*). After 60, 180, and 360 min at 37 °C, the reaction was stopped by the addition of 60 μL of acetic acid. The samples were frozen in liquid nitrogen, lyophilized, and applied to amino acid analysis.

Formation of Inhibitor/Enzyme Complexes. To determine the inhibitor activities of lentil isoinhibitor/bovine proteinase complexes, inhibitor solutions (5×10^{-5} M) and enzyme solutions (5×10^{-5} M in active enzyme), both in 0.2 M TRIS−HCl buffer, pH 7.6, 66 mM in $CaCl_2$, were mixed at a ratio of 1:1 (v/v). The mixtures, prepared in triplicate, were kept at ambient temperature for 15 min. Then, their inhibitor activities were determined in diluted aliquots.

To detect the complexes by pore-gradient PAGE and gel-permeation HPLC, inhibitor solutions (5×10^{-5} M) and enzyme solutions (5×10^{-5} M), both in 0.2 M TRIS−HCl buffer, pH 7.2, 0.1 M in NaCl, were mixed at a ratio of 1:0.5, 1:1, and 1:2 (v/v). In addition, ternary mixtures containing the inhibitor, BT, and BCT at a ratio of 1:1:1 (v/v/v) were prepared accordingly. The mixtures were kept at ambient temperature for 30 min and then analyzed.

Pore-Gradient PAGE. Slab gels for anodic pore-gradient PAGE (258 × 125 × 0.5 mm) were prepared according to Görg et al. (*20*) to give a gradient of 5−15% total acrylamide concentration (cross-linking factor, C, 3%) in 0.375 M TRIS-HCl buffer, pH 8.8. Using an electrode solution of 25 mM TRIS/192 mM glycine, pH 8.3, electrophoreses were run at 5 °C for 10 min at 700 V and 10 mA maximum, followed by ∼150 min at 700 V and 35 mA maximum.

Gels for cathodic pore-gradient PAGE (258 × 125 × 0.5 mm) were prepared accordingly to give a gradient of 5−20% total acrylamide concentration (C 3%) in 0.18 M acetic acid−KOH buffer, pH 5.0. Using an electrode solution of 35 mM β-alanine, adjusted to pH 4.5 with acetic acid (*21*), electrophoreses were run at 5 °C for 30 min at 1000 V and 10 mA maximum, followed by 270 min at 1000 V, 30 mA and 25 W maximum.

Samples of 5−20 μL were applied per slot. Proteins were stained with Coomassie Brilliant Blue G-250 (*22*). Inhibition of BT and BCT was detected by the negative staining technique (*23*) and that of HT and HCT by the inhibitor overlay membrane technique (*24*), using Z-Gly-Gly-Arg-TFMCA and Ala-Ala-Phe-MCA membranes, respectively.

Table 1. Inhibitor Activities and Inhibitor Constants of Four Lentil Isoinhibitors[a]

inhibitor	HT	BT	HCT	BCT	PT
LCI-1.7	0.71 (3.6×10^{-8})	1.06 (7.9×10^{-9})	1.75 (1.3×10^{-8})[b]	0.67 (2.1×10^{-7})	0.54 (2.6×10^{-7})
LCI-2.2	0.73 (1.9×10^{-8})	1.00 (9.0×10^{-9})	1.72 (3.5×10^{-8})[b]	0.76 (1.7×10^{-7})	0.58 (1.7×10^{-7})
LCI-3.3	0.60 (3.8×10^{-8})	0.64 (4.1×10^{-8})	1.41 (6.8×10^{-9})[b]	0.58 (2.6×10^{-7})	0.37 (7.5×10^{-7})
LCI-4.6	0.54 (5.6×10^{-8})	0.93 (2.4×10^{-8})	1.35 (2.1×10^{-8})[b]	0.55 (2.5×10^{-7})	0.40 (6.1×10^{-8})

[a] Values of inhibitor activity are mol active enzyme maximally inhibited per mol inhibitor; values in parentheses are inhibitor constants in M (calculated for EI). [b] Calculated for E_2I complexes.

Gel-Permeation HPLC. Chromatography was performed at ambient temperature on a column (4 × 500 mm) packed with Fractogel TSK HW-50 (S), using 0.2 M TRIS−HCl buffer, pH 7.2, 0.1 M in NaCl, at a flow rate of 0.05 mL/min as the eluent. The column was calibrated with trypsin inhibitor from bovine pancreas and from bovine lung, limabean inhibitor, bovine ribonuclease, myoglobulin from sperm whale, Kunitz soybean inhibitor, bovine trypsin and α-chymotrypsin, papain, porcine pepsin, and porcine thyroglobulin (void volume), with 5−100 μg of protein dissolved in 100 μL of the eluent. The proteins were detected by absorbance at 220 nm.

RESULTS AND DISCUSSION

The inhibitor activities of the four lentil isoinhibitors, calculated from the initial slope of the inhibition curves (*5*) and expressed on a molar basis, are compiled in **Table 1**. The figures represent maximum inhibitor activities (i.e., the amount of enzyme inhibited when enzyme is present in excess). Due to the shape of the inhibition curves, the inhibitor activities at equimolar inhibitor/enzyme ratios were somewhat smaller (see **Table 3**). The four inhibitors generally inhibited ∼1 mol of BT per mol of inhibitor, whereas HT and in particular PT were inhibited to a smaller extent. On the other hand, the inhibitors clearly inhibited >1 mol of HCT per mol of inhibitor, whereas the inhibition of BCT was similar to that of HT.

The differences in action against PT, HT, and BT can be explained by differences in inhibitor constants (i.e., different affinities of the inhibitors toward the three trypsins). The inhibitor constants, calculated according to Green and Work (*25*), are included in **Table 1**. To explain the differences in action against HCT and BCT, other reasons must be considered, because one molecule of inhibitor clearly bound and thereby inhibited more than one molecule of HCT in all cases. Therefore, the inhibitor constants of the HCT/inhibitor complexes were calculated using the equation $K_i = ([E]^3/([E_{tot}] - [E]))^{0.5}$, where $[E]$ is the amount of free enzyme present at equilibrium and $[E_{tot}]$ the total amount of enzyme present in the incubation mixture (*7*), which takes into consideration the formation of a complex consisting of two molecules of enzyme and one molecule of inhibitor. The inhibitor constants of the lentil inhibitors, $6.8 \times 10^{-9} - 7.5 \times 10^{-7}$ M, were within the range reported for other Bowman−Birk inhibitors, for example, for soybean BBI ($K_i = 3.3 \times 10^{-7}$ M for BT) (*26*) and lima bean inhibitor LBI IV ($K_i = 8.4 \times 10^{-11}$ M for BT) (*27*). Two of the lentil inhibitors, LCI-1.7 and LCI-4.6, inhibited BT competitively and BCT noncompetitively, as determined with casein as the substrate using the Lineweaver−Burk plot (*28*) (results not shown).

To find out the amino acid residues that participate in binding the human and bovine proteinases, the respective amino acid residues were chemically modified (**Table 2**). Modification of arginine residues with BDT and CHD strongly suppressed the inhibition of HT and BT in all four lentil inhibitors. Whereas the inhibition of HT was almost completely abolished by BDT modification, some residual inhibitor activity was observed against BT. Modification with CHD was less effective, independent of the inhibitor and the trypsin inhibited. Inhibitor activities against HCT and BCT were not or not severely influenced by those reagents. The somewhat larger influence of BDT, the larger molecule, is thought to be caused by its larger influence on the native conformation of the inhibitors or by direct steric hindrance. In evaluating the effect of modification on inhibitor activity, it must be considered that the data listed in **Table 2** were obtained as differences between two enzymatic determinations, which naturally resulted in larger deviations. The above results clearly indicated that all four lentil inhibitors contained arginine at the trypsin-reactive site. The inhibition of the chymotrypsins was independently caused by a second, chymotrypsin-reactive site. Thus, the lentil isoinhibitors behaved as other Bowman−Birk inhibitors. In accordance with these results, modification of lysine residues with MA produced no considerable effect on inhibitor activities. Lysine and arginine are the two amino acids that are characteristic of the trypsin-reactive site of proteinase inhibitors (*1*−*3*).

Table 2. Inhibitor Activities of Lentil Isoinhibitors after Chemical Modification (% of Untreated, Determined in Triplicate)

	modification		residual activity against			
inhibitor	of	with[a]	HT	BT	HCT	BCT
LCI-1.7	Arg	BDT	1	8	76	68
		CHD	12	12	99	116
	Lys	MA	83	100	109	120
	Tyr[b]	TNM	11	14	33	7
LCI-2.2	Arg	BDT	0	1	130	97
		CHD	36	31	112	100
	Lys	MA	104	98	108	92
	Tyr	TNM	103	93	43	31
LCI-3.3	Arg	BDT	1	17	123	79
		CHD	20	34	115	81
	Lys	MA	94	108	113	86
	Tyr	TNM	64	94	25	24
LCI-4.6	Arg	BDT	7	21	59	85
		CHD	17	17	101	96
	Lys	MA	83	85	87	160
	Tyr[b]	TNM	7	13	13	5

[a] BDT, 2,3-butanedione trimer; CHD, 1,2-cyclohexanedione; MA, maleic anhydride; TNM, tetranitromethane. [b] In CHD-modified inhibitors.

Modification of tyrosine residues with TNM clearly suppressed the inhibition of HCT and BCT in all four lentil inhibitors. The inhibition of HT and BT was not influenced by that modification (for LCI-1.7 and LCI-4.6, the data must be compared with those observed after CHD modification, because CHD-modified inhibitors were reacted with TNM due to lack of material). Tyrosine, phenylalanine and leucine are amino acids for which chymotrypsins are specific, and that have been found at chymotrypsin-reactive sites of proteinase inhibitors (*1*−*3*). The assumption that tyrosine was the amino acid at the chymotrypsin-reactive site of the lentil inhibitors could not be confirmed in all cases (see below). In some inhibitors, a tyrosine residue seemed to be located close to the reactive site, thus influencing inhibitor activity after modification by direct steric hindrance or by causing conformational changes.

To check the results of the chemical modification experiments, three of the lentil isoinhibitors were enzymatically modified and then degraded with a carboxypeptidase after

performic acid oxidation. By enzymatic modification, i.e., by incubation with catalytic amounts of enzyme under conditions where the enzyme/inhibitor complex is unstable, the peptide bond after the P_1 amino acid (Schechter-Berger notation) (*29*) at the reactive site was cleaved by the respective enzyme (*16*). Degradation with carboxypeptidase then liberated the P_1 amino acid in addition to the C-terminal amino acid(s). Lentil inhibitor LCI-1.7 thus resulted in arginine after incubation with BT and degradation with carboxypeptidase Y, and in leucine after incubation with BCT (alanine was found as original C-terminal amino acid). Likewise, LCI-3.3 yielded arginine and phenylalanine (original C-terminus arginine), and LCI-4.6 arginine and leucine (original C-terminus alanine) after incubation with BT and BCT, respectively. Thus, all four lentil inhibitors contained arginine at the trypsin-reactive site.

Whereas the present study indicated leucine as P_1 amino acid at the chymotrypsin-reactive site, sequencing of LCI-1.7 unambiguously resulted in tyrosine at that position (Weder, J. K. P.; Hinkers, S.; unpublished), thus confirming the results of the chemical modification experiment. The discrepancy between the two experiments may be explained by a microheterogeneity at the chymotrypsin-reactive site of LCI-1.7, with the Leu-X bond more susceptible under the conditions of incubation with catalytic amounts of BCT at a low pH. Such microheterogeneities are known to occur in Bowman−Birk inhibitors (e.g., at the chymotrypsin-reactive site of the limabean protease inhibitors LBI IV/IV′) (*2*). Another explanation may be the location of a leucine residue in a part of the inhibitor molecule that is under tension in its native conformation, such that the Leu−X bond is cleaved by BCT even under acidic conditions. The chymotrypsin-reactive sites of LCI-3.3 and LCI-4.6 contained phenylalanine and leucine, respectively, and that of LCI-2.2 probably tyrosine. The P_1 amino acid combinations found in the present study are rare within the ~50 Bowman−Birk-type inhibitors sequenced so far (*1*−*3*). Both LCI-1.7 and LCI-2.2 are so-called Arg-Tyr inhibitors, as it is the case for VAI, the protease inhibitor from common vetch seeds (*Vicia angustifolia* L. var. *Segetalis* Koch) (*30*). Inhibitor LCI-3.3 is an Arg-Phe inhibitor, as is ALI DE-3, the proteinase inhibitor DE-3 from apple-leaf seeds (*Lonchocarpus capassa* Rolfe) (*31*). An Arg-Leu inhibitor, as LCI-4.6, has not been sequenced so far. However, an inhibitor of this type, TFI-N2, has been demonstrated to occur in fenugreek seeds (*Trigonella foenum-graecum* L.) (*32*).

To demonstrate the independent binding of trypsin and chymotrypsin at the two reactive sites and to obtain information on the binding site for the amounts of HCT exceeding one molecule per molecule of inhibitor, equimolecular inhibitor/enzyme mixtures were tested with regard to their ability to bind additional enzyme (**Table 3**). The 1:1 mol/mol mixture of LCI-1.7 and BT, LCI-1.7/BT, with its trypsin-reactive site occupied by BT, did not inhibit HT and BT but inhibited HCT and BCT, though to a different extent. The same results were obtained with the other respective mixtures, LCI-2.2/BT, LCI-3.3/BT, and LCI-4.6/BT. The corresponding mixtures with BCT (LCI-1.7/BCT, LCI-2.2/BCT, and so on), with their chymotrypsin-reactive sites occupied by BCT, did not inhibit additional BCT, but inhibited HCT as well as HT and BT. The ternary mixtures of inhibitor, BT, and BCT (LCI-1.7/BT/BCT, LCI-2.2/BT/BCT, and so on), with both reactive sites occupied, did not inhibit HT, BT, and BCT but inhibited HCT, though less than the binary mixtures. These results, with the exception of those for HCT, are in accordance with the usual behavior of Bowman−Birk-type inhibitors with two different and locally separated reactive sites, one for trypsin and one for chymotrypsin, that are independent of each other.

Table 3. Inhibitor Activities of Lentil Isoinhibitor/Bovine Proteinase Mixtures against Human and Bovine Proteinases[a]

		free LCI	LCI/BT		LCI/BCT		LCI/BT/BCT	
inhibitor	enzyme	(a)	(b)	(c)	(b)	(c)	(b)	(c)
LCI-1.7	HT	0.58	0	0.00	66	0.47	0	0.00
	BT	0.80	2	0.02	65	0.69	0	0.00
	HCT	0.79	42	0.73	25	0.44	17	0.29
	BCT	0.67	83	0.56	2	0.01	0	0.00
LCI-2.2	HT	0.59	0	0.00	78	0.57	0	0.00
	BT	0.80	0	0.00	65	0.65	0	0.00
	HCT	0.73	41	0.70	22	0.37	19	0.33
	BCT	0.50	47	0.36	0	0.00	0	0.00
LCI-3.3	HT	0.56	0	0.00	90	0.54	0	0.00
	BT	0.59	0	0.00	78	0.50	0	0.00
	HCT	0.81	57	0.81	29	0.41	16	0.22
	BCT	0.43	71	0.41	0	0.00	0	0.00
LCI-4.6	HT	0.51	0	0.00	76	0.41	0	0.00
	BT	0.67	0	0.00	49	0.46	0	0.00
	HCT	0.74	24	0.32	19	0.26	16	0.22
	BCT	0.42	53	0.29	0	0.00	0	0.00

[a] LCI/BT, LCI/BCT, and LCI/BT/BCT are mol/mol inhibitor/enzyme mixtures; (a) values are mol active enzyme per mol inhibitor bound at a molar ratio of 1:1; (b) values are percent activity of the mixture related to the maximum activity of the free inhibitor determined in triplicate; (c) values are mol active enzyme per mol inhibitor additionally bound by the mixture.

The results obtained with HCT and the ternary mixtures can be explained by binding of the amounts of HCT additionally inhibited (0.22−0.33 mol/mol inhibitor, **Table 3**, LCI/BT/BCT, column c) at the chymotrypsin-reactive sites due to the higher chymotrypsin concentrations. The chymotrypsin-reactive sites were not completely occupied by BCT in equimolecular mixtures (only 0.42−0.67 mol of BCT were bound per mol inhibitor at a molar ratio of 1:1, **Table 3**, free LCI, column a), thus HCT could be bound and might also displace some BCT due to the smaller inhibitor constants (**Table 1**). In case of the binary mixtures, HCT was inhibited less than BCT in mixtures with BT, for example 42 versus 83% for LCI-1.7/BT (**Table 3**, LCI/BT, column b), whereas HCT was inhibited more than BCT in mixtures with BCT, for example 25 versus 2% for LCI-1.7/BCT (**Table 3**, LCI/BCT, column b). These results indicated that some HCT was bound at the trypsin-reactive sites of all inhibitors, which were occupied in the BT/inhibitor complexes, but free in BCT/inhibitor complexes. However, the amount of total chymotrypsin (HCT + BCT) bound exceeded 1.00 mol/mol only for LCI-1.7; 0.67 mol of BCT was bound per mol of LCI-1.7 at a molar ratio of BCT/LCI-1.7 = 1:1, and 0.44 mol of HCT was additionally bound.

No indications could be found for the existence of a third binding site, at which the amounts of HCT exceeding 1 mol/mol inhibitor (**Table 1**) might be bound. In case of a third binding site, the amount of HCT bound should be independent of the conditions at the trypsin-reactive site (free or occupied). Moreover, the total amount of enzymes bound never exceeded 2 mol/mol of inhibitor; the maximum amount bound summed up to 1.76 mol/mol for LCI-1.7/BT/BCT (0.80 mol of BT and 0.67 mol of BCT bound in the mixture and 0.29 mol of HCT additionally bound). As a consequence, the amounts of inhibited HCT exceeding 1 mol/mol of inhibitor can only be explained by an atypical binding at the wrong site, the trypsin-reactive site.

To check the possibility of an atypical binding of BCT at the trypsin-reactive site, which might be possible due to the similarity of BCT and HCT, or of such a binding of BT at the

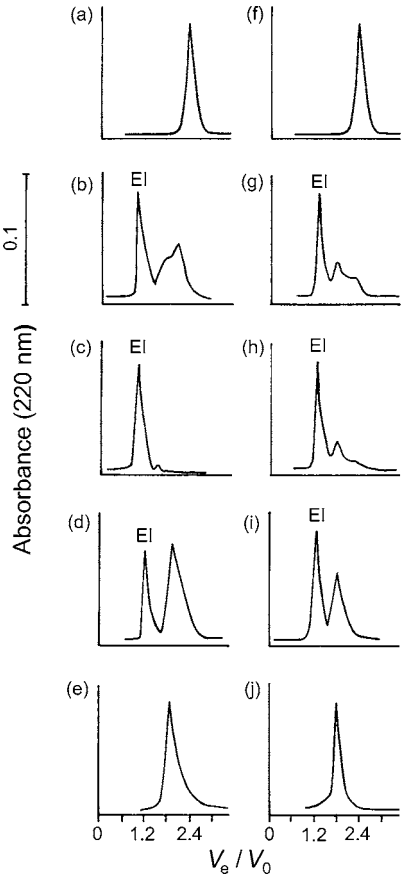

Figure 1. Gel-permeation HPLC patterns of lentil isoinhibitor LCI-2.2/bovine proteinase mixtures: column (4 × 500 mm) of Fractogel TSK HW-50 (S), eluted with 0.2 M TRIS–HCl buffer, pH 7.2, 0.1 M in NaCl (0.05 mL/min, ambient temperature); (a), (f) LCI-2.2; (b) LCI-2.2/BT (1:0.5, mol/mol); (c) LCI-2.2/BT (1:1, mol/mol); (d) LCI-2.2/BT (1:2, mol/mol); (e) BT; (g) LCI-2.2/BCT (1:0.5, mol/mol); (h) LCI-2.2/BCT (1:1, mol/mol); (i) LCI-2.2/BCT (1:2, mol/mol); (j) BCT; EI, enzyme/inhibitor complex; V_e, elution volume; V_0, void volume.

chymotrypsin-reactive site, which has been demonstrated for two inhibitors from Italian red lentils, LCI-1 and LCI-4 (*6*), mixtures of the inhibitors with BT, BCT, or BT and BCT, at different molar ratios, had been applied to pore-gradient PAGE and gel-permeation HPLC to detect the complexes formed. Only binary complexes, for example LCI-1.7/BT or LCI-1.7/BCT, were found in mixtures of inhibitor and BT or BCT, respectively. No indications of the existence of ternary complexes such as LCI/BT_2 or LCI/BCT_2 were observed, even when BT or BCT were present in excess (2 mol/mol of inhibitor).

The results obtained by gel-permeation HPLC with LCI-2.2 and BT or BCT are shown in **Figure 1** as examples. In increase in the portion of enzyme from 0.5 mol (b and g) to 1 mol (c and h) per mol inhibitor decreased the portion of free inhibitor and increased the portion of inhibitor/enzyme complex (EI in **Figure 1**). In evaluating the HPLC patterns, it must be considered that always the same amount of total protein (inhibitor and enzyme) had been applied, thus reducing the portion of inhibitor with increasing amounts of enzyme. Furthermore, a shift of free LCI-2.2 toward a lower elution volume was observed in samples in which BT was present. This shift was probably caused by temporary interactions between LCI-2.2 and BT. A further increase in enzyme (2 mol/mol of inhibitor, d and i) increased only the portion of free enzyme, whereas not even traces of an E_2I complex were formed. Ternary complexes, such as LCI-1.7/BT/BCT, were only detected when both bovine enzymes were present in the mixture. Thus, HCT was the only enzyme that could be bound at a reactive site usually not specific for that enzyme, and the four isoinhibitors from Syrian local small lentils studied here behaved differently in their reaction with human and bovine proteinases compared to the inhibitors from Italian red lentils mentioned above.

In conclusion, the results of the present study clearly demonstrate that the four isoinhibitors from Syrian local small lentils are members of the Bowman−Birk inhibitor family. They contain two independent reactive sites, one for trypsin and one for chymotrypsin. This is in accordance with the results of their biochemical characterization (inhibitor activities; amino acid composition, in particular high cystine content; and molecular mass) (*4*). All four inhibitors contain arginine at their trypsin-reactive site. Inhibitors LCI-1.7 and LCI-2.2 contain tyrosine, inhibitor LCI-3.3 contains phenylalanine and LCI-4.6 leucine 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 (0.54−0.73 and 0.55−0.76 mol/mol, respectively, **Table 1**), and ∼1 mol of BT (0.64−1.06 mol/mol) are inhibited per mol of inhibitor. On the contrary, clearly >1 mol of HCT is inhibited per mol of inhibitor (1.35−1.75 mol/mol). The type of inhibition (competitive for BT, noncompetitive for BCT) as well as the data on inhibitor constants ($6.8 \times 10^{-9} - 7.5 \times 10^{-7}$ M) are those known for other Bowman−Birk inhibitors. Inhibition of PT, studied in parallel to some extent, shows greater similarities to HT than to BT and even greater similarity to BCT, in particular with respect to inhibitor constants. The inhibition of >1 mol of HCT/mol inhibitor is caused by an atypical binding at the trypsin-reactive site of all four inhibitors. The ∼2.5-fold higher inhibition of HCT compared to BCT is caused by a combination of two effects: the additional binding of HCT at the trypsin-reactive site and the low inhibition of BCT.

In summary, the two lentil varieties, the Syrian local small lentil studied here and the Italian red lentil studied earlier, are able to bind enzymes also at the wrong reactive site, though differences occur in detail between the varieties. Within one variety, no differences have been found so far in their reaction with these enzymes.

As a nutritional and also analytical consequence, determinations of inhibitor activities in raw or processed lentils with BT, the usual test enzyme, result in too low data with respect to the effect of the inhibitors in the human digestive tract. Whereas HT is inhibited only somewhat less than BT (mean lentil inhibitor activity against HT is 73% of that against BT), HCT is inhibited nearly twice as much as BT (mean lentil inhibitor activity against HCT is 176% of that against BT). Similar relations have also been found for other legume species, such as common beans (*Phaseolus vulgaris* L.), winged beans (*Psophocarpus tetragonolobus* DC.), chickpeas (*Cicer arietinum* L.), and peas (*Pisum sativum* L.) (*33*). During processing, alterations in action against the human enzymes are paralleled by those against the bovine ones (*34*). The inhibitors can be inactivated by adequate processing, whereas active inhibitors would pass the stomach unaltered because they are stable against pepsin and the low pH. Thus, to evaluate the effect of legume proteinase inhibitors on human beings, human enzyme preparations should be used for their determinations or a suitable conversion factor should be considered, if bovine enzymes are used, for example multiplying with 2 to consider the difference in action on BT and HCT for lentils (see above).

ABBREVIATIONS USED

Ala-Ala-Phe-MCA, L-alanyl-L-alanyl-L-phenylalanine-4-methylcoumarinyl-7-amide; BCT, bovine chymotrypsin; BDT, 2,3-butanedione trimer; BT, bovine trypsin; CHD, 1,2-cyclohexanedione; HCT, human chymotrypsin; HPLC, high-pressure liquid chromatography; HT, human trypsin; LCI, *Lens culinaris* inhibitor; MA, maleic anhydride; PAGE, polyacrylamide gel electrophoresis; PT, porcine trypsin; TNM, tetranitromethane; TPCK, *N*−*p*-tosyl-L-phenylalanine chloromethyl-ketone; TRIS, tris(hydroxymethyl)aminomethane; Z-Gly-Gly-Arg-TFMCA, carbobenzoxy-glycyl-glycyl-L-arginine-4-trifluoromethylcoumarinyl-7-amide.

ACKNOWLEDGMENT

We thank Mohan C. Saxena (ICARDA, Aleppo, Syria) for providing the lentil sample, the late Peter Lehnert (Gastroenterologisches Labor der Medizinischen Klinik Innenstadt der Universität München) for supplying the human duodenal juice, and A. Mödl and C. Dölling for performing the amino acid analyses.

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Received for review August 6, 2003. Revised manuscript received September 23, 2003. Accepted October 9, 2003. Parts of this study have been presented at the 1st European Conference on Grain Legumes, Angers, France, 1−3 June 1992, and at the 2nd International Workshop 'Antinutritional Factors (ANFs) in Legume Seeds', Wageningen, The Netherlands, 1−3 December 1993. Financial support of the work by the Deutsche Forschungsgemeinschaft is gratefully acknowledged.

JF0305682