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Isolation and Characterisation of Four Trypsin-Chymotrypsin Inhibitors from Lentil Seeds†

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Abstract: Twenty-three proteinase inhibitors were isolated from Syrian local small lentils (*Lens culinaris*) by ammonium sulphate fractionation of the acidic extract followed by affinity chromatography on anhydrotrypsin-Sepharose. They all inhibited human and bovine trypsin and chymotrypsin. Three inhibitors (LCI-1·7, -3·3 and -4·6) were separated and purified to homogeneity by anion exchange chromatography and preparative isoelectric focusing (IEF) with immobilised pH gradients; a fourth (LCI-2·2) required additional reversed-phase high-pressure liquid chromatography. The four inhibitors were similar in their amino acid composition, with high cystine and aspartic acid/asparagine content, and lack of free sulphydryl groups, methionine and tryptophan. The calculated minimum number of amino acid residues per molecule, the calculated molecular masses confirmed by gel liquid chromatography, gel-permeation high-pressure liquid chromatography and sodium-dodecylsulphate polyacrylamide gel electrophoresis, and the isoelectric points determined by IEF (immobilised pH gradients and carrier ampholytes) were 84, 77, 68 and 60 residues per molecule, 9200, 8500, 7200 and 6750, and 5·26, 5·88, 6·80 and 7·80 for LCI-1·7, -2·2, -3·3 and -4·6, respectively. All four inhibitors inhibited human trypsin less than bovine trypsin, and human chymotrypsin more than the bovine enzyme. All these properties are in accordance with the classification of the four lentil inhibitors as members of the Bowman-Birk proteinase inhibitor family. © 1998 Society of Chemical Industry.

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Key words: trypsin inhibitor; chymotrypsin inhibitor; inhibition of human enzymes; lentil; *Lens culinaris*; isolation; characterisation

INTRODUCTION

Proteinase inhibitors are widely distributed in plant foodstuffs and have been found in many legumes (Belitz and Weder 1990). The demonstration of the inhibition of trypsin (EC 3.4.21.4, species not given) by lentil seed extracts was first reported by Jaffé (1950) and that of chymotrypsin (EC 3.4.21.1) by Mansfeld *et al* (1959). In a comparative study of the inhibitory activity of the seeds of 18 legumes, lentils have been found to inhibit human chymotrypsin (HCT) much more than bovine chymotrypsin (BCT), human trypsin (HT) and bovine trypsin (BT) (Belitz *et al* 1982). Furthermore, the occurrence of four isoinhibitors in all the 38 lentil samples tested (Weder *et al* 1985), the isolation and characterisation of the principal inhibitor from a local market lentil sample (Weder *et al* 1983) and that of two trypsin-chymotrypsin inhibitors from Italian red lentils (Mueller and Weder 1989) have been reported. The latter two inhibitors inhibit 1 mol of HT, more than

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1 mol of BT and HCT and less than 1 mol of BCT per mol of inhibitor (Weder and Mueller 1989). The amounts of bound enzyme exceeding 1 mol per mol of inhibitor react with the 'wrong' sites: BT with the chymotrypsin-reactive and HCT with the trypsin-reactive one. The demonstration of a larger number of isoinhibitors in lentil inhibitor preparations using more advanced separation techniques, similar to the number of isoinhibitors occurring in fenugreek seeds (Weder and Haußner 1991a), and the differences in mode of action against the four enzymes (HT, BT, HCT and BCT) between three isoinhibitors from fenugreek (Weder and Haußner 1991b) caused the authors to re-investigate the reaction between the human and bovine proteinases and the lentil inhibitors with the example of one representative of each of the four inhibitor groups. The demonstration of a large number of lentil inhibitors, the isolation of four inhibitors representing four of the five inhibitor groups and their characterisation are described in this paper.

EXPERIMENTAL

Materials

Syrian local small lentils (*Lens culinaris* Medik, ssp *microsperma*, ILL 4401) were obtained from ICARDA (Aleppo, Syria). Human duodenal juice was from a local hospital. All other materials were the same as described earlier (Weder and Haußner 1991a,c).

Inhibitor determinations

Inhibitor activities were determined with synthetic substrates, N^{α}-benzoyl-L-arginine 4-nitroanilide for the trypsins and glutaryl-L-phenylalanine 4-nitroanilide for the chymotrypsins, and commercial bovine enzymes or human duodenal juice (Mueller and Weder 1989). Activities were expressed as mg of active enzyme (determined by active-site titration) totally inhibited by 1 g inhibitor preparation. The batches used contained 55·5 and 70·6% active BT and BCT, respectively.

Isolation procedures

Ground seeds were extracted with 0·125 M sulphuric acid and the proteins fractionated with ammonium sulphate (20–80% saturation, Mueller and Weder 1989). The resulting LCI-C (*L culinaris* inhibitor-crude) was applied to affinity chromatography on anhydrotrypsin-Sepharose (Weder and Haußner 1991a) yielding LCI-A (*L culinaris* inhibitor-affinity chromatographed), which was separated by anion exchange chromatography on Whatman DE-52 (Weder and Haußner 1991c). Selected fractions were applied to preparative isoelectric focusing (IEF) with immobilised pH gradients (IPG) using the canal technique (Weder and Haußner 1991c). Crude LCI-2·2 was purified by reversed-phase high-pressure liquid chromatography (RP-HPLC) on ODS-Hypersil RP-18 (5 μm, Bischoff, Leonberg, Germany, column 4·6 × 240 mm) eluted with a gradient of acetonitrile in 0·01 M triethylammonium formate (TEAF) buffer pH 6·0 at 60°C and 1·5 ml min^{-1} (isocratic 13·75% acetonitrile in TEAF buffer for 2 min, linear gradient of 13·75 → 20·75% acetonitrile in 38 min).

Analytical electrophoreses and chromatographies

The IEF with carrier ampholytes (CA-IEF) and with IPG (IEF-IPG), protein staining and inhibitor detection were carried out as described previously (Weder and Haußner 1991a); inhibitors of human enzymes were detected with fluorogenic substrate membranes (Weder *et al* 1993). Amino acid analyses, gel liquid chromatography (GLC), gel-permeation HPLC (GP-HPLC) and sodium-dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a pore-size gradient (SDS-PPAGE) were performed as given elsewhere (Weder and Haußner 1991c).

RESULTS AND DISCUSSION

Syrian local small lentils were ground and extracted with 0·125 M sulphuric acid. The extract was brought to pH 7·6, and the inhibitor-containing protein fraction (LCI-C) was precipitated with ammonium sulphate from 20 to 80% saturation. The LCI-C preparation was separated into inhibitors (LCI-A) and other proteins by affinity chromatography on anhydrotrypsin-Sepharose to avoid enzymatic modification, ie splitting of a peptide bond in the reactive site of the inhibitors. Determination of inhibitor activity against HT, BT, HCT and BCT in the eluate of the anhydrotrypsin-Sepharose column demonstrated that all inhibitors were bound to the column and were eluted in one peak after changing the eluent from 0·05 M Tris/HCl buffer, pH 7·6, to 0·01 M HCl (pH 1·9). Thus, no specific chymotrypsin inhibitor was present in LCI-C which would not bind to the anhydrotrypsin column. From 1 kg of lentil seed meal, 20·3 g LCI-C and 771 mg LCI-A were obtained. Total inhibitor enrichment was about 600-fold; recovery was 50%. The HT was inhibited less than BT by the seed extract, LCI-C and -A, whereas HCT was inhibited much better than BCT (Table 1). The amount of HCT inhibited by 100 g raw lentils, a usual amount, if not cooked appropriately, is about 1300 mg or more than half to more than the whole amount of HCT produced daily in human beings (the amount of HT

TABLE 1
Inhibitor activities of lentil seeds and inhibitor preparations (mg enzyme inhibited g^{-1} sample)

Sample	*Inhibitor activity against:*			
	HT	*BT*	*HCT*	*BCT*
Seeds	2·4	2·9	12·7	1·4
LCI-C	66	76	326	35
LCI-A	1530	1900	7080	1030
LCI-DE1	1130	1400	6100	600
LCI-DE2	2300	3050	11 280	1130
LCI-DE3	1500	1830	7450	1080
LCI-DE4	1790	2180	9560	1090
LCI-DE5	1600	2260	9700	1100
LCI-DE6	330	570	2800	340
LCI-1·7	1780	2500	4690	1700
LCI-2·2	1820	2610	5100	2250
LCI-3·3	1880	2070	4910	2040
LCI-4·6	1830	2470	5360	2040

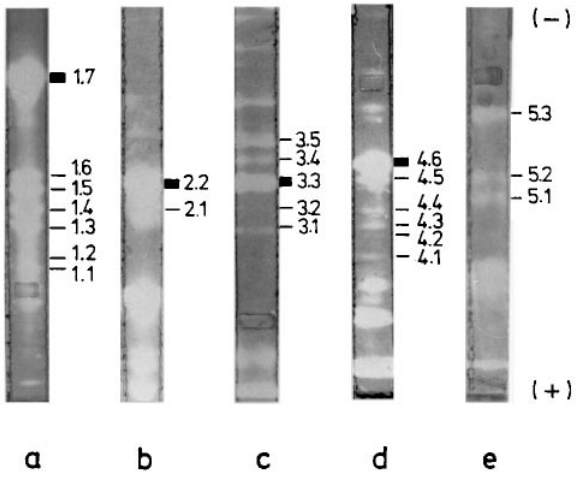

Fig 1. Isoelectric focusing of lentil inhibitors in immobilised pH gradients. (a) IEF-IPG pH 4·5–5·5, 70 µg LCI-A, 1·1 to 1·7: LCI-1·1 to 1·7 (pI 4·80, 4·93, 4·99, 5·07, 5·13, 5·28 and 5·32, respectively). (b) IEF-IPG pH 5·0–7·0, 70 µg LCI-A, 2·1 and 2·2: LCI-2·1 and -2·2 (pI 5·77 and 5·85, respectively). (c) IEF-IPG pH 6·0–8·0, 80 µg LCI-A, 3·1 to 3·5: LCI-3·1 to -3·5 (pI 6·48, 6·61, 6·77, 6·93 and 7·03, respectively). (d) IEP-IPG pH 7·0–9·0, 50 µg LCI-A, 4·1 to 4·6: LCI-4·1 to -4·6 (pI 7·19, 7·35, 7·46, 7·60, 7·71 and 7·83, respectively). (e) IEF-IPG pH 7·5–8·5, 100 µg LCI-A, 5·1 to 5·3: LCI-5·1 to -5·3 (pI 8·15, 8·24 and 8·45, respectively). Staining for BT inhibition.

inhibited is only one-eighth to one-fourth of that amount). The difference in action against human and bovine proteinases is in accordance with the behaviour of other lentil varieties (Weder *et al* 1985). Since this difference was maintained in the course of the isolation, in particular during affinity chromatography, the presence of a specific chymotrypsin inhibitor responsible for the so much higher inhibition of HCT can be excluded. IEF of LCI-A with smooth IPG demonstrated the presence of 23 inhibitors in lentil seeds, which all inhibited BT (Fig 1), HT, BCT and HCT (results not shown). The inhibitors are arranged in five groups to indicate the relationships between these inhibitors, the four inhibitors demonstrated by PAGE in the same cultivar (designated LCI-1, -2, -3 and -4; Weder *et al* 1985) and the two out of four isoinhibitors isolated earlier from Italian red lentils (LCI-1 and -4; Mueller and Weder 1989). The group of acidic isoinhibitors LCI-1.1 to -1·7 (pI 4·80–5·32) correspond to the acidic isoinhibitor LCI-1 from Italian red lentils (pI 5·35). Similarly, the weakly acidic isoinhibitors LCI-2·1 and -2·2 (pI 5·77 and 5·85), the neutral isoinhibitors LCI-3·1 to -3·5 (pI 6·48–7·03) and the weakly basic isoinhibitors LCI-4·1 to -4·6 (pI 7·19–7·83) correspond to the isoinhibitors LCI-2 (pI 6·00), -3 (pI 6·75) and -4 (pI 7·70), respectively, from Italian red lentils. A more basic isoinhibitor, corresponding to LCI-5·1 to -5·3 (pI 8·15–8·45) had not been demonstrated in Italian red lentils.

The separation of LCI-A by anion exchange chromatography resulted in six to eight peaks detected by protein absorbance and inhibitor activity (Fig 2). All peaks exhibited a higher inhibition of HCT than of BCT (Fig 2c) and also of BT than of HT (Fig 2b) by the eluate (half the amount of eluate was tested for human enzyme inhibition). The pooled fractions (DE1–6) were analysed by CA-IEF with protein and inhibitor staining. Despite the clear separation of the peaks detected after chromatography, CA-IEF demonstrated the presence of the same inhibitor in different fractions (Fig 2d–f), thus indicating the strong association tendency of the inhibitors (cf also Weder and Haußner 1991c). The four dominating inhibitors, LCI-1·7, -2·2, -3·3 and -4·6, were clearly visible in the LCI-A track (Fig 2e, lane A). Fractions DE2–5 were selected to isolate LCI-4·6, -3·3, -2·2 and -1·7 by preparative IEF-IPG at pH 7·5–8·5, 6·0–8·0, 5·0–7·0 and 5·0–6·0, respectively. The CA-IEF pH 2–11 (results not shown) and HPLC (Fig 3) showed that LCI-4·6, -3·3 and -1·7 were homogeneous, whereas LCI-2·2c required HPLC purification to yield LCI-2·2. Inhibitor activities of LCI-DE fractions and purified inhibitors are included in Table 1. The differences in specific inhibitor activity against HCT between LCI-DE fractions and purified inhibitors are thought to be related to the different composition of human duodenal juice containing various chymotrypsins (HCT IA, IB and II) of different stability and/or being differently inhibited (Weder 1986). Repeated determinations showed that the results of HCT inhibitor determinations varied considerably, as already observed earlier with other samples (Belitz *et al* 1982, Weder and Haußner 1991c). No specific HCT inhibitors nor inhibitors with unusually high HCT inhibitor activity could be detected in the LCI-DE fractions by electrophoresis. Inhibitor yields were 75% for LCI-DE fractions and 7–36% for preparative IEF-IPG. In total, 8·2 mg LCI-1·7, 1·0 mg LCI-2·2, 4·5 mg LCI-3·3 and 8·5 mg LCI

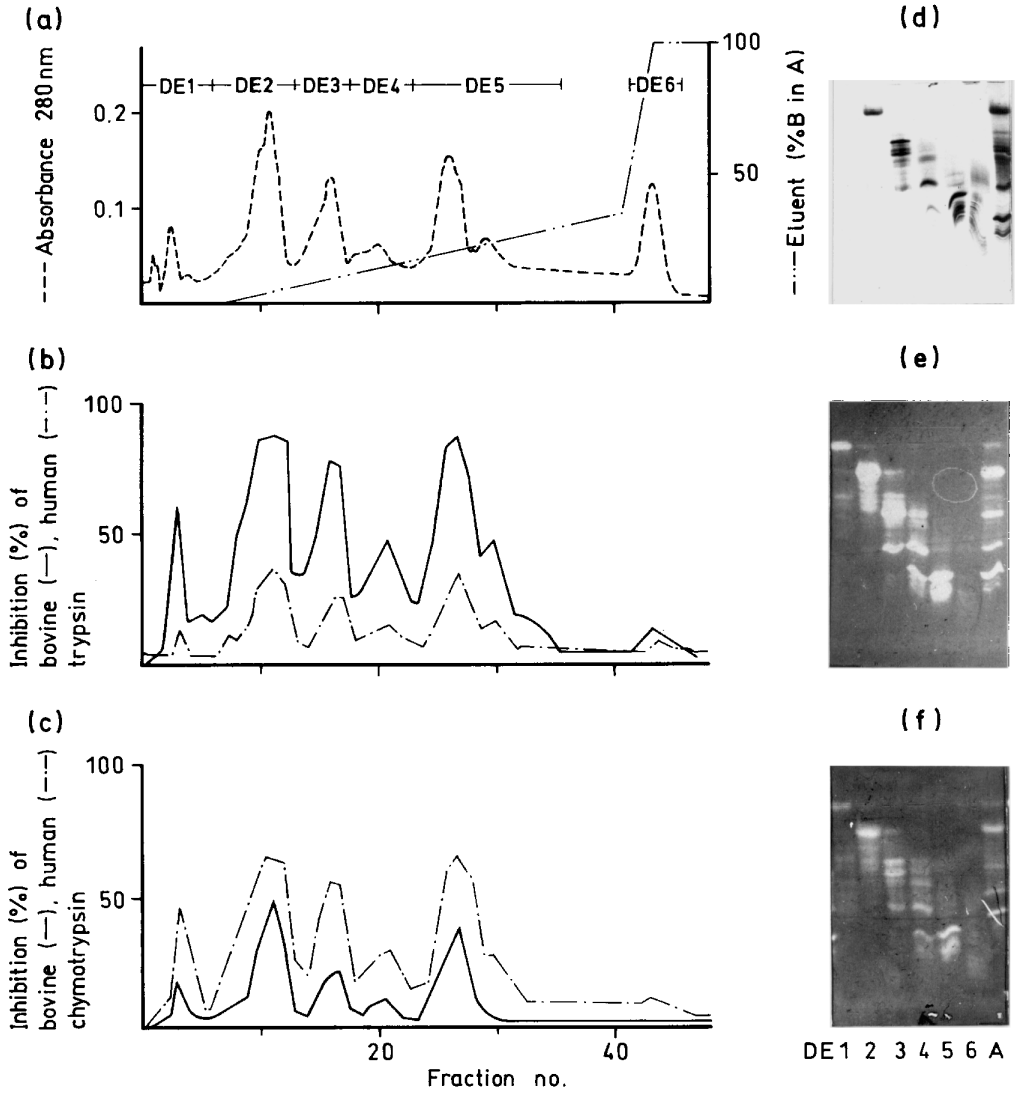

Fig 2. Anion exchange chromatography of LCI-A on Whatman DE-52. (a) Protein profile, 25 mg LCI-A applied; eluent A, 76 mM Tris/HCl buffer pH 9·0; eluent B, 0·3 M NaCl in eluent A; DE1–6, fractions pooled. (b) Trypsin inhibition profile, BT by 100 µl, HT by 50 µl eluate. (c) Chymotrypsin inhibition profile, BCT by 100 µl, HCT by 50 µl eluate. (d–f) CA-IEF pH 2–11 of pooled fractions (DE1–6) and LCI-A (A), staining for proteins, 20 µg DE1–6, 40 µg LCI-A (d), for BT inhibitors, 10 µg each (e), and for BCT inhibitors, 10 µg DE1–6, 20 µg LCI-A (f).

4·6 were obtained from 290, 299, 714 and 190 mg LCI-A, respectively.

The four lentil inhibitors were similar in their amino acid composition (Table 2). They were rich in half-cystine (no free sulphydryl groups, ie no cysteine could be detected with Ellman's reagent) and aspartic acid/asparagine, and lacked methionine, tryptophan and also for two of them (LCI-3·3 and -4·6), also isoleucine. The calculation of the minimum number of amino acid residues per molecule led to 60–84 residues and molecular masses (M_r) of 6750–9200. The M_r determined by other techniques (GLC, GP-HPLC and SDS-PPAGE) confirmed these minimum values (Table 3). In addition, isoelectric points were determined by IEF-IPG (Fig 1) and CA-IEF (results not shown; means are given in Table 3).

On the basis of the isolated representatives, the inhibitors of different isoinhibitor groups differ slightly in specific inhibitor activity (Table 1), molecular mass (Table 3) and amino acid composition (Table 2), of which the differences in half-cystine or disulphide content are the most interesting features in relation to the general structure of Bowman-Birk inhibitors (Belitz and Weder 1990). Furthermore, they differ in the amino acid present in the reactive site against chymotrypsin, with tyrosine occurring in LCI-1·7, phenylalanine in LCI-3·3 and leucine in LCI-4·6 (J K P Weder and R Kahleyß 1989, unpubl). On the other hand, they all contain arginine in the reactive site against trypsin, and they can bind human chymotrypsin additionally at the trypsin-reactive site (J K P Weder and R Kahleyß 1989, unpubl). The differences within each group are probably

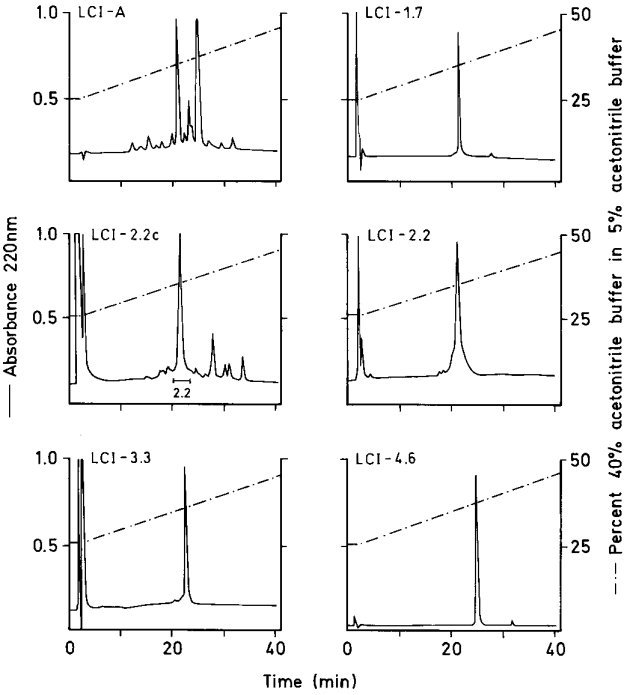

Fig 3. RP-HPLC of lentil inhibitors. ODS-Hypersil RP-18 (4·6 × 240 mm) eluted with acetonitrile in 10 mM triethylammonium formate buffer pH 6·0 at 60°C and 1·5 ml min^{-1}; 10–20 μg applied; LCI-2·2c, crude LCI-2·2.

very small, since they are not separated by PAGE (Weder *et al* 1985). Also, not all lentil inhibitors may exhibit this heterogeneity, since the four inhibitors from Italian red lentils are not separated into a larger number of isoinhibitors by IEF (Mueller and Weder 1989).

CONCLUSIONS

Because of their high cystine content (five to seven disulphide bonds), their M_r values and their ability to inhibit trypsin and chymotrypsin, the four lentil inhibitors belong to the Bowman-Birk (or soybean proteinase) inhibitor family. This was confirmed by the demonstration of two reactive sites in these inhibitors and by the homology in primary structure of one of the inhibitors (LCI-1·7) to other Bowman-Birk inhibitors (papers in preparation). Members of the Bowman-Birk inhibitor family are characterised by a M_r of 6000–10 000, high cystine content (generally seven disulphide bridges, but five and six have also been demonstrated; Weder and Haußner 1991c) and two reactive sites, usually one for trypsin and one for chymotrypsin (Belitz and Weder 1990). The amount of HT inhibited by all the four lentil inhibitors described herein was about

TABLE 2
Amino acid composition of four lentil isoinhibitors[a]

Amino acid	*LCI-1·7*			*LCI-2·2*			*LCI-3·3*			*LCI-4·6*		
	a	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*	*a*	*b*	*c*
Asx	12·0	9·98	10	13·2	10·13	10	11·8	8·05	8	13·8	8·39	8
Thr	5·7	4·71	5	6·3	4·87	5	5·8	3·94	4	6·3	3·83	4
Ser	9·4	7·76	8	9·2	7·06	7	8·3	5·66	6	7·6	4·61	5
Glx	11·0	9·11	9	10·3	7·92	8	7·2	4·94	5	6·9	4·22	4
Pro	5·7	4·70	5	6·3	4·86	5	5·8	3·94	4	7·2	4·37	4
Gly	3·8	3·14	3	2·4	1·86	2	10·4	7·12	7	3·1	1·88	2
Ala	6·2	5·14	5	6·7	5·15	5	6·0	4·11	4	6·6	4·03	4
Cystine/2	17.2	14·28	14	15·7	12·03	12	17·8	12·12	12	17·5	10·64	10
Val	6·0	5·00	5	6·6	5·04	5	5·7	3·87	4	6·7	4·06	4
Met	ND	0	0	ND	0	0	ND	0	0	ND	0	0
Ile	2·2	1·80	2	1·0	0·78	1	0·3	0·18	0	ND	0	0
Leu	2·0	1·69	2	1·6	1·25	1	1·7	1·17	1	1·5	0·91	1
Tyr	2·6	2·14	2	3·1	2·36	2	2·8	1·90	2	4·7	2·85	3
Phe	1·6	1·34	1	1·6	1·25	1	1·9	1·27	1	1·7	1·02	1
His	4·4	3·62	4	4·8	3·66	4	4·4	2·98	3	5·0	3·06	3
Lys	6·0	4·96	5	6·3	4·87	5	5·8	3·95	4	6·7	4·06	4
Arg	4·2	3·50	4	4·9	3·79	4	4·5	3·05	3	5·0	3·07	3
Trp	ND	0	0	ND	0	0	ND	0	0	ND	0	0
Total	100·0		84	100·0		77	100·0		68	100·3		60

[a] a, mol%, means of four determinations from two hydrolyses; b, calculated residues per molecule, based on minimum molecular mass; c, residues per molecule to the nearest integer (for cystine/2 nearest even number); ND, not detectable (<0·1 mol%).

TABLE 3
Characteristic data of four lentil isoinhibitors

Inhibitor	*LCI-1·7*	*LCI-2·2*	*LCI-3·3*	*LCI-4·6*
Inhibitor activity ratio				
HT/BT	0·71	0·70	0·91	0·74
HCT/BCT	2·76	2·27	2·41	2·63
Molecular mass				
Amino acid analysis	9200	8500	7200	6750
GLC	7700	7450	7050	7350
GP-HPLC	8600	7900	7400	7150
SDS-PPAGE	10700	11100	9400	8100
Isoelectric point	5·26	5·88	6·80	7·80

80% of that of BT, and that of HCT was about 250% of that of BCT (Table 3). Thus, the inhibitors proved to be suitable representatives for studying the differences in action displayed by the lentil seed extract on a molecular level. The results of this work will be presented in a following paper.

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