

Note

Characterization of a Novel Legumin α -Amylase Inhibitor from Chickpea (*Cicer arietinum* L.) Seeds

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A proteinaceous α -amylase inhibitor (CLAI) was purified from *Cicer arietinum* seeds. It had a molecular mass of 25.947 kDa and inhibited α -amylases from plants and mammals. Analysis of the amino acid sequence of a polypeptide from CLAI showed that it was different from other known α -amylase inhibitors, but had high identity to legumins from *Cicer arietinum* (100%) and *Vicia faba* var. *minor* (90%).

Key words: chickpea; α -amylase inhibitor; inhibitory activity; amino acid sequence

Leguminous plants have become a popular research source, due to the abundance of proteins and peptides involved in plant defense.^{1,2} Among these proteins are lectins, arcelins, chitinases, β -1,3-glucanases, defensins, digestive enzyme inhibitors, and some storage proteins.^{1,2} α -Amylase inhibitors, one class of digestive enzyme inhibitors, naturally occur in many food plants, and are particularly abundant in cereals and legumes.^{3,4} Many inhibitors of mammalian and insect α -amylases have been isolated from different plant sources and subsequently characterized. These inhibitors show remarkable structural variety, leading to different modes of inhibition and different specificity profiles against several α -amylases.^{1–3} α -Amylase inhibitors may be classified by their tertiary structure into six different classes: lectin-like, knottin-type, cereal-type, Kunitz-like, thaumatin-like, and γ -purothionin-like.¹ So far, the continuing discovery of new α -amylase inhibitors suggests that discoveries remain to be made.³

Chickpea (*Cicer arietinum* L.) is the third important pulse crop, grown mainly in arid and semi-arid regions. α -Amylase inhibitors have been detected in chickpea seeds.⁵ However, to date, there is little information concerning their identification and characterization. In this study, a legumin α -amylase inhibitor in chickpea seeds was characterized and its inhibitory activities towards several α -amylases were measured.

The proteinaceous α -amylase inhibitor was extracted from chickpea seed powder with tris-buffered saline (10 mM Tris-HCl, pH 8.0, containing 500 mM NaCl,

2 mM phenylmethanesulphonyl fluoride, 1% β -mercaptoethanol, and 10 mM ethylene diamine tetraacetic acid disodium salt). The extract was incubated at 4 °C for 3 h, and then was centrifuged at 12,000 $\times$ g for 10 min. The supernatant was pooled and then heated in a water bath at 70 °C for 5 min to destroy endogenous amylolytic activity.⁶ The resulting turbid solution was centrifuged (12,000 $\times$ g for 10 min), and the supernatant was used as a source of crude α -amylase inhibitor.

The protein concentration was determined by the method of Bradford,⁷ using bovine serum albumin as standard. α -Amylase inhibitory activities were measured according to Ishimoto and Chrispeels,⁶ with some modifications. Twenty-five microliters of human salivary α -amylase (0.03 AU, the unit of α -amylase activity defined as the amount of enzyme that liberates 1 μ mol/min of maltose) preparation⁵ and 25 μ l the α -amylase inhibitor extract were mixed and preincubated at 37 °C for 30 min prior to the addition of 400 μ l of substrate solution (1% soluble starch solution in 0.1 M phosphate buffer solution, pH 6.9, containing 20 mM NaCl and 0.1 mM CaCl₂). After 10 min, the reaction was stopped by the addition of 250 μ l dinitrosalicylic acid, followed by boiling for 10 min in a water bath. After the addition of 3 ml of distilled water, the solution was mixed and allowed to stand at room temperature for 15 min, and then A₅₄₆ was measured. Distilled water (25 μ l) instead of the α -amylase inhibitor solution was used as the control. The α -amylase inhibitory activity was calculated as follows:

Inhibitory activity (%)

$$= (A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}} \times 100$$

Purification of crude α -amylase inhibitor extracts from chickpea seeds was conducted by ammonium sulfate precipitation (ASP) according to Giri and Kachole.⁸ The extracts were subjected to ammonium sulphate fractionation (0–40%, fraction I; 40–60%, fraction II; 60–80%, fraction III; and 80–100%, fraction IV) at 0 °C. Four fractions were found, and each of the fractions was collected by centrifugation (12,000 $\times$ g, 15 min, 4 °C). The precipitate was dialyzed (Mr exclusion limit < 14 kDa) in ultrapure water at 4 °C for 24 h.

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Abbreviations: ASP, ammonium sulfate precipitation; AU, the unit of α -amylase activity; CLAI, a legumin α -amylase inhibitor from *Cicer arietinum*; IEC, ion exchange chromatography; MALDI-TOF-TOF-MS, matrix-assisted laser desorption/ionization time of flight mass spectrometry; RPLC, reversed phase liquid chromatography; TFA, trifluoroacetic acid; UPLC/MS-MS, ultra performance liquid chromatography-tandem-mass spectrometry

Fractions I and II were found to have no inhibitory activity, and fractions III and IV to have inhibitory activity of 73.5% and 17.4% respectively. Hence, the combination of fractions III and IV was mixed and concentrated with PEG6000 at 4 °C, and then lyophilized and stored at -20 °C for further purification.

The combination of fractions III and IV was further purified by ion exchange chromatography (IEC) on ÄKTA prime (GE Healthcare, Uppsala, Sweden). The fractions with inhibitory activity were dissolved in 20 mM Tris-HCl (pH 8.0) and ultrafiltered (0.22 μ m, Elite Analytical Instruments, Dalian, China). A 2-ml sample solution (15 mg/ml) was loaded onto the column (Resource Q, 6 ml bed volume, GE Healthcare, Uppsala, Sweden) pre-equilibrated with the same buffer (elution buffer A: 20 mM Tris-HCl, pH 8.0; elution buffer B: 20 mM Tris-HCl, pH 8.0, containing 1.0 M NaCl). The sample was injected at a flow rate of 0.7 ml/min, and elution was performed at a flow rate of 1.0 ml/min with an elution gradient of 0 to 1.0 M NaCl. Several principal peaks were eluted. Each eluted fraction was collected separately by 2 ml per tube according to elution volume. Only two tubes from one peak were detected to have inhibitory activity (65.4% and 55.4%), while the others showed no/lower inhibitory activity. Hence, the two fractions with inhibitory activity were pooled, lyophilized, and stored at -20 °C for further purification.

Further purification of the two fractions with inhibitory activity from IEC was performed by reversed phase liquid chromatography (RPLC) on an ÄKTA purifier 10 (GE Healthcare, Uppsala, Sweden). The fractions were dissolved in distilled water, and 100 μ l sample solution (50 μ g/ml) was loaded onto the column (Resource RPC, 3 ml bed volume, GE Healthcare, Uppsala, Sweden) pre-equilibrated with 5% acetonitrile containing 0.05% trifluoroacetic acid (TFA) (eluent A, distilled water with 0.05% TFA in it; eluent B, acetonitrile, containing 0.05% TFA). The sample was injected at a flow rate of 0.5 ml/min, and elution was performed at a flow rate of 1.0 ml/min. The elution gradient was 5% to 100% solvent B. The eluted fraction corresponding to each of the peaks was collected separately. Seven principal peaks were eluted, the inhibitory activities being 15.6%, 19.9%, 2.3%, 29.6%, 74.1%, 49.0%, and 67.6% (Fig. 1). When solutions of the last three peaks with higher inhibitory activity were freeze-dried, only the one from peak 5 had visible substance. Hence, the lyophilizate of peak 5 was used as purified α -amylase inhibitor (CLAI), and was stored at -20 °C for further study.

The molecular mass of the purified sample (CLAI) from RPLC was determined by matrix-assisted laser desorption/ionization time of flight mass spectrometry (AutoFlexII MALDI-TOF-TOF, Bruker Daltonics, Bremen, Germany) equipped with a nitrogen laser (337 nm). The sample was dissolved in distilled water (containing 0.01% TFA). α -Cyano-4-hydroxy cinnamic acid was used as the matrix compound. The spectrometer was operated in reflection mode with delayed extraction, and the acceleration voltage in the ion source was 20 kV. In this case, a single protein peak was detected, and the result indicated that CLAI has a molecular mass of 25.947 kDa.

The amino acid sequence of CLAI was determined by ultra performance liquid chromatography-tandem mass

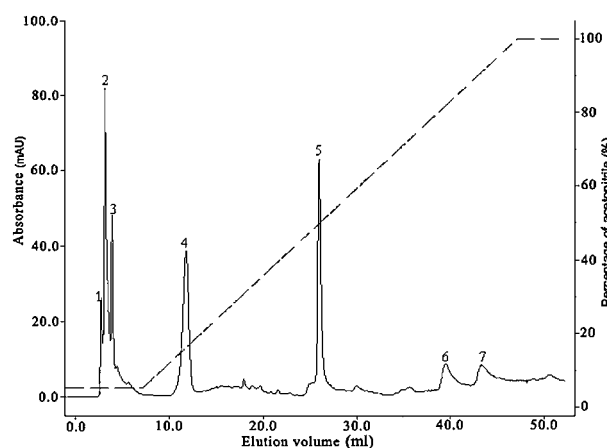


Fig. 1. Reversed Phase Liquid Chromatography of the Fractions with α -Amylase Inhibitory Activity from IEC.

The solid line expresses the absorbance at 280 nm. The dashed line displays the linear gradient percentage of acetonitrile from 5% to 100% in the mobile phase.

spectrometry (UPLC/MS-MS). It was performed by the National Centre for Biomedical Analysis, Beijing, China. After the sample was digested with trypsin,¹⁾ the polypeptide fragments were determined by UPLC/MS-MS (Waters, Milford, MA). PLGS v2.3 and Mascot search soft⁹⁾ were used to analyze the data. The resulting sequence alignment and calculation of percentage identities were conducted with the UNIPROT database using the NCBI-blastp program. The amino acid sequence comparisons of a peptide fragment of CLAI are shown in Table 1. Higher identities of the amino acid sequence of a polypeptide fragment of CLAI were obtained against Q9SMJ4, a legumin from *Cicer arietinum*, with 100% sequence identity, against Q99304/Q03971, a legumin from *Vicia faba* var. *minor*, with 90% identity, and as well as with P04405/Q549Z4, a glycinin from *Glycine max*, with 84% identity. However, the molecular mass of Q9SMJ4 was 56.260 kDa, while that of CLAI was 25.947 kDa, suggesting that CLAI is most likely a subunit of the legumin (Q9SMJ4). According to the UniProt Knowledgebase, the legumin (Q9SMJ4) from *Cicer arietinum* consists of alpha and beta subunits, but no property of enzyme activity has been reported for it. To our knowledge, a basic subunit of legumin in the red kidney bean has been reported to have proteinase inhibitory activity,¹⁹⁾ but no legumin has been reported to have α -amylase inhibitory activity. This suggests that CLAI is the first legumin reported to have α -amylase inhibitory activity. Because UniProtKB/Swiss Prot is a minimally redundant database, our data do not qualify to obtain an individual accession number for the protein sequence data of CLAI. Hence, the protein sequence data of CLAI will appear in the UniProt Knowledgebase under accession no. Q9SMJ4, which is already used in the Uniprot database for the submission of the legumin, alpha and beta subunits from *Cicer arietinum*.¹⁰⁾ However, the α -amylase inhibitory activity of CLAI will be labeled underneath the legumin (Q9SMJ4).

To determine identity between the amino acid sequences of the polypeptide fragment of CLAI and all the α -amylase inhibitors of six classes (lectin-like, knottin-type, cereal-type, Kunitz-like, thaumatin-like,

Table 1. Alignment of the Amino Acid Sequence of CLAI with Members of the Legumin Family

PIR code	Sequence	Residue number	Source	Identity (%)	Reference
CLAI	LHQNIGSSSSPDIYNPQAGR		<i>Cicer arietinum</i>		
Q9SMJ4	LHQNIGSSSSPDIYNPQAGR	20	<i>Cicer arietinum</i>	100	(10)
Q99304	LRLNIGSSSSPDIYNPQAGR	20	<i>Vicia faba</i> var. <i>minor</i>	90	(11)
Q03971	LRLNIGSSSSPDIYNPQAGR	20	<i>Vicia faba</i> var. <i>minor</i>	90	(11)
P04405	LRQNIGQNSSPDIYNPQAG	19	<i>Glycine max</i>	84	(12)
Q549Z4	LRQNIGQNSSPDIYNPQAG	19	<i>Glycine max</i>	84	(13)
Q41036	LRLNIGPSSSPDIYNPEAGR	20	<i>Pisum sativum</i>	80	(14)
P02857	LRLNIGPSSSPDIYNPEAGR	20	<i>Pisum sativum</i>	80	(15)
Q9T0P5	LRLNIGPSSSPDIYNPEAGR	20	<i>Pisum sativum</i>	80	(15)
P15838	LRLNIGPSSSPDIYNPEAGR	20	<i>Pisum sativum</i>	80	(16)
P04776	LRHNIGQTSSPDIYNPQAG	19	<i>Glycine max</i>	78	(17)
Q96475	LRHNIGQSTSPDAYNPQAGR	20	<i>Lupinus angustifolius</i>	75	(18)
P11828	LRHNIGQTSSPDIFNPQAG	19	<i>Glycine max</i>	73	(17)

Table 2. Inhibitory Activities of CLAI against Several α -Amylases*

Sources of α -amylases	α -Amylase activity ($\times 10^{-3}$ unit)	Inhibitory activity (%)
Human salivary	7.0 $\pm$ 0.6	73.6 $\pm$ 0.5
<i>Bacillus subtilis</i>	120.9 $\pm$ 16.1	not detectable
Porcine pancreas	89.1 $\pm$ 6.4	80.3 $\pm$ 1.3
Maize	5.0 $\pm$ 0	67.3 $\pm$ 7.0
<i>Aspergillus oryzae</i>	148.4 $\pm$ 35.4	15.2 $\pm$ 0.8

*Data are expressed as the mean $\pm$ standard deviation, and are the means of four replicates.

and γ -purothionins-like),^{1,3)} we also compared the new sequence with the sequences of all the α -amylase inhibitors of the six classes. Reduced degrees of sequence similarity in a range of 0–19% identity were observed, indicating that CLAI is different from other known α -amylase inhibitors in sequence. Due to the classification of α -amylase inhibitors based on their tertiary structure,¹⁾ further data on CLAI's full-length amino acid sequence and tertiary structure should be obtained to determine which class of the six classes it belongs to.

The inhibitory activity of CLAI against α -amylases of various origins was determined, and the results are shown in Table 2. CLAI did not inhibit the α -amylase from *Bacillus subtilis*, and had low inhibitory activity (15.2%) against α -amylase from *Aspergillus oryzae*. However, the inhibiting activities of CLAI against α -amylases from maize, porcine pancreas, and human salivary were 67.3%, 80.3%, and 73.6% respectively. This indicates that CLAI was capable of inhibiting α -amylases of plants and mammals, but incapable of inhibiting α -amylases from bacteria and fungi. A purified α -amylase inhibitor from kidney bean has been reported to show a similar inhibition pattern.²⁰⁾ α -Amylase inhibitor phaseolamin from kidney bean, which can hinder the digestion of complex carbohydrates, has been used as a dietary supplement in promoting and supporting weight loss.²¹⁾ Hence, this novel legumin α -amylase inhibitor (CLAI) from chickpea seeds is potentially beneficial in controlling obesity and other human health complications.

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