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THE COMPLETE AMINO ACID SEQUENCE OF A MAJOR WHEAT PROTEIN INHIBITOR OF α-AMYLASE

NIZAR KASHLAN and MICHAEL RICHARDSON

Department of Botany, University of Durham, Durham City, DH1 3LE, U.K.

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Key Word Index—*Triticum aestivum*; Gramineae; wheat; α-amylase; protein inhibitor; amino acid sequence.

Abstract—The complete amino acid sequence of one of the major wheat protein iso-inhibitors of α-amylase was determined. The sequence was deduced from analysis of fragments and peptides derived from the protein by cleavage with cyanogen bromide and by digestion with trypsin, chymotrypsin, thermolysin and the *Staphylococcus aureus* V8 protease. The molecule consists of a single polypeptide chain of 123 residues. Both serine and alanine were found in position 65, and further minor examples of microheterogeneity were observed in four other residues.

INTRODUCTION

The endosperm of wheat seeds is known to contain a number of closely related albumin proteins which are inhibitors of the α-amylase enzymes from a wide spectrum of animals [1, 2]. These proteins have been separated into three major families of iso-inhibitors by gel filtration on Sephadex G-100 [3, 4]. The family with the smallest MW (12 000) has become known as the 0.28 family from the electrophoretic mobility (relative to bromophenol blue) of its major component [5], while the iso-inhibitors of MW 24 000 are known as the 0.19 family [6]. The third family of iso-inhibitors which have a MW of 60 000 has not been so well characterized as those of the 0.19 and 0.28 families, and their electrophoretic pattern is still to be established [2]. The proteins of the MW 60 000 family and the MW 24 000 (0.19) family undergo a reversible dissociation in the presence of dissociating agents such as guanidine HCl or sodium dodecyl sulphate to yield protomer sub-units with a MW close to 12 000 [4].

Although they are different in their MWs and exhibit different molar stoichiometric ratios on binding to amylase [7], it has been suggested that the iso-inhibitors of the 0.28 and 0.19 families are closely related [2]. Some measure of the degree of homology between these two groups of proteins has been provided by preliminary amino acid sequence studies. Redman [8, 9] purified three iso-inhibitors of the 0.28 family and showed that they contained identical sequences in their 24 N-terminal amino acids. Subsequently Petrucci *et al.* [10] found that nine of the first 24 N-terminal residues in the 0.19 inhibitor were homologous with those in the 0.28 inhibitor.

The present paper presents the complete amino acid sequence of one of the iso-inhibitors of the 0.28 (MW 12 000) family.

RESULTS AND DISCUSSION

The complete amino acid sequence of the wheat α-amylase iso-inhibitor CIII is shown in Fig. 1 together with the details of the CNBr fragments and the overlapping peptides from which it was deduced. This is the first report of the complete amino acid sequence for any of the wheat albumin proteins which inhibit α-amylase. The only region of the sequence which lacks support from good overlapping segments (3 or more residues) of peptides and fragments is the sequence of residues 116–120, but the sequence presented in Fig. 1 is entirely compatible with the results of the amino acid analysis, and is in agreement with the tentative ordering of the CNBr fragments suggested by Redman [8] and the identification of valine as the C-terminal residue [8, 11].

The highly sensitive DABITC micro-sequence method was particularly useful in the identification of the TRP residues in peptides X1, H1, H9, V3 and V9. Also this method was used in conjunction with the specificity of digestion by the *S. aureus* protease to resolve the identification of the acid/amide residues in those peptides (X3, X4, X5, H6, H7, H9, V2, V3, V9) where the placement could not be unambiguously assigned by the dansyl-Edman method and consideration of the electrophoretic mobilities.

Treatment of iso-inhibitor CIII with cyanogen bromide led to the expected cleavages at the three methionine residues and generated two CNBr fragments (X3 and X4) with the same terminal residues and very similar amino acid compositions to the CNBr-1 and CNBr-3 fragments reported by Redman [8] for the 0.28 albumin M. In addition, however, the excess of cyanogen bromide used in this work produced anomalous cleavages of the $Trp_{(6)}$–$Cys_{(7)}$ and $Trp_{(117)}$–$Ala/Cys_{(118)}$ bonds leading to the formation of four additional fragments (X1, X2, X5 and X6) which clearly represent modified forms of the CNBr-4 and CNBr-2 fragments of Redman [8]. This type of anomalous cleavage of tryptophanyl peptide bonds by an excess of cyanogen bromide in the presence of 70% formic acid has also been reported previously in the cytochromes [12]. On the other hand, it is perhaps noteworthy that there was no evidence for any hydrolysis of the $Asp/Asn_{(8)}$–$Pro_{(9)}$ by the 70% formic acid in contrast to its effects on the same peptide bond in other proteins [13].

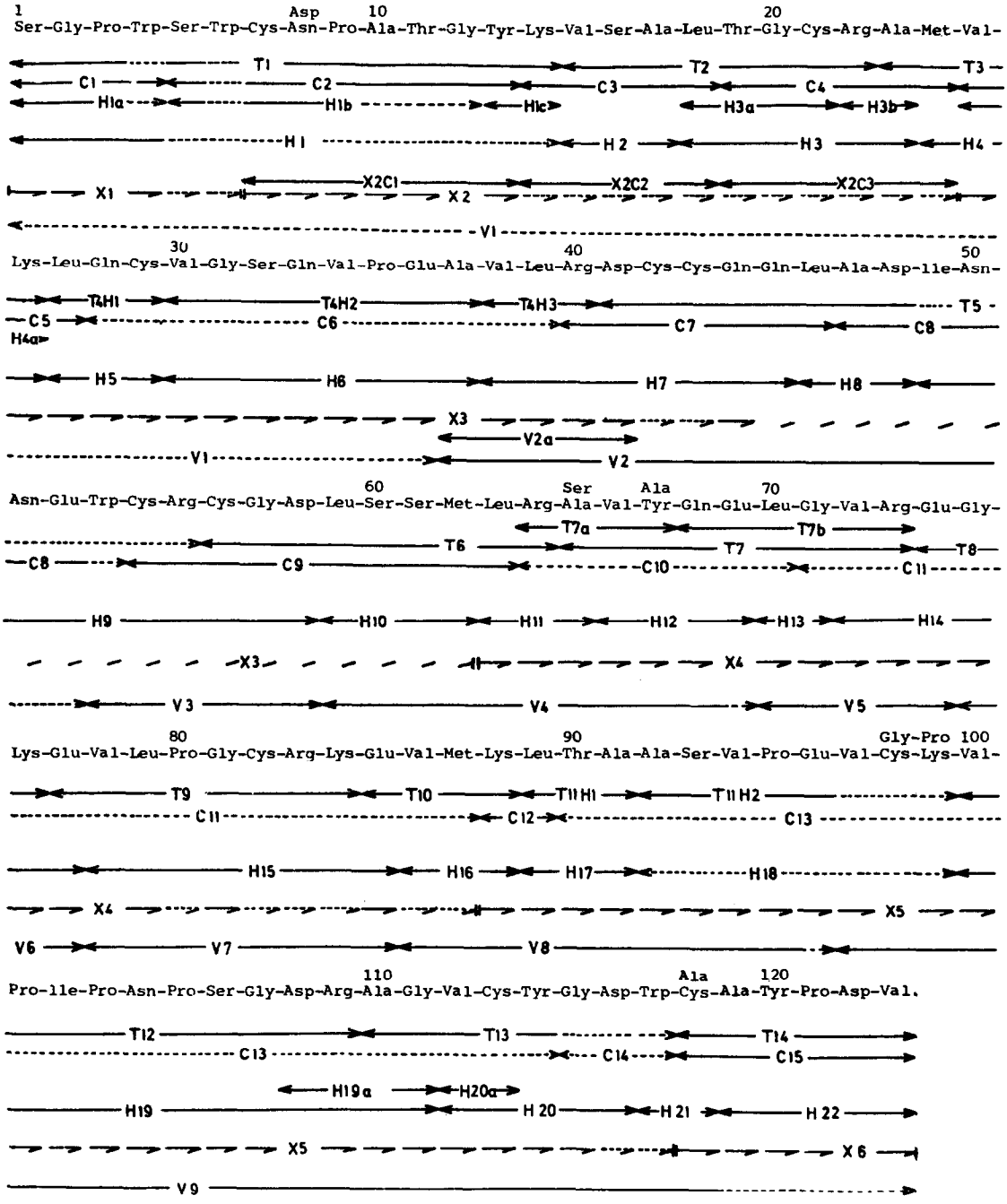

Fig. 1. The amino acid sequence of the wheat protein α-amylase iso-inhibitor CIII. T = trypsin peptides, C = chymotryptic peptides, H = thermolytic peptides, V = peptides from digestion with *S. aureus* V_8 protease, X = fragments resulting from cleavage with cyanogen bromide; → results from DABITC method applied to large fragments, ↔ peptides sequenced by the DABITC method and/or the dansyl-Edman procedure: ←→ peptides/residues which were not sequenced or yielded unsatisfactory results.

The protein contains 123 amino acids which corresponds to an approximate MW of 13400. A more accurate MW cannot be calculated owing to the degree of microheterogeneity exhibited by this protein. The value obtained is in agreement with the previous estimates of 12500 made for the 0.28 albumin inhibitor by gel filtration [3, 4], the values of 13300–14500 from sedimentation equilibrium studies [5, 14], and the figures of 12900–13400 obtained from amino acid analyses [1, 8, 11, 15].

The sequence of the first 24 amino acids in iso-inhibitor CIII is identical with that reported by Redman [9] for the N-terminal CNBr peptides from three inhibitors of the 0.28 family with the exception that residue 8 was usually present as asparagine although small amounts of the deamidated form were also observed.

This work also confirmed the previous suggestion based on the amino acid analysis of CNBr fragments [8] that the Glu/Gln residues in the 0.28 inhibitor are located in the middle of the peptide chain. In fact, all 12 of the Glu/Gln amino acids in the protein were found to be restricted to the central region between residues 28 and 96 (Fig. 1). The sequence of the protein was also notable for being completely lacking in phenylalanine and histidine residues. This is in agreement with most previous amino acid analyses carried out on this family of wheat albumins where both amino acids were absent [1, 5, 8, 11, 15] or only present in very small amounts [14].

Only one residue (position 65) in the protein exhibited significant polymorphism, with equal amounts of Ser and Ala being found in this position in the various peptides and fragments covering the region. Two iso-inhibitors with such high sequence homology, i.e. differing only in these very similar amino acids, would not be separable by the conventional methods of protein purification employed in this investigation, nor would they separate on polyacrylamide gel electrophoresis (PAGE). The other examples of microheterogeneity which were detected all occurred in much smaller amounts, with the minor component in each case being present below 5%. The minor components observed were in position 67 (Ala), 98 (Gly), 99 (Pro) and 118 (Ala). The existence of traces of such polymorphism is perhaps not surprising in view of the multiplicity of iso-inhibitors which have been reported in the 0.28 family [2, 5]. The traces of Asp detected in position 8 in addition to the Asn were possibly the result of deamidation.

Further work is in progress to determine the position of the single residue of reducing sugar which is thought to be present at the binding site for α-amylase [7, 10], and also the location of the five intramolecular disulphide bridges in this protein which appear to be essential for the inhibitory activity [2, 8, 10].

EXPERIMENTAL

Purification of α-amylase inhibitor. The CIII iso-inhibitor of α-amylase was isolated from wheat (*Triticum aestivum* var. Flanders) flour by a combination of the methods of refs. [16] and [17] as described previously [18]. Wheat seeds were ground in a Brook Laboratory mill using a 0.1 mm sieve. The wheat flour obtained was homogenized in 1 kg batches with 2 l. of chilled (1°) H_2O in a Waring blender for 3 min. The homogenate was centrifuged at 2000 *g* for 15 min in an MSE 4 L centrifuge to remove the coarse solids and then the supernatant clarified by a further centrifugation at 9000 g for 20 min in an MSE High Speed 18. The clear supernatant (1.6 l.) was concd to 300 ml by rotary evaporation *in vacuo* at 50°. This conc extract of wheat albumin protein was then heated at 70° for 10 min to ppt. the majority of the unwanted proteins which were removed by centrifugation at 6000 *g* for 20 min. A calculated vol. of EtOH was added slowly to the clear supernatant to give a 70% soln. This was stirred for 2 hr at room temp. and the resulting ppt. removed by centrifugation, before the concn of the soln was raised to 90% by the slow addition of a further calculated vol. of EtOH. The ppt. which formed on standing at 4° for 8 hr was collected by centrifugation at 9000 *g* and dissolved in a minimal vol. of 50 mM pyridine–HOAc buffer, pH 6.5, and dialysed extensively against the same buffer at 4°.

Samples (80 ml) of the dialysed inhibitor extract were subjected to gel filtration chromatography on a column (6.8 × 140 cm) of Sephadex G-75 equilibrated and eluted with 50 mM pyridine–HOAc buffer, pH 6.5. Three peaks (A–C) of inhibitory activity were obtained, the elution vols of which corresponded with the apparent MWs of 60 000, 24 000 and 12 000 previously reported for the α-amylase inhibitors in wheat [4].

The third peak, C (MW 12 000), was collected, lyophilized and dissolved in 30 ml 50 mM Tris–HCl buffer, pH 8.4. This soln was loaded onto a column (2.5 × 23 cm) of DE-52 Cellulose equilibrated with the same buffer. The column was then washed with 4 column vols of the starting buffer before a linear gradient of 0–0.1 M NaCl (1 l. of each) was applied. This procedure yielded 5 (CI–CV) major peaks of protein (280 nm absorption), the third of which (CIII) possessed weak inhibitory activity against hog pancreatic α-amylase. The fractions comprising the centre of this peak were collected, dialysed extensively against H_2O and lyophilized.

Iso-inhibitor CIII obtained in this way gave a single band of mobility 0.30 (relative to bromophenol blue) when subjected to PAGE at pH 8.3 [19].

Assay of inhibitor. The α-amylase inhibitory activity was assayed by the 3,5-dinitrosalicylic acid method of Bernfeld [20] using hog pancreatic α-amylase.

Sequence determination. After reduction and *S*-carboxymethylation [21], the protein was cleaved with CNBr as described in ref. [22]. The resulting fragments were separated by gel filtration on a column (3.2 × 200 cm) of Sephadex G-75 equilibrated and eluted with 0.05 M pyridine–HOAc buffer, pH 5.4. The CNBr fragments contained in the first 2 peaks to elute from this column were further purified by ion-exchange chromatography on columns (1.6 × 30 cm) of CM-52 Cellulose equilibrated with 5 mM NaOAc buffer at pH 3.5, as described in ref. [8] except that a linear gradient of 0–0.17 M NaCl (250 ml of each) was used.

Samples (20–30 mg) of the reduced and *S*-carboxymethylated protein were digested separately with 2% quantities of trypsin, chymotrypsin, thermolysin and the *Staphylococcus aureus* V_8 protease as described in ref. [23]. One of the CNBr fragments (X2) was digested with chymotrypsin, and certain of the larger tryptic peptides (T4 and T11) were redigested with thermolysin.

Mixtures of peptides resulting from the enzyme digestions were fractionated by gel filtration on columns (1 × 190 cm) of Biogel P-2 and Sephadex G-50 (fine) in 0.05 M pyridine–HOAc buffer at pH 5.4, high voltage paper electrophoresis at pH 6.5 and 1.9 and by PC [22].

The fragments resulting from cleavage of the protein with CNBr and certain of the larger peptides were subjected to micro-sequence analysis using the 4-N,N-dimethylaminoazobenzene-4′-isothiocyanate (DABITC)/phenylisothiocyanate double coupling method [24]. The amino acid sequences of the other peptides were determined by the dansyl-Edman procedure [22]. Amino acid analyses of the whole protein, the CNBr fragments and certain peptides were obtained using a Locarte amino acid analyser.

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