Structural Studies of Wheat Monomeric and Dimeric Protein Inhibitors of α -Amylase

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Two wheat monomeric protein inhibitors of α -amylase with mol.wt. 12000, designated inhibitors 0.28 and 0.39 according to their gel-electrophoretic mobilities, showed almost identical circular-dichroism spectra in both the far and near u.v. at different pH values as well as in the presence or absence of dissociating and reducing agents. Both inhibitors (0.28 and 0.39) were readily inactivated by reduction of the five disulphide bridges present in each inhibitor molecule. These properties are very similar to those exhibited by the wheat dimeric protein inhibitor of α -amylase with mol.wt. 24000, designated inhibitor 0.19 according to its gel-electrophoretic mobility. The *N*-terminal sequence of the 0.19 inhibitor was determined without separating its subunits and compared with that of the 0.28 inhibitor reported by Redman [(1976) *Biochem. J.* **155**, 193–195]. Peptide ‘maps’ from tryptic digests of reduced and carboxymethylated inhibitors 0.19 and 0.28 were compared. One molecule of reducing sugar is covalently bound per inhibitor-0.19 protomer and inhibitor-0.28 molecule. The results obtained strongly support previous findings indicating the structural equivalence of inhibitor 0.28 with each inhibitor-0.19 protomer and the common phylogenetic origin of these protein α -amylase inhibitors from wheat kernel.

The kernel of polyploid wheats and phylogenetically related Triticinae species contains a number of protein components capable of inhibiting many α -amylases (1,4- α -D-glucan glucanohydrolase, EC 3.2.1.1) from different origins (Buonocore *et al.*, 1977). These protein inhibitors are albumin in nature and belong to three main inhibitor families with mol.wts. 60000, 24000, and 12000 (Deponte *et al.*, 1976). The 12000-mol.wt. inhibitor family consists of five components with a closely related structure as well as a similar inhibitory behaviour towards amylases of different origins (Silano *et al.*, 1973; Redman, 1975, 1976). The main components of the 12000-mol.wt. protein family have been coded, according to their gel-electrophoretic mobilities relative to Bromophenol Blue, as 0.28 and 0.39. The 24000-mol.wt. inhibitor family, whose main component has a gel-electrophoretic mobility of 0.19, is more heterogeneous and consists of ten closely related components with different electrophoretic mobilities at alkaline pH values (Deponte *et al.*, 1976). The 0.28 and 0.39 inhibitors are monomers, whereas the molecular weight of inhibitor 0.19 falls to about 12500 in 6M-guanidinium chloride or in 1% sodium dodecyl sulphate (Silano *et al.*, 1973). Gel electrophoresis in dissociating solvents has revealed

a very slight difference between the molecular weights of the two inhibitor-0.19 protomers (Silano *et al.*, 1973). When dissociating agents are removed, the inhibitor reassociates and regains its amylase-inhibitory activity (Petrucci *et al.*, 1974; Deponte *et al.*, 1976). Although differing in their molecular weights, inhibitors 0.19 and 0.28 appear to be somewhat related. Both inhibitors (0.19 and 0.28) produced markedly similar denaturation thermograms, with identical denaturation temperature at 93°C (Silano & Zahnley, 1978). The amino acid compositions of inhibitors 0.28 and 0.19 are not greatly different. The structural equivalence of inhibitor 0.28 with each inhibitor-0.19 protomer is also suggested by the fact that 2 mol of inhibitor 0.28 and only 1 mol of inhibitor 0.19 bind each mol of *Tenebrio molitor* L. (yellow mealworm) α -amylase to form amylase-inhibitor complexes (Silano *et al.*, 1977). Finally, the inhibition patterns of inhibitors 0.19 and 0.28 towards amylases from different origins prompted some authors (Shainkin & Birk, 1970; Silano *et al.*, 1973) to suggest that inhibitor 0.28 might actually be contained in inhibitor 0.19.

The present paper deals with physicochemical studies of inhibitors 0.19, 0.28 and 0.39. We sought to clarify structural features to elucidate the relationship between these wheat albumins and to

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establish whether these proteins are coded by structurally related genes.

Experimental

Materials

Sodium dodecyl sulphate and guanidinium chloride were supplied by Schwarz-Mann, Orangeburg, NY, U.S.A.; iodoacetate was from Koch-Light Laboratories, Colnbrook, Bucks., U.K. Trypsin [twice-crystallized, 1-chloro-4-phenyl-3-L-tosylamidobutan-2-one ('TPCK')-treated] was purchased from Worthington, Freehold, NJ, U.S.A., and iodo[2-¹⁴C]acetic acid from The Radiochemical Centre, Amersham, Bucks., U.K. Bio-Gel P2 was from Bio-Rad Laboratories, Richmond, CA, U.S.A., and Sephadex G-100 from Pharmacia, Uppsala, Sweden. Dithiothreitol was supplied by Pierce, Rockford, IL, U.S.A., and 5,5'-dithiobis-(2-nitrobenzoate) by Calbiochem, Los Angeles, CA, U.S.A. All the reagents used in the sequence studies were from Beckman, Palo Alto, CA, U.S.A. The reagents for polyacrylamide-gel electrophoresis, including Aniline Blue-Black, were obtained from Canalcio Industrial Corp., Rockville, MD, U.S.A., and all the other reagents were of analytical grade, from Carlo Erba, Milano, Italy. Materials for radioautography were from Kodak, Rochester, NY, U.S.A.

Purification of albumin inhibitors

Inhibitor 0.19 was purified from hexaploid wheat (*Triticum aestivum*) kernel (pure variety Mentana) by the method of Sodini *et al.* (1970); inhibitors 0.28 and 0.39 were obtained from the same source by the method of Cantagalli *et al.* (1971).

Optical methods

C.d. measurements were carried out on native and reduced inhibitors in the presence or absence of dissociating agents with a Cary model 60 spectropolarimeter equipped with a model 6002 c.d. accessory. Results are reported as mean residue ellipticity, with units of degree·cm⁻²·dmol⁻¹, taking the mean residue weight for the inhibitors as 108. When present, the concentration of sodium dodecyl sulphate was 1% and that of guanidinium chloride was 5M. Dissociating agents were removed by ultrafiltration with Amicon membranes (UM 10) or by dialysis against water for 72h at 4°C. Dithiothreitol, when present, was 60mM. The protein concentration was determined with a Gilford model 2400 spectrophotometer by the method of Waddell (1956), or by using the absorption-coefficient values reported by Silano *et al.* (1973).

Assay of thiols

Thiol groups of denatured inhibitors were determined, before and after reduction with dithiothreitol, with 5,5'-dithiobis-(2-nitrobenzoate) by the method of Cavallini *et al.* (1966). The half-cystine content was determined as cysteic acid after 48h hydrolysis of the performic acid-oxidized proteins in 6M-HCl (Hirs, 1967), with a BC 200 Bio-Cal amino acid analyser, and referred to the amount of protein hydrolysate applied.

Carboxymethylation and tryptic digestion

Carboxymethylation of inhibitors 0.19 and 0.28 was performed with both unlabelled and 2-¹⁴C-labelled iodoacetic acid as described by Kolb *et al.* (1974). Solutions of carboxymethylated protein inhibitors (10mg/ml) in 0.5% (w/v) NH₄HCO₃ were digested for 4h at 37°C with trypsin (1:50, w/v).

'Fingerprints'

Chromatography and subsequent electrophoresis were as described by Bennett (1967). Peptides were located with the cadmium-ninhydrin reagent (Barrolier, 1957) and those containing histidine and/or tyrosine were identified with Pauly's reagent as suggested by Easley (1965). Radioactively labelled peptides were detected by radioautography.

Sequence studies

The N-terminal sequence of carboxymethylated inhibitor 0.19 was determined in duplicate on 5mg samples with a Beckman sequenator model 890 C by using the fast protein-Quadrol program supplied by Beckman Instruments. Amino acid phenylthiohydantoins were identified and assayed by g.l.c. with a Beckman model GC 65 chromatograph.

Carbohydrate content

The content of reducing sugars was measured by the method of Krystal & Graham (1976) on inhibitor samples treated with 6M-guanidinium chloride for 1h, after removal of guanidinium chloride with a column (0.9cm×20cm) of Bio-Gel P2 equilibrated with 0.2M-acetic acid.

α-Amylase-inhibitory activity

Inhibitory activity towards α-amylase purified from *Tenebrio molitor* L. larvae (Buonocore *et al.*, 1975) was determined as described by Silano *et al.* (1975).

Results

Chemical studies

Half-cystine residues per molecule of inhibitors 0.19, 0.28 and 0.39, determined as cysteic acid after performic acid oxidation, were 17.8, 8.2 and 9.3 respectively. After disulphide reduction, 19.2, 8.1 and 9.4 thiol groups were titrated with 5,5'-dithiobis-(2-nitrobenzoate) per molecule of inhibitors 0.19, 0.28 and 0.39 respectively. No free thiol group was detectable in any of the inhibitors before reduction. Nine radioactive spots were clearly evident in the peptide 'maps' of tryptic digests obtained from inhibitors 0.19 and 0.28 after reduction with dithiothreitol and carboxymethylation with iodo[14 C]acetic acid (Fig. 1). These results are in good agreement with the findings reported by Redman (1975) and indicate that inhibitors 0.28 and 0.39 contain five disulphide bridges per molecule. As inhibitor 0.19 consists of two subunits dissociable in non-reducing conditions (Silano *et al.*, 1973), they also indicate that each inhibitor-0.19 subunit contains five disulphide bridges.

Peptide 'maps' from tryptic digests of inhibitors 0.19 and 0.28 were different (Fig. 1). Seventeen spots were clearly detectable in the 'fingerprint' for inhibitor 0.28; such a value is in good agreement with the theoretical value of 15 spots computed from the amino acid composition (lysine+arginine = 14) (Silano *et al.*, 1973). The 'map' for inhibitor 0.19 showed 20 well-separated spots. As this number is lower than the theoretical value of 23 (lysine+arginine = 22) (Petrucci *et al.*, 1976), it suggests a significant homology between the two inhibitor-0.19 subunits. Such homology was confirmed by determining the *N*-terminal sequence of carboxymethylated inhibitor 0.19 without any previous separation of the two constituent subunits (Fig. 2). Only one amino acid residue was obtained for each degradation step up to cycle 23, where the identification of the detached amino acid residue was impossible. The recovery of the *N*-terminal residue (serine) was close to 60%; such a yield, which is usual for single-chain proteins (Niall, 1973), suggests that this residue was derived from both subunits of inhibitor 0.19. In agreement with the previous reports by Redman (1975, 1976), we found that serine is also the *N*-terminal residue of inhibitor 0.28. We were unable to identify the amino acid phenylthiohydantoin released at the second cycle of the automated Edman procedure with inhibitor 0.19; this product did not give any characteristic peak by g.l.c. before and after silylation and is not arginine or histidine, as was shown by testing the aqueous phase with specific reagents. The *N*-terminal sequences of inhibitors 0.19 and 0.28 show a high degree of homology that is particularly evident if the amino acid sequence of inhibitor 0.19 is shifted by one position after residue 4.

Fig. 1. Line drawing of spots from 'fingerprints' of the tryptic digest of 0.19 (a) and 0.28 (b) inhibitors. Carboxymethylation of 0.19 and 0.28 inhibitors was performed with iodo[14 C]acetic acid. Solutions of carboxymethylated protein inhibitors were digested for 4 h at 37°C with trypsin (1:50, w/w). Chromatography of 1 ml samples was carried out in butan-1-ol/acetic acid/water/pyridine (15:3:12:10, by vol.) for 30 h, followed by electrophoresis in pyridine/acetic acid (pH 3.6) at 50 V/cm for 60 min. □, Ninhydrin spots; ■, radioactive spots; ●, spots containing histidine and/or tyrosine (located with Pauly's reagent).

Inhibitor 0.19 contained 1.7 mol of covalently bound reducing sugars/mol, whereas 0.55 mol of reducing sugars was present per mol of inhibitor 0.28.

Fig. 2. *N*-Terminal sequence of inhibitor 0.19

For comparison the *N*-terminal sequence of inhibitor 0.28, as described by Redman (1976), is also reported. The amino acid sequence of inhibitor 0.19 has been shifted by one position after residue 4 to allow a better overlapping of primary structures of inhibitors 0.19 and 0.28. The amino acid residues coded by codons differing by only one nucleotide are underlined. The *N*-terminal sequence of carboxymethylated 0.19 inhibitor was determined in duplicate on 5 mg samples with a Beckman sequenator model 890 C by using the fast protein-Quadrol program supplied by Beckman Instruments. Amino acid phenylthiohydantoin were identified and assayed by g.l.c.

Incubation for 1 h at room temperature (25°C) of 20 μ M solutions of inhibitors 0.28 or 0.39 at alkaline pH values (up to 10) or at acidic pH values (down to 2) did not affect the inhibitory activity of the two albumins towards *T. molitor* α -amylase. Moreover, it was possible to keep the inhibitors at pH 12 for 1 h with only a 25% loss of activity. Treatment of inhibitors 0.28 and 0.39 with 1% sodium dodecyl sulphate or 5M-guanidinium chloride and removal of the dissociating agents by dialysis against water did not affect the activity of the two inhibitors, but when 60mM-dithiothreitol was present both inhibitors were fully inactivated. Loss of inhibitor-0.28 inhibitory activity after CNBr cleavage or thermal treatment was identical with that described by Petrucci *et al.* (1976) for inhibitor 0.19.

Circular-dichroism measurements

The 0.28 and 0.39 inhibitors showed (Fig. 3) a c.d. spectrum in the far u.v. identical with that reported by Petrucci *et al.* (1976) for inhibitor 0.19. This spectrum was not affected by pH throughout the range 2–12 and changed only slightly on addition of 1% sodium dodecyl sulphate (Fig. 4). According to such a spectrum, about one-third of the residues of inhibitors 0.28 and 0.39 may be in the α -helical form (Greenfield & Fasman, 1969) and about one-sixth in the β -structure (Chen & Yang, 1971). As shown by the decrease of the ellipticity at 222nm (Fig. 4), about one-third of the α -helix of inhibitors 0.28 and 0.39

unfolded in the presence of 5M-guanidinium chloride.

C.d. spectra of inhibitors 0.28 and 0.39 in the region of aromatic side-chain absorption were very similar (Fig. 3). Moreover, these spectra were consistently affected by pH changes in the range 2–12. At pH 2 only one positive band centred at 268nm was evident. As at acidic pH values, tyrosine usually shows ellipticity bands at longer wavelengths, and the two inhibitors lack phenylalanine, such a band might be due to tryptophan contributions. Disulphide contributions appear unlikely, because this band is not detectable in the presence of sodium dodecyl sulphate or guanidinium chloride (Fig. 4). In the pH range 2–6, where carboxy groups are titrated, a new band centred at 286nm appeared, and some increase of the positive band was observed. Protonated tyrosine may contribute to the negative band, thus implying that dissociation of carboxy groups produces asymmetrical environments for some tyrosine residues. Finally, at alkaline pH values, the development of a large negative band at 300nm was observed simultaneously with a shift of the positive band toward longer wavelengths. Such a behaviour is what one would expect from titration of tyrosine residues (Stricklan *et al.*, 1969; Horowitz *et al.*, 1970). All these pH-dependent spectral changes were reversible. As shown in Fig. 4, in the presence of 1% sodium dodecyl sulphate or 5M-guanidinium chloride all the main ellipticity bands disappeared, giving rise for both inhibitors 0.28 and 0.39 to a very

Fig. 3. Circular-dichroism spectra of inhibitors 0.28 (a) and 0.39 (b) at different pH values

Buffers used were 20 mM-glycine adjusted to pH 2 with HCl or to pH 10 and 12 with NaOH, and 0.02 M-sodium acetate/0.02 M-sodium barbital adjusted to pH 4, 6 or 8 with acetic acid. In all cases 0.1 M-NaCl was present. ---, pH 2; ----, pH 6; ····, pH 9; —, pH 10; -·-·, pH 12.

broad band covering the whole range from 250 to 310 nm. Such a picture may be due to the relatively large number of disulphide bridges present in these two inhibitors. This band disappeared when 60 mM-dithiothreitol was added to the inhibitor solutions containing sodium dodecyl sulphate. All the spectral changes observed after addition of dissociating agents in the absence of dithiothreitol were reversed on removal of these chemicals by dialysis.

Discussion

The results reported provide further evidence supporting the close similarity of wheat 0.28 and 0.39 inhibitors of α -amylase. These two proteins with identical molecular weight not only have similar inhibitory behaviour towards α -amylases from

different origins (Silano *et al.*, 1973) and have homologous amino acid sequences of large peptide fragments (Redman, 1975, 1976), but are also characterized by an identical content of disulphide bridges and closely related conformations as monitored by c.d. spectra. Inhibitors 0.28 and 0.39 both have a relatively high percentage (about 50%) of ordered structure. At alkaline pH values or in the presence of dissociating agents, the two inhibitors, as observed with inhibitor 0.19 (Petrucci *et al.*, 1976), undergo significant structural changes associated with only minor losses of inhibitory activity. However, both 0.28 and 0.39 inhibitors, similarly to 0.19 inhibitor, were readily inactivated by treatments that break disulphide bonds, thus indicating that their stability mainly depends on the integrity of their disulphide bridges. Actually as many as five disulphide bridges

Fig. 4. Effect of dissociating agents on circular-dichroism spectra of inhibitors 0.28 (a) and 0.39 (b). Measurements of spectra were carried out at pH 6.0. The buffer was 0.02M-sodium acetate/0.02M-sodium barbital, adjusted to pH 6 with acetic acid. —, No sodium dodecyl sulphate or guanidinium chloride; ----, 1% sodium dodecyl sulphate; - · - ·, 5 M-guanidinium chloride.

are present per molecule of the 0.28 and 0.39 inhibitor as well as per each subunit of inhibitor 0.19. As shown by the *N*-terminal sequence and by the 'fingerprint' of the tryptic digest of carboxymethylated inhibitor 0.19, the two 0.19-inhibitor subunits have a relatively high degree of homology in their primary structures. A significant homology has also been observed between the *N*-terminal sequences of inhibitors 0.19 and 0.28, and similar amounts of covalently bound reducing sugars were found in inhibitor 0.28 and each inhibitor-0.19 protomer. Finally, c.d. spectra in the far u.v. of inhibitors 0.19 and 0.28 at different pH values in the presence or absence of dissociating agents were identical. These data are in line with the previous findings (Shainkin & Birk, 1970; Silano *et al.*, 1973, 1977; Silano & Zahnley, 1978), suggesting the structural equivalence of inhibitor 0.28 with each inhibitor-0.19 protomer. In fact the large differences observed between 'fingerprints' of tryptic digest and near-u.v. c.d. spectra of 0.19 and 0.28 inhibitors may result from the rather limited differences observed in their amino acid compositions (Silano *et al.*, 1973).

In conclusion, the overall information available on highly purified α -amylase inhibitors from wheat kernel strongly support the view that these inhibitors have originated by a common ancestral gene through replication and mutation (Buonocore *et al.*, 1977).

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