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Interaction of Wheat Monomeric and Dimeric Protein Inhibitors with α-Amylase from Yellow Mealworm (*Tenebrio molitor* L. Larva)

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The highly purified α-amylase from *Tenebrio molitor* L. larva (yellow mealworm) reversibly combines with two closely related homogeneous glycoprotein inhibitors, one dimeric (termed 'inhibitor 0.19') and one monomeric (termed 'inhibitor 0.28'), from wheat flour. As established by means of difference spectroscopy and kinetic studies, molar combining ratios for the amylase–inhibitor-0.19 and amylase–inhibitor-0.28 complexes were 1:1 and 1:2 respectively. Two amylase–inhibitor-0.19 complexes with slightly different retention volumes on Bio-Gel P-300 and only one amylase–inhibitor-0.28 complex were observed. Dissociation constants of the amylase–inhibitor-0.19 and amylase–inhibitor-0.28 complexes were 0.85 nM and 0.13 nM respectively. A strong tendency of both complexes to precipitate under an ultracentrifugal field was observed; the minimum molecular weight calculated for the two complexes under such conditions was approx. 95000. The two complexes showed difference spectra indicating involvement of structurally related or identical tryptophyl side chains in the binding of inhibitors 0.28 and 0.19 to the amylase. A model summarizing the main features of the inhibition of the insect amylase by the two wheat protein inhibitors is proposed.

Of the several species of insects that attack stored wheat and wheat products, *Tenebrio molitor* L. larva (yellow mealworm) has a very high amylase activity (Silano *et al.*, 1975). *T. molitor* larva only contains one molecular form of amylase that has been purified to homogeneity and proved to be an acidic (pI 4.0) Ca^{2+}-glycoprotein that is irreversibly inactivated by removal of Ca^{2+} ions and activated by addition of Cl^- (Buonocore *et al.*, 1975, 1976*b*). The starch-degradation pattern of this insect enzyme is typical of an α-amylase (1,4-α-D-glucan glucanohydrolase, EC 3.2.1.1). The amylase has a denaturation temperature of 70.5°C and a denaturation enthalpy of 25.1 J/g (Silano & Zahnley, 1978). Such a high denaturation enthalpy for a single-chain protein with mol.wt. 68000 having very low α-helix and disulphide content indicates a significant stabilizing effect of the Ca^{2+} present in the amylase molecule.

The *T. molitor* amylase is effectively inhibited by a number of water-soluble protein components (albumins) from the wheat kernel, including those termed, according to their gel-electrophoretic mobility relative to Bromophenol Blue, inhibitors 0.28 and 0.19 (Silano *et al.*, 1973, 1975). The inhibitor-0.28 albumin is a monomer with mol.wt. 12000, whereas the molecular weight of inhibitor 0.19 is about 24000 in non-dissociating solvents (Silano *et al.*, 1973). The inhibitor-0.19 albumin consists of two non-covalently bound subunits whose molecular weight is not significantly different from that of inhibitor 0.28. The inhibitor-0.19 protomers dissociate in the presence of 6 M-guanidine hydrochloride or in 1% SDS, but they reassociate on removal of the dissociating agent; after such a treatment the dimer still exhibits its amylase-inhibitory activity (Silano *et al.*, 1973; Petrucci *et al.*, 1976). Gel-electrophoretic, 'fingerprinting' and sequencing studies of inhibitor 0.19 have confirmed that its two subunits are similar, although not identical (Petrucci *et al.*, 1978). The isoelectric points of inhibitors 0.28 and 0.19 are 6.2 and 7.1 respectively. Both inhibitors 0.19 and 0.28 produce markedly similar denaturation thermograms, with denaturation temperature at 93°C (Silano & Zahnley,

Abbreviations used: inhibitors 0.28 and 0.19, α-amylase protein inhibitors from wheat kernel with mol.wt. 12000 and 24000 and gel-electrophoretic mobilities, relative to Bromophenol Blue, 0.28 and 0.19, respectively; SDS, sodium dodecyl sulphate.

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1978), which reflects the large content of α-helix (about 40%) and disulphide cross-linking (5 disulphide bonds per protomer of 12000 mol.wt.) (Petrucci *et al.*, 1976, 1978). The two inhibitors are glycoproteins bearing 1 mol of reducing sugar per 12000-mol.wt. protomer, and exhibit an almost identical denaturation enthalpy and resistance to extreme pH treatments (Petrucci *et al.*, 1976, 1978). These findings indicate that, in spite of the differences observed in amino acid compositions and sequences (Redman, 1975; Petrucci *et al.*, 1978), inhibitor 0.28 and the two subunits of inhibitor 0.19 strongly resemble each other.

Only preliminary data about the interaction of inhibitors 0.19 and 0.28 with *T. molitor* amylase are available (Buonocore *et al.*, 1977; Silano *et al.*, 1977). Affinity-chromatography studies by Buonocore *et al.* (1975) have shown that the binding of the insect amylase to Sepharose-coupled wheat albumins is effectively reversed by maltose or gelatinized starch. A 1 mol:1 mol complex is formed between amylase and the 0.19 inhibitor (Buonocore *et al.*, 1976*a*). Denaturation temperatures of the insect amylase in the presence of inhibitor 0.19 or 0.28 are 10°C and 14°C, respectively, above the observed denaturation temperature for the free enzyme, thus indicating that inhibitor 0.28 has a higher stabilizing effect of the amylase than inhibitor 0.19 (Silano & Zahnley, 1978). Interaction of inhibitor 0.19 with crude human salivary amylase has been studied by Granum & Whitaker (1977).

The present paper deals with physico-chemical and kinetic studies carried out to clarify further the interaction mechanism of *T. molitor* amylase with inhibitors 0.28 and 0.19. These studies also provide a unique model of highly specific and reversible monomer–monomer and monomer–dimer protein interactions.

Experimental

Materials

The α-amylase from *T. molitor* larva was obtained in a homogeneous form as described by Buonocore *et al.* (1975), and the 0.19 and 0.28 albumin inhibitors were purified from wheat kernel as described by Sodini *et al.* (1970) and Cantagalli *et al.* (1971) respectively. Bio-Gel P-300 was from Bio-Rad Laboratories, Richmond, CA, U.S.A. Acrylamide and bisacrylamide were supplied by BDH Chemicals, Poole, Dorset, U.K. Reference proteins for gel-filtration experiments were purchased from Sigma Chemical Co, St. Louis, MO, U.S.A., and starch was obtained from Connaught Laboratories, Toronto, Ont., Canada.

Amylase activity and inhibition kinetics

The amylase assay was performed by the method of Nelson (1944) as described by Buonocore *et al.* (1976*b*). Amylase assay in the presence of maltose was carried out with the iodine-staining method (Robyt & Whelan, 1968). The standard assay of amylase inhibition by inhibitor 0.19 was carried out in the presence of bovine serum albumin (25 μg/ml) by preincubating, in a final volume of 0.9 ml, the amylase with the inhibitor for 20 min at 37°C in 20 mM-sodium cacodylate/HCl buffer, pH 5.3, containing 10 mM-NaCl and 0.1 mM-$CaCl_2$; residual amylase activity was then tested after addition of starch (0.1 ml of a 20 mg/ml solution). Amylase inhibition by inhibitor 0.28 was measured by the same procedure, except that the cacodylate/HCl buffer used had pH 5.8.

The amylase-inhibition kinetics were studied by applying to the amylase–protein-inhibitor systems the approach suitable for mutual depletion systems (Edsall & Wyman, 1958; Tanford, 1961; Webb, 1963) characterized by strong binding of the inhibitors to the amylase and by partial dissociation of the enzyme–inhibitor complexes after addition of starch (Bieth, 1974). The equation

$$\frac{[I_0]}{1-a} = \frac{1}{a}\frac{K_i\,(\text{app.})}{n} + \frac{[E_0]}{n} \qquad (1)$$

where $[I_0]$ and $[E_0]$ represent the initial concentrations of inhibitor and enzyme respectively, a is the fraction of total enzyme not bound to the inhibitor and n the number of equivalent and independent binding sites of the enzyme for the inhibitor, was used for calculating both n and K_i (app.). As the enzyme–inhibitor complexes are inactive (see Fig. 4*d*), values of a were determined as v_i/v_0 ratios, where v_i is the initial rate of enzymic activity in the presence of inhibitor and v_0 the corresponding rate in the absence of inhibitor. Moreover, from the equation (Bieth, 1974)

$$K_i\,(\text{app.}) = K_i\left(1 + \frac{[S_0]}{K_m}\right) \qquad (2)$$

where $[S_0]$ is the initial substrate concentration and K_m is 1.8 mg/ml (Buonocore *et al.*, 1976*b*), true K_i values were obtained.

Protein concentration

This was determined colorimetrically by the Lowry method as modified by Hartree (1972), with bovine serum albumin as standard, and spectrophotometrically by using the $A^{1\%}_{1cm}$ at 280 nm (8.1, 12.6 and 16.6 for *T. molitor* amylase, 0.19 and 0.28 inhibitors respectively) or by the method of Waddel (1956).

Gel-filtration studies

Complexes between amylase and either inhibitor were purified by chromatography on Bio-Gel P-300, which, as compared with Bio-Gel P-100 and P-200,

gave a better separation. Enzyme and inhibitor were incubated for 30min at 25°C in the cacodylate buffer at the pH value used for the standard inhibition assay. The mixture was then loaded on a Bio-Gel P-300 column (2.5cm × 40cm) and eluted with the appropriate buffer at a flow rate of 10ml/h. Eluate was tested for A_{280} and for enzymic and inhibitory activity. Pooled fractions were concentrated by ultrafiltration through Amicon UM-10 membranes and tested for protein content as described by Hartree (1972).

Gel electrophoresis

Disc electrophoresis was performed in 0.05M-Tris/0.383M-glycine buffer, pH8.5, as described by Davis (1964). Enzymic and inhibitory activity of the protein eluted from the gels was assayed as described by Buonocore *et al.* (1976*b*). Polyacrylamide-gel electrophoresis in SDS was carried out as described by Laemmli (1970).

Optical methods

The u.v. difference spectra were recorded between 380 and 240nm on a Cary 118 spectrophotometer by using a tandem cell thermostatically maintained at 30°C. Enzyme and inhibitor were incubated in the cell for 30min in the cacodylate buffer of the appropriate pH before recording the spectrum. The c.d. measurements were made under identical experimental conditions with a Cary 60 spectropolarimeter equipped with a 6002 CD accessory.

Sedimentation analyses

Sedimentation-velocity runs were carried out at 20°C in a Beckman E analytical ultracentrifuge for 2h at 56100rev./min. Sedimentation-equilibrium runs were performed at 12°C in the same apparatus for 72h at 19160rev./min. The cacodylate buffer utilized for inhibition assays was also used in these runs.

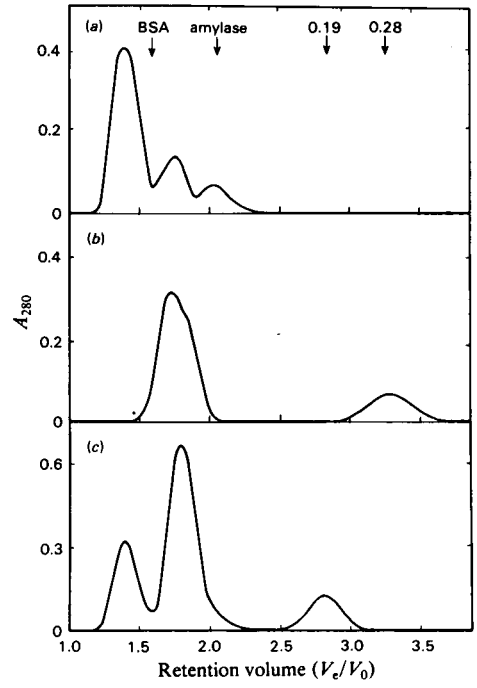

Fig. 1. *Gel filtration of mixtures (4ml) containing T. molitor amylase and inhibitor 0.19 and/or 0.28*

The mixtures were incubated for 30min at 25°C before being loaded on a Bio-Gel P-300 column (2.5cm × 40cm). The proteins were eluted at a flow rate of 10ml/h with 20mM-sodium cacodylate/HCl buffer, containing 10mM-NaCl and 0.1mM-$CaCl_2$; buffer pH was 5.3 in (*a*) and 5.8 in (*b*) and (*c*). As indicated by arrows, retention volumes of bovine serum albumin (BSA), *T. molitor* amylase, inhibitors 0.19 and 0.28, separately chromatographed, were 1.6, 2.05, 2.8 and 3.25 × V_e/V_0 respectively. (*a*) Filtration of mixture of amylase (38nmol) and inhibitor 0.19 (34nmol); (*b*) filtration of mixture of amylase (27nmol) and inhibitor 0.28 (100nmol); (*c*) filtration of mixture of amylase (82nmol) and inhibitors 0.19 (90nmol) and 0.28 (100nmol).

Results

Gel-filtration studies

Three peaks of absorbance at 280nm were separated by filtration on a Bio-Gel P-300 column of an approximately equimolar mixture of amylase (38nmol/4ml) and inhibitor 0.19 (34nmol/4ml) after 30min incubation at room temperature (Fig. 1*a*). As shown by its retention volume and by polyacrylamide-gel-electrophoretic pattern, the third peak consisted of free amylase (about 6nmol). It appears that the amylase and inhibitor 0.19 had reacted in a 1:1 molar stoichiometric ratio. The retention volume of pure amylase from the Bio-Gel matrix was 2.05 × V_e/V_0, which is higher than the value of 1.6 expected on the basis of its molecular weight. Gel-electrophoretic patterns of the first and second peaks were identical; they contained an intermediate band relative to the amylase–inhibitor-0.19 complex in addition to bands corresponding to free enzyme and inhibitor, thus indicating that partial dissociation of the enzyme–inhibitor complex occurs during the electrophoretic run. The presence of both enzyme and inhibitor in the intermediate band was shown by assaying, as described by Buonocore *et al.* (1976*a*), the protein eluted from the gel for enzymic and inhibitory activity. Gel electrophoresis in the presence of SDS confirmed the presence of both inhibitor 0.19 and

amylase in the first and second peaks of Fig. 1(*a*) and of amylase alone in the third peak. Thereafter we will refer to the first peak as amylase–inhibitor-0.19 complex A, and to the second one as amylase–inhibitor-0.19 complex B. Retention volumes of amylase–inhibitor-0.19 complexes A and B remained unchanged when separately reloaded on the Bio-Gel column, whereas the two complexes were eluted as a single peak from a Sephadex G-100 column (Buonocore *et al.*, 1976*a*). Such behaviour may be related to the stronger affinity that the amylase exhibited for the Sephadex matrix as compared with the Bio-Gel matrix. The different retention volumes of the two amylase–inhibitor-0.19 complexes from the Bio-Gel column do not necessarily imply any difference in their molecular weights, and they might as well be conformational isomers exhibiting a slightly different binding affinity for the Bio-Gel matrix. These two complexes also were undistinguishable by gel electrophoresis in buffer systems with or without dissociating agents.

When a 4ml mixture of amylase (27nmol) and inhibitor 0.28 (100nmol) was incubated for 30min at room temperature and then filtered on the Bio-Gel P-300 column, two A_{280} peaks were separated (Fig. 1*b*). The presence of the amylase–inhibitor-0.28 complex in the first peak was demonstrated by polyacrylamide-gel electrophoresis in the absence or presence of SDS. The amylase–inhibitor-0.28 complex exhibited a retention volume identical with that of the amylase–inhibitor-0.19 complex B. The second peak of Fig. 1(*b*) consisted of free 0.28 inhibitor (40nmol). As no free amylase peak could be detected in the elution pattern, it was concluded that amylase and inhibitor 0.28 had reacted in a 1:2 molar stoichiometric ratio. These findings were confirmed by filtering on the Bio-Gel column five mixtures containing amylase and inhibitor 0.28 in molar ratios varying from 1:0.5 to 1:3. No peak of free inhibitor was observed in the elution patterns of mixtures with enzyme/inhibitor ratios 1:0.5, 1:1 and 1:2, whereas a peak of free inhibitor 0.28 was clearly evident for mixtures with enzyme/inhibitor ratios 1:2.5 and 1:3. At all the enzyme/inhibitor ratios tested only one amylase–inhibitor-0.28 complex peak could be observed. Similarly, Silano & Zahnley (1978) reported the presence, in the thermograms of equimolar mixtures of inhibitor 0.28 and amylase, of two peaks, one corresponding to free enzyme and the other to amylase–inhibitor-0.28 complex. When amylase and inhibitor 0.28 were mixed in a 1:2 molar ratio, only the peak of the complex was evident in the thermogram (J. C. Zahnley & V. Silano, unpublished work).

When a 4ml mixture of amylase (82nmol), inhibitor 0.19 (90nmol) and inhibitor 0.28 (100nmol) was incubated for 30min at room temperature and then filtered on the Bio-Gel column, three A_{280} peaks were observed (Fig. 1*c*). As shown by gel electrophoresis, the first peak only consisted of the amylase–inhibitor-0.19 complex A, whereas the second peak contained both amylase–inhibitor-0.28 complex and amylase–inhibitor-0.19 complex B; free 0.19 inhibitor (54nmol) was present in the third peak, and no free inhibitor 0.28 was detectable in the eluate. Therefore no evidence supporting the formation of a ternary inhibitor-0.19–amylase–inhibitor-0.28 complex was obtained.

Difference-spectroscopy studies

Difference spectra of amylase–inhibitor-0.28 and amylase–inhibitor-0.19 complexes were recorded in the 240–380nm wavelength region for mixtures of enzyme and either inhibitor. From a qualitative standpoint, the difference spectra were identical, exhibiting two maxima at 290–291 and 284–285nm, and a shoulder peak, less evident for the amylase–inhibitor-0.19 complex, at 274–275nm (Fig. 2). Maximal absorbance difference was obtained with mixtures containing 1mol of inhibitor 0.19 or 2mol of inhibitor 0.28 per mol of amylase (Fig. 3). Dependence of extent of spectral perturbation at 291nm on inhibitor concentration strongly suggests

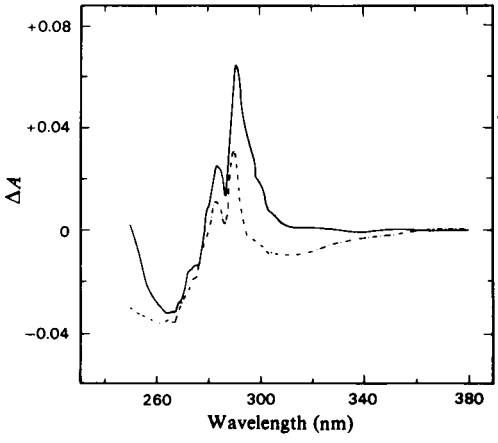

Fig. 2. *Typical difference spectra induced by addition of inhibitor 0.28* (——) *or 0.19* (–·–·–·–) *to T. molitor amylase*

Amylase and either inhibitor were mixed in a tandem cell in 20mM-sodium cacodylate/HCl buffer containing 10mM-NaCl and 0.1mM-$CaCl_2$; buffer pH was 5.8 (amylase–inhibitor-0.28 system) or 5.3 (amylase–inhibitor-0.19 system). The mixtures were incubated for 30min at 30°C, then the difference spectrum was recorded against separate solutions of enzyme and inhibitor contained in the reference tandem cell. Protein concentrations in the cells were: amylase, 10.8μM; inhibitor 0.28, 20.7μM; inhibitor 0.19, 10.25μM.

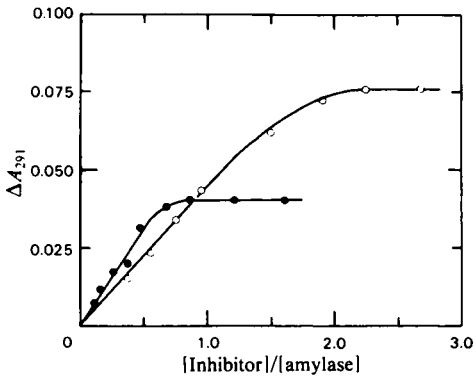

Fig. 3. *Dependence of spectral perturbation at 291 nm on inhibitor/amylase molar ratio*
Absorbance differences at 291 nm were plotted against inhibitor-0.28/amylase (○) or inhibitor-0.19/amylase (●) molar ratio.

that titration of the enzyme by the two inhibitors occurred under the experimental conditions chosen. Molar-absorption-coefficient differences at 291 nm were $3.8 \times 10^3 M^{-1} \cdot cm^{-1}$ and $7.1 \times 10^3 M^{-1} \cdot cm^{-1}$ for amylase–inhibitor-0.19 and amylase–inhibitor-0.28 complexes respectively. The difference spectra observed are very similar to the solvent perturbation difference spectra of *N*-acetyltryptophan ethyl ester solutions induced by addition of maltose, dimethyl sulphoxide or other perturbants, as well as to the difference spectra of pig pancreatic amylase in the presence of 29 mM-maltose (Herskovits, 1967; Elodi *et al.*, 1972). However, no detectable difference spectrum of *T. molitor* amylase (28 μM) was observed in the presence of maltose up to 100 mM concentration.

Inhibition kinetics

Shainkin & Birk (1970) first showed that maximal inhibition of *T. molitor* amylase by wheat

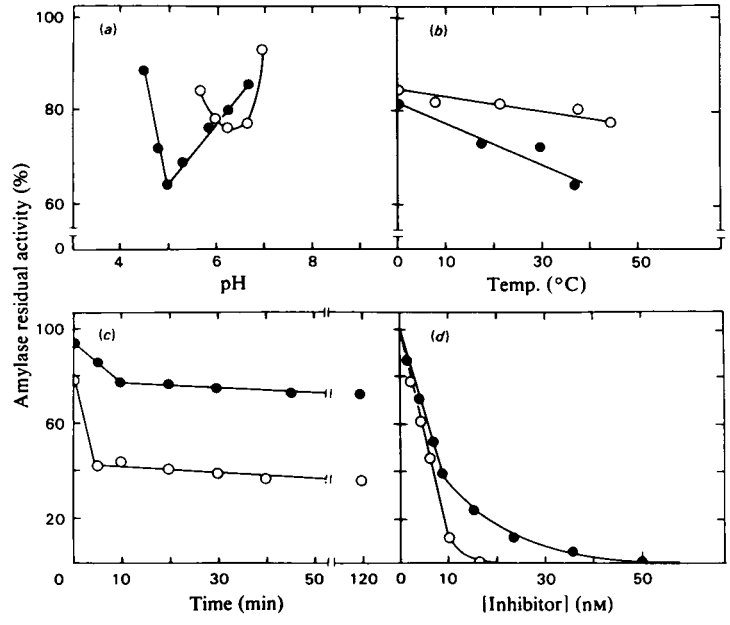

Fig. 4. *Effect of preincubation conditions [(a) pH, (b) temperature and (c) time] and of (d) inhibitor concentration on T. molitor amylase inhibition by inhibitors 0.28* (○) *and 0.19* (●)
(*a*) Amylase (1.4 nM) was preincubated in the presence of bovine serum albumin (25 μg/ml), in a final volume of 0.9 ml, with either inhibitor 0.28 (5.6 nM) or 0.19 (7.0 nM) for 20 min at 37°C in 20 mM-sodium cacodylate/HCl buffer solutions of different pH values, containing 10 mM-NaCl and 0.1 mM-$CaCl_2$. Residual amylase activity was tested by Nelson's (1944) method after addition of 0.1 ml of starch (20 mg/ml); (*b*) amylase and either inhibitor were preincubated, at the protein concentration indicated in (*a*), for 20 min in the cacodylate buffer of pH 5.3 or 5.8 for assay of inhibition by inhibitor 0.19 or 0.28, respectively, at temperature varying from 0 to 45°C; (*c*) amylase and either inhibitor were preincubated, at the protein concentration indicated in (*a*), at 37°C in the cacodylate buffer of pH 5.3 (inhibitor 0.19) or 5.8 (inhibitor 0.28); residual amylase activity was tested at the times indicated; (*d*) amylase (1.1 nM) was preincubated with different amounts of either inhibitor for 20 min at 37°C in the cacodylate buffer of pH 5.3 (inhibitor 0.19) or 5.8 (inhibitor 0.28); residual enzymic activity was then tested.

albumin inhibitors was only obtained after preincubation of enzyme and inhibitor before addition of starch. The dependence of the amylase inhibition by inhibitor 0.19 on enzyme–inhibitor preincubation conditions, including time, pH and temperature, was shown by Buonocore *et al.* (1976*a*). Similar results were obtained for amylase inhibition by inhibitor 0.28 under the same experimental conditions as adopted for inhibitor 0.19 (Fig. 4). Maximal inhibition of amylase by inhibitor 0.28, compared with that by inhibitor 0.19, required a higher preincubation pH (6.2 instead of 5.0) (Fig. 4*a*), and a shorter preincubation time (about 4 min instead of 10 min) (Fig. 4*c*); in the range 0–40°C the inhibitory activity of inhibitor 0.28 was only slightly affected by preincubation temperature (Fig. 4*b*). Preincubation pH chosen for standard inhibition assay with inhibitor 0.28 was 5.8, where enzymic activity is maximal and inhibitory activity is as much as 80%. Moreover, as for the inhibition assay with inhibitor 0.19, standard preincubation temperature and time were 37°C and 20 min respectively. Amylase residual activity in the presence of increasing amounts of the two inhibitors is shown in Fig. 4(*d*). Complete inhibition of the enzyme, showing that the two enzyme–inhibitor complexes are inactive, was obtained under appropriate conditions. Previous inhibition assays with inhibitor 0.19 (Buonocore *et al.*, 1976*a*), showing an incomplete inhibition of the amylase, were carried out at a lower inhibitor concentration. When inhibitors 0.19 (4.8 nM) and 0.28 (1.6 nM) were simultaneously tested with the amylase (3.3 nM) at pH 5.8, a full additivity of the inhibitory activities was observed.

Time-dependence of starch hydrolysis after addition of the starch to amylase–inhibitor-0.19 and amylase–inhibitor-0.28 mixtures as well as to pure amylase is shown in Fig. 5. In the presence of the inhibitors, the production of reducing sugars after starch addition became linear after a short log phase; in the absence of the inhibitors, the production of reducing sugars was linear through all the time-range tested. Such a kinetic trend was more evident with the amylase–inhibitor-0.19 system than with the amylase–inhibitor-0.28 one and, for both systems, it was less evident at lower starch concentrations. As these findings are consistent with a partial dissociation of both complexes induced by starch addition, and the inhibitors strongly bind the enzyme (see Figs. 3 and 4*d*), eqn. (1) (see the Experimental section) was utilized to calculate the apparent dissociation constant [K_i (app.)] and the number of mol of each inhibitor bound per mol of enzyme (n). With such a procedure, a 1:1 molar stoichiometric ratio for the amylase–inhibitor-0.19 complex and a 1:2 ratio for the amylase–inhibitor-0.28 complex were derived from the plot shown in Fig. 6. Application of such a kinetic approach to the amylase–inhibitor-0.28 system implies that the two inhibitor sites on the enzyme are equivalent and independent, and that binding of one inhibitor-0.28

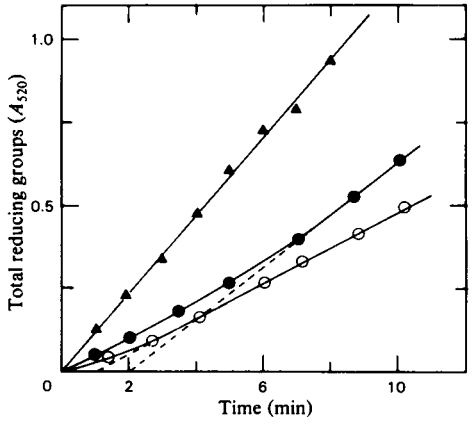

Fig. 5. *Time-dependence of starch hydrolysis in the absence* (▲) *and in the presence of inhibitors 0.28* (○) *or 0.19* (●)

T. molitor amylase (3.8 nM) was preincubated with inhibitor 0.28 (5.2 nM) or 0.19 (7.5 nM) under standard conditions; after addition of starch (6 mg/ml final concn.) the release of reducing sugars was tested at the times indicated with Nelson's (1944) method.

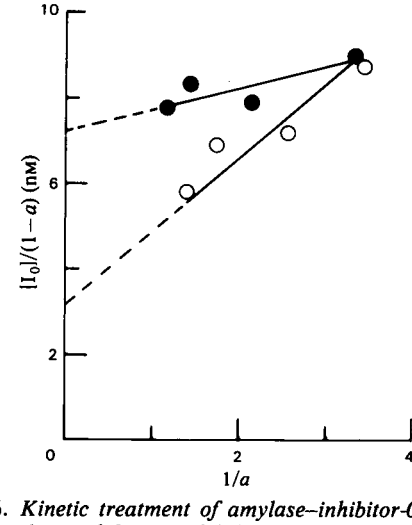

Fig. 6. *Kinetic treatment of amylase–inhibitor-0.28* (○) *and amylase–inhibitor-0.19* (●) *mutual depletion systems*

The plot allows calculation of number of inhibitor molecules bound per molecule of enzyme and apparent dissociation constants according to eqn. (1). Amylase concentration was 7.2 nM.

molecule decreases the enzymic activity to 50%. Although these assumptions might not take into account all the actual features of the enzyme–inhibitor-0.28 interaction, the binding ratio obtained with such a treatment is in excellent agreement with that derived from gel filtration and differential spectroscopy and calorimetry studies. Moreover, from the eqn. (2) (see the Experimental section) the true K_i values of 0.85×10^{-9}M (amylase–inhibitor-0.19 complex) and 0.13×10^{-9}M (amylase–inhibitor-0.28 complex) were obtained. The K_i value of the amylase–inhibitor-0.19 system is significatively lower than that of 3×10^{-7}M found by Buonocore *et al.* (1976*a*) using classical Michaelis–Menten kinetics, which has also been applied to other amylase–protein-inhibitor systems (Militzer *et al.*, 1946; Saunders & Lang, 1973). Anyhow it should be pointed out that the data obtained with the Michaelis–Menten treatment are not reliable, because this kinetic approach cannot be applied to mutual-depletion systems.

Physical properties of amylase–inhibitor complexes

In agreement with previous results by Buonocore *et al.* (1976*a*), amylase–inhibitor-0.19 complex A showed a strong tendency to precipitate during equilibrium-sedimentation runs. The minimum molecular weight ranged in three separate runs between 87000 and 108000, with an average value of 95000. From the velocity-sedimentation experiments a $s^0_{20,w}$ was calculated for this complex of 6.5S, significantly higher than the value of 4.2S obtained with the insect amylase or bovine serum albumin (used as a control). Values of $s^0_{20,w}$ for 0.19 and 0.28 inhibitors were 2.6 and 1.6S respectively (Petrucci *et al.*, 1974). The amylase–inhibitor-0.28 complex showed in both equilibrium- and velocity-sedimentation experiments a behaviour closely related to that of the amylase–inhibitor-0.19 complex A. These findings are consistent with the other results indicating a 1:1 molar combination ratio for the amylase–inhibitor-0.19 complex A and a 1:2 ratio for the amylase–inhibitor-0.28 complex. As the amylase–inhibitor-0.19 complex B was not available in adequate amounts for physical characterization, further studies are needed to obtain direct evidence of a 1:1 molar combination ratio for this complex. However, all the available data seem to exclude the possibility of other combination ratios.

Circular-dichroism spectra in the far and near u.v. of both amylase–inhibitor-0.19 complex A and amylase–inhibitor-0.28 complex were very similar to those of the enzyme (Buonocore *et al.*, 1976*b*). The u.v.-absorption spectra of the two complexes both showed a maximum at 278nm, with absorption coefficients ($A^{1\%}_{1cm}$) of 9.2 (amylase–inhibitor-0.19 complex A) and 12.0 (amylase–inhibitor-0.28 complex).

Discussion

We have shown that the wheat proteins termed inhibitors 0.19 and 0.28 reversibly inhibit the amylase from *T. molitor* larva giving 1:1 and 1:2 (molar ratios) amylase–inhibitor complexes respectively. These stoichiometric ratios have been confirmed by kinetic, spectral, gel-filtration and differential-calorimetry studies. It appears that the amylase has at least two binding sites for the 0.28 inhibitor (one for each molecule). Moreover, as the 0.19 inhibitor is a dimeric protein consisting of two non-identical monomers, both closely related to the inhibitor-0.28 molecule, the 1:1 molar combination

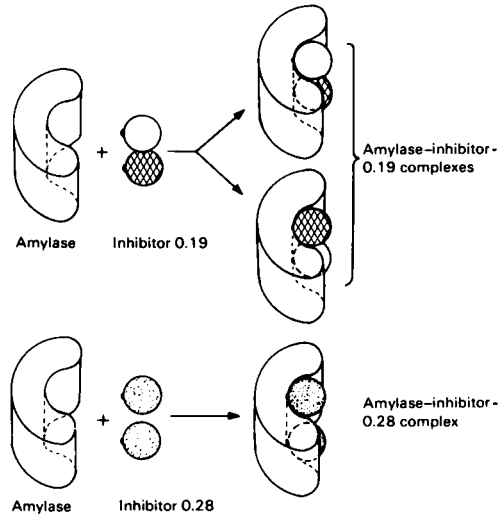

Fig. 7. *Hypothetical schematic representation of the complexes formed between inhibitor 0.19 or 0.28 and T. molitor amylase*

For analogy with the model of pig pancreatic amylase presented by Simon *et al.* (1974), *T. molitor* amylase has been drawn with a semi-cylindrical cavity capable of accommodating either starch or the protein inhibitors. Each inhibitor protomer of mol.wt. 12000 is represented as a sphere; the black dot attached to each sphere indicates the reducing-sugar moiety. The scheme shows that the enzyme molecule has two binding sites for the inhibitors (one for each protomer), although in binding of inhibitor 0.19 one site might be silent. It is speculated that the complex-formation involves binding of the sugar moiety of the inhibitors to the enzyme; the complex is further stabilized by secondary protein–protein bonding forces. Assuming that the two non-identical inhibitor-0.19 subunits can both interact, although not simultaneously, with each amylase binding site, the formation of two slightly different amylase–inhibitor-0.19 complexes, both containing 1 mol of enzyme/mol of inhibitor, is expected.

ratio observed for the amylase–inhibitor-0.19 complex suggests that the amylase might also have two binding sites for the 0.19 inhibitor (one for each subunit) (Fig. 7). The qualitatively identical difference spectra of amylase–inhibitor-0.28 and amylase–inhibitor-0.19 complexes indicate that similar groups are involved in the enzyme–inhibitor interaction. As we failed to show the presence of a ternary inhibitor-0.28–amylase–inhibitor-0.19 complex, we conclude that the binding to the amylase of each inhibitor prevents the binding of the other. The molar-absorption-coefficient difference of amylase in the presence of inhibitor 0.28 is twice that observed in the presence of inhibitor 0.19; therefore it can be suggested that one of the two binding sites of the amylase molecule for inhibitor 0.19 is silent and/or that reactive tryptophan residues involved in the enzyme–inhibitor binding are located on the inhibitors and one of the two inhibitor-0.19 subunits lacks such residues. Actually, the inhibitor-0.28 molecule has a higher content of tryptophan residues than does inhibitor 0.19, and two molecules of inhibitor 0.28 should have more freedom than two linked subunits of an inhibitor-0.19 molecule of adjusting themselves to complementary parts of the amylase molecule, and of possibly inducing conformational changes in the enzyme structure. The hypothesized lack of expression of one binding site of the amylase for inhibitor 0.19 provides acceptable explanations for the higher affinity for the insect amylase of the monomeric inhibitor (0.28) than the dimeric one (0.19); such a higher affinity is indicated by the lower dissociation constant of the amylase–inhibitor-0.28 complex, by the higher thermal-stabilization effect of the enzyme displayed by inhibitor 0.28, and by the lower susceptibility of amylase inhibition by inhibitor 0.28 to starch addition. We have not been able to provide any evidence supporting the formation of a 1:1 amylase–inhibitor-0.28 complex, but this point deserves further investigation. Assuming that the two non-identical 0.19 subunits are both able to interact, although not simultaneously, with each amylase binding site, the presence of two non-equivalent inhibitor-0.19–amylase complexes with a 1:1 molar stoichiometric ratio and of only one amylase–inhibitor-0.28 complex can be inferred from the model of Fig. 7. Therefore, such a model is consistent with the results obtained by gel-filtration studies that showed the existence of two slightly different, non-interconvertible, amylase–inhibitor-0.19 complexes and of only one amylase–inhibitor-0.28 complex. The model does not imply significant structural differences between binding sites of inhibitor-0.19 subunits and the inhibitor-0.28 molecule, being in line with the structural similarity of these polypeptide chains.

As indicated by the affinity-chromatography experiments (Buonocore *et al.*, 1975), maltose decreases the affinity of the amylase for both inhibitors 0.28 and 0.19. This effect might indicate that the binding of the two protein inhibitors and of maltose takes place at related sites of the amylase molecule, although the possibility that maltose acts by stabilizing an amylase conformation less favourable to the binding of the inhibitors cannot be ruled out. As each inhibitor-0.28 molecule and inhibitor-0.19 subunit contain one residue of reducing sugar (Petrucci *et al.*, 1978), it can be speculated that amylase–inhibitor-complex formation involves the binding of the inhibitor sugar residue at the same amylase sites where maltose binds. Such a hypothesis is consistent with the higher affinity for the amylase of both inhibitors 0.28 and 0.19, as compared with maltose (Buonocore *et al.*, 1976*a*). In fact, assuming that the formation of both amylase–maltose and amylase–inhibitor complexes involves the binding of similar carbohydrate residues to the enzyme, it is obvious that only the amylase–protein-inhibitor complexes are stabilized by secondary protein–protein bonding forces. The peculiar importance of ionic bonds for the stabilization of the amylase–inhibitor complexes is clearly shown by the fact that no amylase inhibition was detectable at pH values where both the enzyme and the inhibitors carry the same net electric charge.

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