BIOCHEMICAL AND NUTRITIONAL SIGNIFICANCE OF WHEAT ALBUMIN INHIBITORS OF α-AMYLASE[1]

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ABSTRACT

Molecular properties of α-amylase albumin inhibitors from the kernel of polyploid wheats and phylogenetically related *Triticinae* species as well as those of inhibited α-amylases are described. Main biochemical features of amylase-albumin inhibitor systems that offer a unique model of specific and reversible protein-protein interaction are also outlined. Moreover, nutritional significance under some circumstances of these naturally occurring inhibitors is discussed in relation to their active presence in foods. The possibility of using albumin inhibitors of α-amylase as therapeutic agents in persons with diabetes, obesity, and related diseases is critically evaluated.

MOLECULAR PROPERTIES

Figure 1 summarizes the main structural information available on albumin amylase inhibitors from wheat as well as the known and inferred relationships among different inhibitor groups (1). The kernel of polyploid wheats appears to contain a number of protein components capable of inhibiting many α-amylases of different origins. The biologic role of such a large number of closely related protein molecules in the kernel has been discussed recently (1). These protein inhibitors are albumin in nature and belong to three main isoinhibitor families with molecular weights of 60,000, 24,000, and 12,000 (1–11). The albumin isoinhibitor families with molecular weights of 24,000 and 12,000 also have been coded as 0.19 and 0.28 families, respectively (from the gel electrophoretic mobilities of the main components).

An interesting point is the relationship among the three albumin inhibitor families (Fig. 1). The 0.19 albumin as well as all components of the albumin family with molecular weights of 24,000 dissociate in the presence of guanidine hydrochloride or sodium dodecyl sulfate into two subunits with molecular weights close to 12,000 (11). A preliminary investigation of the 60,000-mol wt albumin fraction has shown that up to 80% of its protein components undergo reversible dissociation into subunits with molecular weights of 12,000 (11). Figure 1 also shows that when inhibitor dissociation takes place in the presence of reducing agents, the inhibitory activity is not restored on removal of the dissociating and reducing agents, even though molecular weight is restored.

The relationship between the 24,000 and 12,000 inhibitor families has been studied further through direct comparison of the purified 0.19 and 0.28 inhibitors. The inhibition patterns of 0.19 and 0.28 toward amylases of different origins prompted some authors (2,6) to suggest that 0.28 actually might be contained in 0.19. Although different in molecular weights, 0.19 and 0.28 appear closely related. They both produce markedly similar denaturation thermograms,

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with identical denaturation temperature at 93°C (12), and have about 50% of ordered structure (13). At alkaline pH values or in the presence of dissociating agents, the two inhibitors undergo significant structural changes associated with only minor losses of inhibitory activity. Both 0.19 and 0.28, however, are readily inactivated by treatments that break disulfide bonds, thus indicating that their stability depends mainly on the integrity of their disulfide bonds (13). Actually, as many as five disulfide bonds are present per molecule of 0.28 and per each 0.19 subunit (13).

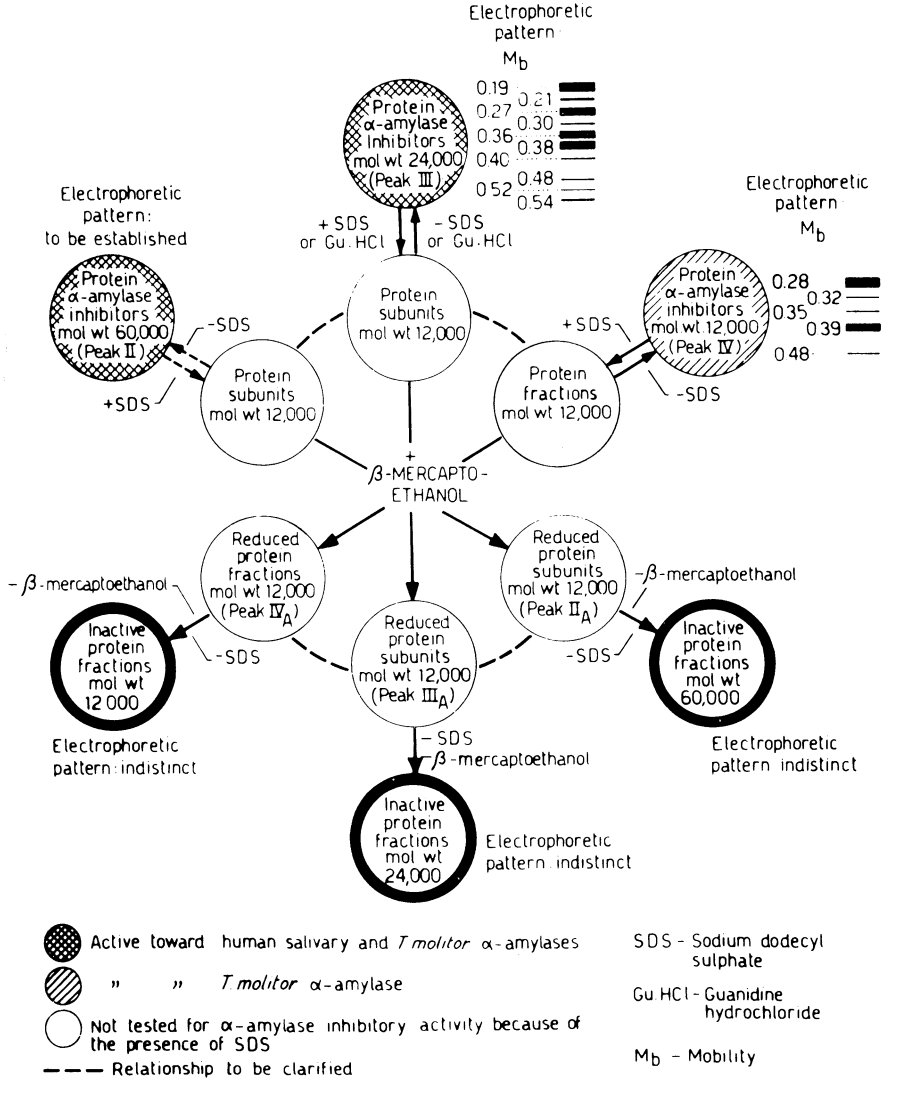

Fig. 1. Known and inferred relationships among α-amylase isoinhibitor families from kernel of hexaploid wheats. (Reproduced from Deponte et al [11]).

A significant homology has been observed between the aminoterminal sequences of 0.19 and 0.28, and 1 mol of covalently bound reducing sugars was found in 0.28 and each 0.19 subunit (13). Finally, circular dichroism in the far ultraviolet spectra of 0.19 and 0.28 at different pH values is identical in the presence or absence of dissociating agents, further confirming the structural equivalence of a 0.28 molecule with each 0.19 subunit (13).

Thus, the information available on highly purified α-amylase inhibitors from wheat kernels strongly supports the view that these inhibitors have originated from a common ancestral gene through replication and mutation (1). α-Amylase inhibitors are present not only in hexaploid wheats but also in genetically related *Triticinae* spp. Several amylase inhibitor fractions with different molecular weights were found in seven tested *Aegilops* spp. (14).

Apart from *Triticum urartu,* which contains only one inhibitor fraction (24,000 mol wt), no detectable amylase inhibitor activity was found in the extracts obtained from the diploid wheats tested. The presence of an inhibitor fraction in *T. urartu* was attributed (14) to some transfer of amylase inhibitor-coding genes from diploid *Aegilops* spp. to diploid *Triticum* spp. The correspondence of the molecular weights of the inhibitor fractions obtained from different *Aegilops* spp. suggests that these fractions all derive from common ancestral genes.

Further investigations are needed to show whether, in agreement with observations in *T. aestivum* (11), the inhibitor fractions in the *Aegilops* spp. with higher molecular weights also are composed of subunits with molecular weights of 12,000. If this is the case, the appearance or disappearance of an inhibitor peak might result from any mutation capable of affecting polymerization properties of the basic peptide units of the amylase inhibitors.

Therefore, in the simplest hypothesis, all of the inhibitor types found up to now in *Triticinae* spp. might derive from a limited number of ancestral genes coding for peptide units with molecular weights of 12,000, and the distribution of the inhibitor fractions in diploid *Aegilops* spp. might indicate a duplication of these ancestral genes followed by divergence of duplicated genes through mutation. Tetraploid *Triticum* spp. (*T. turgidum* and *T. timopheevi*) exhibited amylase inhibitor patterns of equal or higher complexity than did diploid *Aegilops* and *Triticum* spp., suggesting that different diploid *Aegilops* spp. could have contributed inhibitor-coding genes to tetraploid wheats. Vittozzi and Silano (14) suggested that such a mixed contribution might have occurred through repeated hybridizations among different *Triticum* and *Aegilops* amphiploids, in agreement with the hypothesis of the polyphyletic origin of polyploid wheats. Moreover, a significant homology has been found between the amylase inhibitor-coding genes of the wheat D genome and those of the *Aegilops* D genome, thus confirming that *A. squarrosa* is the donor of the D genome to *T. aestivum* (14).

INHIBITION SPECIFICITY

Silano et al (15) have tested the inhibitory activity of the albumin fractions with molecular weights of 60,000, 24,000, and 12,500 toward 58 animal amylases and a number of α-amylases from cereal species (*Triticum monococcum, T. durum, T. aestivum, Hordeum vulgare, Zea mays, Secale cereale, Oryza sativa,*

and *Panicum miliaceum*) or from immature and germinated wheat kernels. On the basis of the effectiveness with which the three albumin fractions inhibit their activities, the amylase preparations tested were divided into susceptible, partially susceptible, and resistant amylases. Susceptible amylases, which are inhibited by any of the three albumin fractions, are found mainly in insects that attack wheat and in marine species. Partially susceptible amylases, which are inhibited by only one or two of the three albumin fractions, are present in a few avian and mammalian species, including man. Resistant amylases are largely distributed in cereal, avian, and mammalian species, as well as in insect species that usually do not attack wheat grain or wheat flour products. At no stage of development was wheat α-amylase inhibited (16).

Applebaum (17) showed that the addition of a crude protein extract from wheat bran to a synthetic diet adversely affected development and greatly increased mortality of *Tenebrio molitor* larvae. He attributed such effects to the in vivo inhibition of the insect amylase by wheat protein inhibitors. Pace et al[2] has confirmed these findings recently, showing that limited amounts of highly purified α-amylase inhibitors in the diet largely slow down larval development. The results of Silano et al (15) also support the hypothesis that albumin inhibitors occurring naturally in wheat might provide a measure of insect resistance. They found that most of the insects that attack wheat grain and flour seem to have high amylase activities and amylases inhibited by wheat albumins, whereas insect species that normally do not feed on wheat have relatively low amylase activities and amylases resistant to inhibition by wheat albumins.

PURIFIED SUSCEPTIBLE α-AMYLASES

To study amylase-inhibitor interaction, the amylases from *T. molitor* L. larvae (the so-called yellow mealworm), chicken pancreas, and octopus digestive gland were purified by an affinity method in which crude extracts are filtered through a Sepharose-wheat inhibitor column and the retained enzymes eluted with maltose (18). These amylases are Ca^{2+}-glycoproteins, which behave as typical α-amylases (19,20). The enzymes are irreversibly inactivated by the removal of Ca^{2+} ions and activated by the presence in the assay mixture of Cl^- ions. Properties of these purified susceptible amylases are shown in Table I.

[2]Unpublished data, W. Pace, A. Rab, and V. Silano.

TABLE I
Properties of α-Amylases Purified by Affinity Chromatography on Wheat Inhibitor Columns[a]

Source of Amylase	Mol Wt	pI	Number of Isoenzymes	Optimal pH for Activity	Optimal Temperature for Activity (°C)	Activated by Cl^-
Tenebrio molitor L. larvae	68,000	4.0	1	5.8	37	yes
Chicken pancreas	53,000	5.0	2	7.5	37	yes
Octopus digestive gland	45,000	...	2	7.2	30	yes

[a]After Buonocore et al (19,20).

AMYLASE-INHIBITOR INTERACTION

When tested at the pH optimal for activity, purified yellow mealworm amylase is inhibited by both 0.19 and 0.28 inhibitors (21,22), whereas the amylases from chicken pancreas and octopus digestive gland are inhibited only by 0.19. At pH 5.5, however, 0.28 is almost as effective as 0.19 in inhibiting amylase from chicken pancreas (23). At pH 6.2, which corresponds to the isoelectric point of the inhibitor, maximal inhibition of yellow mealworm amylase by 0.28 has been obtained after a short preincubation of the enzyme with the inhibitor before addition of starch (Table II). Longer preincubation times are needed to obtain maximal inhibition by 0.19 of amylases from yellow mealworm, chicken pancreas, and octopus digestive gland (Table II) (21–23). These effects indicate that the enzyme inhibition reaction is much slower than is starch hydrolysis. The optimal pH for the inhibition of amylases from yellow mealworm, chicken pancreas, and octopus digestive gland by 0.19 is intermediate between the isoelectric points of enzyme and inhibitor. Almost no inhibition takes place at pH values at which both enzyme and inhibitor carry the same net charge.

Kinetic results obtained applying the kinetic treatment of Bieth for slowly reacting reversible enzyme-inhibitor systems to 0.19-yellow mealworm and 0.19-chicken pancreas amylase systems are consistent with a 1:1 molar stoichiometric combination ratio. The dissociation constants (K_i) of these two complexes are 0.9 nM and 5.0 nM, respectively (Table II) (22,23). The K_i of the 0.28-yellow mealworm amylase system is much lower, and kinetic data indicate that 2 mol of 0.28 bind 1 mol of the insect amylase (Table II). Maltose (an α-amylase end product and inhibitor) partly prevents amylase inhibition by 0.19 and, even though at a lower degree, by 0.28 (21–23). For some amylases, such an effect has been observed at maltose concentrations that inhibit the enzyme only slightly.

AMYLASE-INHIBITOR COMPLEXES

As shown in Fig. 2, on incubation of approximately equimolar mixtures of enzyme and inhibitor, 0.19 forms two stable complexes with amylases from

TABLE II
Main Biochemical Features of Three Amylase-Albumin Inhibitor Systems[a]

α-Amylase-Albumin Inhibitor System	Optimal pH for Inhibition	Optimal Temperature for Inhibition (°C)	Enzyme-Inhibitor Preincubation Time for Maximal Inhibition (min)	Dissociation Constant K_i (nM)
Tenebrio molitor L. amylase (0.19) inhibitor	5.3	37	10	0.9
T. molitor L. amylase (0.28) inhibitor	6.2	37	5	0.1
Chicken pancreas amylase (0.19) inhibitor	6.0	30	30	5.0

[a]After Buonocore et al (22,23) and Silano et al (24).

yellow mealworm and chicken pancreas, both containing 1 mol of enzyme per mole of inhibitor (22,23). Under the same conditions, the 0.28 inhibitor with the insect amylase gives only one complex with a molar stoichiometric ratio of 1 mol of enzyme/2 mol of inhibitor (22). The difference between the two 1:1 0.19-amylase complexes is not clearly understood; they might be conformational isomers exhibiting a slightly different binding affinity for the Bio-Gel® matrix on which they are separated. The formation of a ternary complex 0.19-yellow mealworm–0.28 amylase has been ruled out by means of gel filtration studies on mixtures containing the two inhibitors and the enzyme. Moreover, both 0.19 and 0.28 are able to displace each other when added to a preformed enzyme-inhibitor complex. The 0.28- and 0.19-yellow mealworm amylase combination ratios calculated from gel filtration studies are not only in agreement with those obtained from kinetic studies but also with those obtained from differential scanning calorimetry (12).

THE MODEL

The available data have prompted Silano et al (24) to suggest the scheme of Fig. 2, which summarizes the information available on the interaction between

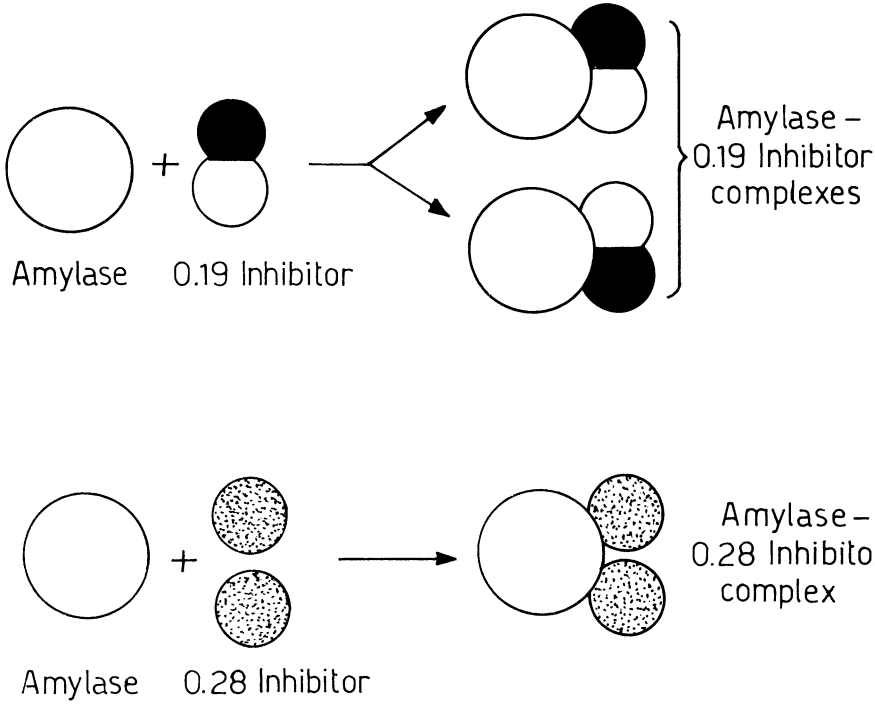

Fig. 2. Hypothetical scheme summarizing information available on interaction between yellow mealworm α-amylase and monomeric (0.28) and dimeric (0.19) protein inhibitors from wheat kernel. (Reproduced from Silano et al [24]).

yellow mealworm α-amylase and monomeric (0.28) or dimeric (0.19) albumin inhibitors from wheat kernel. The scheme points out that the enzyme molecule has two binding sites for the inhibitors (one for each protomer of 12,000 mol wt), and that each 0.19 subunit and 0.28 molecule have one binding site for the amylase.

As the two 0.19 subunits are not identical and both can interact with each amylase binding site, the formation of two nonidentical amylase-0.19 complexes, which contain 1 mol of enzyme per mole of inhibitor, is expected. Two molecules of 0.28 should have more freedom in adjusting themselves to complementary parts of the amylase molecule than should two subunits linked in a 0.19 molecule.

TABLE III
Weight Gain and Starch Availability in Rats Fed a Ration Containing Starch With Inclusion of α-Amylase Inhibitors[a]

Diet	Number of Animals	Average Daily Weight Gain (g/rat/day)	Starch Availability (%)
Basal	6	0.27	...
Basal + starch	6	1.52	100
Basal + 2% inhibitors	5	1.00	58
Basal + 4% inhibitors	6	1.19	74
Basal + 8% inhibitors	6	0.85	46
Basal + 4% inactivated inhibitors	6	1.41	91
Basal + 8% inactivated inhibitors	5	1.49	99

[a]Data by Saunders (27).

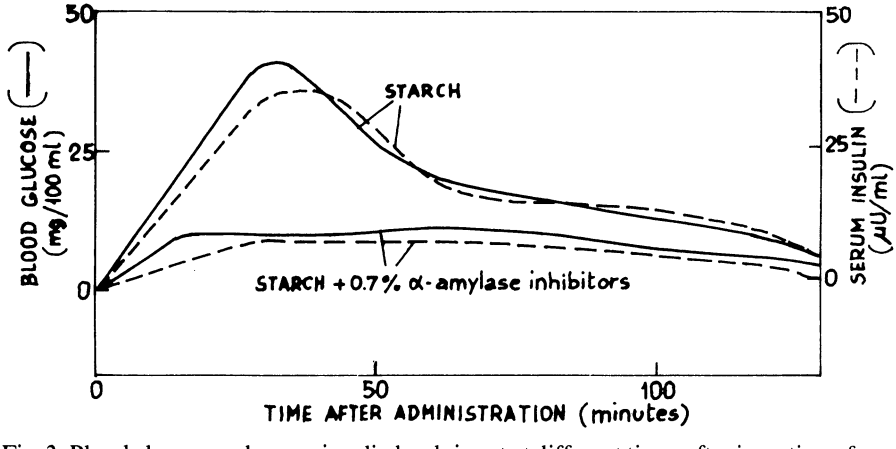

Fig. 3. Blood glucose and serum insulin levels in rat at different times after ingestion of raw starch, with and without inclusion of α-amylase inhibitors. (Modified from Puls and Keup [26]).

This model explains the ability of 0.28, as compared with 0.19, to form a more stable complex with the enzyme without implying significant structural differences between the binding sites of each 0.19 subunit and 0.28 molecule, and is in line with the close structural similarity of 0.28 and each 0.19 subunit. The effect of pH on inhibitory activity points out the importance of protein charge for the interaction to take place.

For all the enzyme-inhibitor systems studied, maximal inhibition has been observed either at the inhibitor isoelectric point or when an opposite electric charge is present on the molecules of enzyme and inhibitor. On the other hand, the ability of maltose to reduce the affinity of the amylases for the two inhibitors suggests that the reducing sugar covalently bound to the inhibitor polypeptide chain plays a main role in the inhibition mechanism.

As studies by Elodi et al (25) indicate, binding of maltose to porcine pancreatic amylase takes place in the active site, producing rather limited structural changes. The sugar part of the inhibitor molecules therefore may bind some part of the active site of the enzyme being stabilized in such a position by strong electrostatic secondary bonding forces between enzyme and inhibitor polypeptide chains.

NUTRITIONAL SIGNIFICANCE

In spite of their sensitivity to pepsin, protein amylase inhibitors from wheat, when supplied in large amounts, can overcome gastric digestion and inhibit α-amylase in man, other mammals, and birds (26–28) (Table III). Hyperglycemia and hyperinsulinemia resulting from raw starch loading in healthy, obese, or diabetic human volunteers are reduced dose dependently by addition of α-amylase inhibitors to the starch load (Fig. 3). When cooked starch is substituted for raw starch, however, α-amylase inhibitors are less effective in slowing down starch digestion.

These findings led Puls and Keup (26) to conclude that inhibitors that are more active than those presently known could be more advantageously used as therapeutic agents to reduce postprandial hyperglycemia and hyperinsulinemia in patients with diabetes mellitus, obesity, hyperlipoproteinemia, and related diseases. In this respect, the results that Macri et al (28) described with chickens are also of interest. They showed that inhibitor preparations resistant to gastric digestion can be used successfully to magnify amylase inhibition in vivo. Adverse effects, however, including pancreatic hypertrophy and a number of histologic changes in the pancreas indicating active degenerative phenomena, have been reported in chickens continuously fed amylase inhibitors from one day after hatching (28).

Protein amylase inhibitors may represent up to 1% of wheat flour. Because of their thermostability, they have been found in bread and in a number of wheat-based human breakfast foods (29–31) and likely are present in other wheat foods such as pasta products and cakes. Therefore, in assessing the nutritional value of the diet, the possibility that amylase inhibition has to be considered in special circumstances should be borne in mind. This may be particularly important for patients with impaired proteolysis and for infants. Moreover, new cereal cultivars obtained through selection or mutation breeding should be controlled with respect to their content of α-amylase inhibitory activity.

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