Phytochemistry, 1977, Vol. 16, pp. 811–820. Pergamon Press. Printed in England

REVIEW

WHEAT PROTEIN INHIBITORS OF α-AMYLASE

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(*Received 20 September 1976*)

Key Word Index—*Triticum*; *Aegilops*; Gramineae; wheat; protein inhibitors; albumins; gliadins; α-amylase; nutrition; coeliac disease; wheat breeding; wheat phylogenesis; insect resistance.

Abstract—The location in the seed, molecular properties and biological role of protein α-amylase inhibitors from wheat are discussed. Inhibition specificity of albumin inhibitors and structural features essential for interaction with inhibited amylases are also examined. The possible significance of these naturally occurring inhibitors in relation to their presence in foods in active form is described. Finally, genetic aspects of the albumin inhibitor production and the possibility of improving nutritional value and insect resistance of wheat by increasing albumin inhibitor content through breeding are outlined.

INTRODUCTION

Alpha-amylase inhibitors naturally occurring in plants or produced by microorganisms can be either low MW compounds or macromolecules. Low MW inhibitors include salicylic acid [1], abscisic acid [2, 3], the antibiotic nojirimycin (5-amino-5-deoxy-D-glucopyranose) [4], a peptide-like compound [5] and an oligosaccharide with MW 1500 produced by certain strains of *Streptomyces* [6]. A number of oligo- and polysaccharidic derivatives with MW in the range 500–8000 very active in inhibiting α-amylases are produced by several *Ampullariella* and *Actinoplanes* species [7]. Among the high MW inhibitors there are enzyme inactivators [8–11] as well as highly specific protein inhibitors. Alpha-amylase inhibitors of a protein nature are present not only in wheat, but also in other genetically related (*Secale cereale* [12] and *Avena sativa* [13]) and unrelated (*Phaseolus vulgaris* [14], *Colocasia esculenta* [15] and *Mangifera indica* [16]) species. Two short reviews on α-amylase inhibitors from plants have been published in 1975 [17, 18].

In this review on protein α-amylase inhibitors from wheat, which have received more attention than any other amylase inhibitor, we try to elucidate the general significance of these naturally occurring inhibitors and the implications of their presence in foods.

Detection and assay

As will be discussed later, the inhibition of α-amylase by protein inhibitors from wheat is a time-dependent reaction. Therefore, it is necessary to preincubate α-amylase and inhibitor, before addition of starch, to observe maximal inhibition. Amylase activity remaining after preincubation has been determined by a variety of methods based on the increase of reducing sugars [19, 20] or decrease in iodine staining power [21]. A semiquantitative screening method on starch–agar gel for detecting α-amylase inhibitors in biological materials has been described by Fossum and Whitaker [22]. Finally, the Bernfeld's dinitrosalicylate method has been adapted for the automated continuous assay of protein α-amylase inhibitors in column effluent by Vittozzi *et al.* [23].

The experimental conditions for the inhibitor assay (such as temperature, pH, ionic strength) have been somewhat arbitrarily chosen, although optimal conditions for enzyme activity have been preferred when they were known. In many instances inhibition activity has been expressed in units based on the amount of inhibitor which inhibits a certain percentage (usually 50% or less) of a fixed amount of α-amylase.

Distribution within the plant

Up to now protein α-amylase inhibitors have been found only in the kernel. Since 1946 Kneen and Sandstedt [24], who first described an α-amylase inhibitor from wheat [25], reported that most of the inhibitor is located in the endosperm. This observation has been confirmed by microscopic studies [26] and by the assay of inhibitor contents in different milling fractions [18]. The linear relationship observed between inhibitor and starch contents of different wheat milling fractions indicates that the inhibitors are closely associated with starch and are probably endospermic in nature. The production of wheat inhibitors with kernel development was followed qualitatively by Sandstedt and Beckord [26] and quantitatively by Pace *et al.* [27]. The inhibitor production starts about 8 days after fertilization and rapidly increases with maturation up to a maximum reached at the full maturity. The inhibitor content rapidly decreases after

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Table 1. Specificities of protein inhibitors from wheat towards α-amylases from different sources

Source of α-amylase	Inhibitor of Kneen *et al.* [24]	AmI_1 [30]	AmI_2 [30]	Inhibitor I [31]	Inhibitor II [31]	0.19 [33]	0.28 [33]
Mammalian (human saliva)	+	–	+	+	nd	+	–
Avian (chicken pancreas)	nd	nd	nd	+	+	+	–
Insect (yellow mealworm)	+	+	+	nd	nd	+	+
Plant (wheat kernel)	–	–	–	nd	nd	–	–
Fungal (*Aspergillus oryzae*)	–	–	–	nd	nd	–	–
Bacterial (*Bacillus subtilis*)	–	–	–	nd	nd	–	–

+ indicates inhibition; – indicates no inhibition; nd not determined.

germination; no detectable amount of inhibitors was found in roots and coleoptiles from germinated seeds [27].

Albumin inhibitors

Early studies on the α-amylase inhibitor from wheat flour carried out by Kneen *et al.* [24, 25, 28, 29] established its protein nature. The inhibitor, extracted from flour with water or aqueous ethanol and precipitated by ammonium sulphate or ethanol, was readily inactivated upon treatment with pepsin and ficin. Moreover, it was non-dialyzable and highly sensitive to the action of a number of oxidizing and reducing agents. It is now well established that the inhibitor preparations used in this work contained a mixture of protein inhibitors. Recently, Shainkin and Birk [30] isolated two protein inhibitors (coded AmI_1 and AmI_2) from an aqueous extract of wheat kernel by ammonium sulphate fractionation and ion-exchange column chromatography. AmI_1 and AmI_2 were electrophoretically homogeneous and differed in their specificity towards α-amylases from different origins (Table 1), in MWs and amino acid compositions (Table 2). By submitting a water extract from wheat flour to DEAE-Sephadex column chromatography, Saunders and Lang [31] isolated two amylase inhibitors (coded I and II) which showed identical MW and inhibitory activity, but different electrophoretic mobility. An inhibitor coded 0.19 from its gel electrophoretic mobility was isolated from a typical albumin preparation from wheat flour by means of DEAE-chromatography and preparative electrophoresis on polyacrylamide gel by Silano and co-workers [32–34]. An α-amylase inhibitor, very likely identical to 0.19, has been very recently purified from a water extract of whole wheat flour by means of alcohol fractionation, ion-exchange chromatography and gel filtration by O'Donnel and McGeeney [35].

Table 2. Amino acid compositions (mol%) of several amylase inhibitors purified from wheat (recalculated where appropriate)

Inhibitor / Reference	AmI_1 [30]	0.28 [33]	0.19 [32]	AmI_2 [30]	Inhibitor I [31]
Amino acid					
Lys	5.2	5.0	2.6	2.5	2.8
His	trace	trace	1.6	1.4	1.4
Arg	6.5	6.4	5.6	6.6	5.4
Asp	7.8	7.5	5.9	6.1	6.4
Thr	1.9	2.7	2.9	2.7	2.8
Ser	7.2	7.3	6.3	5.4	6.7
Glu	8.5	11.4	12.8	10.7	11.6
Pro	9.0	7.6	7.3	8.2	7.7
Gly	9.1	7.8	8.4	8.4	10.4
Ala	9.1	7.8	13.4	13.4	14.9
½Cys	5.7	8.2	6.1	6.9	3.3
Val	12.2	10.8	7.4	7.2	8.4
Met	2.7	2.6	2.0	2.3	1.7
Ile	2.2	1.6	2.4	2.3	2.5
Leu	8.0	8.2	8.3	8.2	8.4
Tyr	3.6	3.3	3.3	4.2	3.6
Phe	trace	trace	1.6	1.8	1.6

AmI_2, inhibitor I, and 0.19 showed similar inhibition patterns (Table 1), almost identical amino acid compositions (Table 2) and MWs all close to 25000. The very close similarities observed among these three inhibitors cannot be explained assuming that the same protein had been isolated independently in different Laboratories. Saunders and Lang [31] showed by means of direct comparison that inhibitor I, 0.19 and inhibitor II have all different electrophoretic mobilities. Moreover, the isoelectric point of AmI_2 was much higher than that of 0.19. These data, along with the findings concerning crude wheat inhibitor preparations [35–37], indicate the existence of several related forms of wheat proteins capable of inhibiting mammalian, insect and other α-amylases. This possibility was confirmed by Deponte *et al.* [38] who submitted to gel filtration on a Sephadex G-100 column the albumins extracted from wheat flour with 0.15 M NaCl and purified by salting out with ammonium sulphate between 0.4 and 1.8 M. Three albumin fractions with apparent MWs 60000, 24000 and 12000 (51, 40 and 9% of the loaded albumin, respectively) were obtained. When submitted to polyacrylamide gel electrophoresis at pH 8.5, the albumin fraction with MW 24000 was highly heterogeneous and ten anodic components (the main of which was the 0.19 inhibitor) were clearly evident. All these components were active in inhibiting human salivary and *Tenebrio molitor* L. α-amylases. Moreover, they showed very similar amino acid compositions and circular dichroism spectra in the far and near UV [38]. As it had already been shown for the 0.19 inhibitor [33], all the components of the albumin fraction with MW 24000 reversibly dissociated in the presence of guanidine hydrochloride or sodium dodecyl sulphate into two subunits with MW very close to 13000 [38]. Deponte *et al.* [38] concluded that most of the components of the albumin fraction with MW 24000 constitute a family of closely related isoinhibitors (coded 0.19 family from the gel electrophoretic mobility of the main component). It appears, therefore, that the AmI_2 of Shainkin and Birk

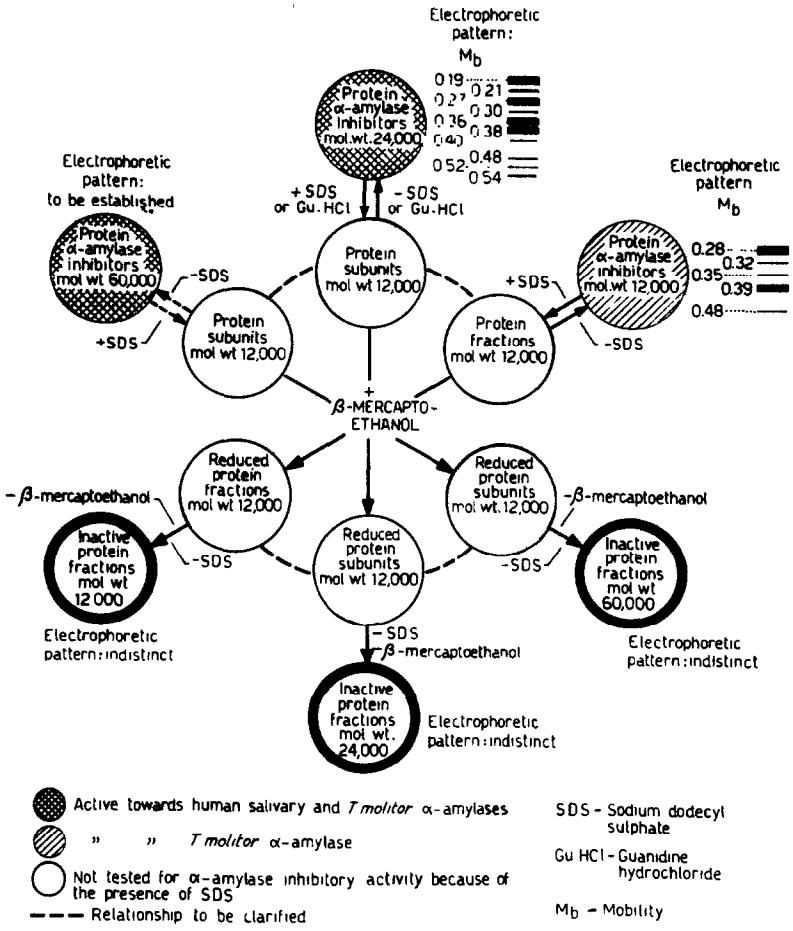

Fig. 1. Known and inferred relationships among the α-amylase isoinhibitor families from kernel of hexaploid wheats. (After Deponte *et al.* [38]).

[30] as well as the inhibitors I and II of Saunders and Lang [31] could very likely be components of the 0.19 albumin family. Actually this could also be the case of the wheat water-soluble protein of Fish and Abbot [39] that, although not tested for amylase inhibitory activity, showed physico-chemical properties similar to those of 0.19. The possibility that the components of the 0.19 albumin family are simply deamidated forms of the 0.19 albumin (the slowest moving component at alkaline pH) has been tested [38] by submitting 0.19 to deamidation in a pH 9.9 Pi buffer. A number of extra bands with greater mobilities were observed by gel electrophoresis in a pH 8.5 buffer system, but no new bands were evident at pH 4.3. Since the components of the 0.19 family had different mobilities at pH 4.3 as well as at pH 8.5, it was concluded that the proteins must differ in ways other than substitution of glutamic or aspartic acid for glutamine or asparagine in the primary structure.

The 0.19 family is not the unique isoinhibitor family in wheat flour albumin. Since 1973 Silano and co-workers [33, 40] showed that the albumin fraction with MW 12000 consists of 5 components which have been individually isolated and characterized. It was suggested [33] that these albumins (coded 0.28 family from the gel electrophoretic mobility of the main component) constitute a family of closely related proteins. This hypothesis has been recently confirmed by Redman [41, 42] who has shown the close homology existing among primary structures of some components of the 0.28 family. As shown in Table 1 and 2, the 0.28 inhibitor is clearly different from 0.19, but very similar to AmI_1 of Shainkin and Birk [30]. It appears that if AmI_1 is not identical to 0.28, they certainly belong to the same family. Evidence showing that the components of this family are not deamidated forms of 0.28 have been reported as well [33].

Although a number of amylase inhibitors have already been isolated from wheat flour, still more inhibitors are to be characterized. In fact, several amylase inhibitor components have been described in the albumin fraction with MW 60000 [36]; a preliminary investigation of this fraction has shown that as much as 80% of its protein components undergoes reversible dissociation into subunits with MW 12000 [38]. Further investigations, possibly using purified inhibitors from this albumin pool, are needed to clarify whether the α-amylase inhibitory activity belongs to the dissociable protein components.

The scheme shown in Fig. 1 summarizes diagrammatically all the information available on albumin amylase inhibitors from wheat as well as the known and inferred relationships among the different inhibitor groups.

Gliadin inhibitors

The only α-amylase inhibitor of a gliadin nature from wheat flour has been described by Strumeyer and Fisher [42] who extracted from commercial wheat gliadin a protein inhibitor active towards several amylases including pig pancreatic α-amylase. Molecular-sieve chromatography gave a MW of 55000 for the inhibitor. Disc-electrophoresis of the purified inhibitor showed two major bands corresponding in electrophoretic mobility to the α-gliadins. Amino acid analysis of the inhibitor showed an extremely high content of glutamine (30%) and proline, but a very low lysine (1%). However, Pace *et al.* [27] were not able to detect any inhibitory activity towards human salivary, *T. molitor* and pig pancreatic α-amylases in a number of purified gliadin fractions including several α-, β- and γ-gliadins, prepared according to the method described by Huebner and Rothfus [44], and the A-gliadin, prepared with the procedure of Kasarda *et al.* [45]. The gliadin fractions were inactive even when tested at concentrations several hundred-fold higher than those usually employed for albumin inhibitors.

In a previous work Strumeyer [12] extracted from wheat flour with 70% ethanol an inhibitor active towards human salivary α-amylase at concentrations one hundred-fold lower than those needed to inhibit pig pancreatic α-amylase. The inhibition behaviour of this preparation is identical to that of the inhibitor of Kneen *et al.* [24], AmI_1, AmI_2 [30], the 0.19 and 0.28 families [46] and the inhibitor of O'Donnel and McGeeney [35].

It appears that further investigations are needed to establish whether amylase inhibitors of a gliadin nature are present in wheat flour. It should be pointed out that the possibility of extracting amylase inhibitors from commercial preparations of wheat gliadin or, even, of wheat gluten is not a proof of their gliadin nature. These preparations, in fact, may even contain 10% or more of wheat albumins. Similarly, it cannot be considered a proof of gliadin nature that amylase inhibitors can be extracted from wheat flour or other wheat products with solvents commonly considered typical for wheat gliadins such as 70% ethanol, dilute acidic solutions and others. These solvents, in fact, extract wheat albumins as well [27]. Moreover, since albumin amylase inhibitors display their action at concentrations as low as 0.01–0.3 μg/ml, the possible presence of active albumin impurities, hardly detectable with the usual homogeneity tests, should be taken into account when isolating gliadin amylase inhibitors.

Evidence indicating differing responses of human α-amylases to inhibition by wheat gliadin has been recently reported by Gertzman [47].

Inhibition specificity

The inhibition specificity of several inhibitors towards six α-amylases is shown in Table 1. The inhibitor of Kneen *et al.* [24] also inhibited several amylases from *Streptomyces* [48] and some insect amylases including those from *Prodenia litura* F. [49] and *Tribolium castaneum* Herbst [50]. This inhibitor was not active towards barley and sorghum α-amylases and barley β-amylase [25]. AmI_1 and AmI_2 also inhibited *Prodenia litura* amylase [30]. Quantitative inhibitory activity of 0.19 and 0.28 towards a number of α-amylases from different origins is reported in Table 3.

The inhibitory activity of the albumin fractions with MWs 60000, 24000 and 12000 towards 58 animal amylases and a number of α-amylases from cereal species (*Triticum monococcum*, *Triticum durum*, *Triticum aestivum*, *Hordeum vulgare*, *Zea mais*, *Secale cereale*, *Oryza sativa* and *Panicum miliaceum*) or from immature and germinated wheat kernels was also tested [46]. On the basis of the effectiveness with which the 3 albumin fractions inhibit their activities, the amylase preparations tested were divided into susceptible, par-

Table 3. Inhibition of amylases from mammalian, avian, insect and marine species by 0.19 and 0.28 wheat albumins

Species		Amount of inhibitor (ng) that gives 30% inhibition of 1 amylase unit*	
Scientific name	Trivial name	0.19	0.28
Homo sapiens	man (saliva)	387	No inhibition
Canis familiaris L.	dog	495	Not tested
Felis domesticus	cat	744	Not tested
Cavia cobaya	guinea pig	No inhibition	No inhibition
Gallus gallus L.	chicken	433	No inhibition
Coturnix c. clturnix L.	quail	566	Not tested
Meleagris g. gallopavo L.	turkey	No inhibition	Not tested
Passer Italiae Vieill.	sparrow	No inhibition	No inhibition
Calandra oryzae L.	rice weevil	86	38
Tenebrio molitor L.	yellow mealworm	212	74
Tribolium confusum Duv.	confused flour beetle	36	38
Blattella germanica L.	german cockroach	386	496
Periplaneta americana L.	american cockroach	368	68
Oryzaephilus surinamensis L.	sawtoothed grain beetle	14	30
Sepia officinalis L.	a variety of cuttlefish	139	88
Cardium tuberculatum L.	red nosed cockle	52	120
Cytherea chione L.	cock	42	162
Natica hebraea Martyn	snail	89	Not tested
Murex trunculus L.	murex	101	Not tested
Maja verrusoca M. Edwards	a variety of crab	72	Not tested
Octopus vulgaris Lam.	a variety of octopus	50	No inhibition

* One Amylase Unit is the amount of enzyme that gives 50% hydrolysis of the added starch under described experimental conditions [46]. The 0.19 and 0.28 inhibitors were tested at maximum concentration of 1 μg/ml in the incubation mixture.

tially susceptible and resistant. Susceptible amylases, inhibited by any of the 3 albumin fractions, were found mainly in insects that attack wheat and in marine species. Partially susceptible amylases, inhibited by only one or two of the 3 albumin fractions, were present in a few avian and mammalian species including man. Resistant amylases were largely distributed in cereal, avian, and mammalian species as well as in insect species that do not usually attack wheat grain or wheat flour products. At no stage of development was wheat α-amylase inhibited. The albumin fraction with MW 12000 was the most effective in inhibiting insect amylases, but it was inactive towards avian and mammalian amylases. The albumin fraction with MW 24000 was the most effective in inhibiting amylases from marine, avian, and mammalian species.

Although valid interspecies comparisons of amylases are difficult because amylase activity and action pattern are easily affected by experimental condition, it appears that only certain amylases have groupings which can combine with the amylase protein inhibitors from the wheat kernel. This suggests that the inhibitors do not act by competing with starch for the active site of the enzyme; in this case we would expect all the amylases to be affected.

Features essential for activity

Although the effect of a number of chemical and physical treatments on albumin inhibitors has been studied, little is known on the inhibitor structural features essential for their activity. Inactivation of the inhibitor of Kneen *et al.* [24] by a number of oxidizing and reducing agents has been reported [28]. Both AmI_1 and AmI_2 were inactivated [30] by digestion with pepsin and pronase, but were not affected by carboxypeptidase A and B or by 0.02 M HCl. AmI_1 lost its activity when submitted to digestion with trypsin and chymotrypsin, whereas AmI_2 was poorly affected by these enzymes. Both inhibitors were inactivated upon reduction with mercaptoethanol, and did not lose activity after treatment in 6.4 M urea. Esterification with methanol–HCl or carboxymethylation at different pH values caused a stronger impairment of the inhibitory activity of AmI_2 towards human salivary amylase than towards *T. molitor* amylase. Cyanogen bromide treatment, at controlled concentrations, removed all the activity of AmI_2 towards the salivary amylase, but had little effect on the activity of AmI_2 or AmI_1 towards *T. molitor* amylase. The inhibition by 0.19 of the mammalian amylase, but not that of the insect amylase, was adversely affected by treatment with CNBr (1:100 ratio of methionine residues to CNBr), thus confirming the results obtained with AmI_2 by Shainkin and Birk [30]. While these authors suggested that the polypeptide chain of AmI_2 consisted of AmI_1 *plus* an additional peptide segment, Silano *et al.* [33] proposed that AmI_2, like 0.19, contained two subunits, one of which was AmI_1 or another member of the 0.28 family. This hypothesis implies that the components of the 0.19 family (which are all two subunits proteins), and possibly the albumin inhibitors with higher MWs, actually contain some of the components of the 0.28 family. Fingerprinting and sequencing studies of the subunits of the high MW albumin inhibitors are needed to verify this hypothesis. At the moment, it should be remembered that the amino acid compositions of the components of the 0.28 and 0.19 families, although not identical, are very similar.

As shown by circular dichroism measurements in the far UV, the 0.19 inhibitor is a protein with about 50% of ordered structure [34]. Significant and largely reversible changes have been observed in the aromatic CD spectrum of 0.19 at alkaline pH values or in the presence of sodium dodecyl sulphate. These changes, which were associated with only a partial loss of inhibitory activity, indicate that ionizable tyrosine groups contribute significantly to the ellipticity bands of 0.19 in the near UV. Incubation for 1 hr at room temperature of a 12 μM solution of 0.19 at alkaline pH values (up to 11.7) or at acidic pH values (down to 3.0) did not affect the inhibitory activity of 0.19 towards both human salivary and *T. molitor* α-amylases. The 0.19 inhibitor activity was resistant to trypsin and thermal treatments, but it was completely destroyed by incubation with pepsin or by reduction of disulphide bonds. Incubation of the 0.19 inhibitor in 1% sodium dodecyl sulphate and removal of the dissociating agent caused only a minor loss of activity.

Inhibition reaction

Kneen and Sandstedt [24, 25] first claimed the reversibility of the inhibition of human salivary α-amylase by wheat protein inhibitor. After addition of ethanol up to a 70% concentration to a mixture of amylase and inhibitor, the amylase was precipitated and the inhibitor remained in solution. When the precipitated enzyme was redissolved and tested again with the inhibitor, it appeared that the alcohol precipitation had not destroyed the sensitivity of the amylase to the inhibitor. Kinetic evidences for the reversibility of the inhibition of chick pancreas α-amylase by several albumin inhibitors (I, II, and 0.19) and of *T. molitor* amylase by 0.19 were reported by Saunders and Lang [31] and Buonocore *et al.* [51], respectively. Moreover, the activity of human salivary and chick pancreatic α-amylases inhibited by 0.19 was largely restored by addition of maltose. Further evidence for the reversibility of α-amylase inhibition by wheat albumins came from the affinity chromatography studies by Buonocore *et al.* [52]. These authors showed that a number of α-amylases from different origins (human saliva, chick pancreas, octopus digestive gland and *T. molitor* larvae) are effectively retained by a Sepharose-coupled inhibitor column and that such a binding is reversed by maltose or gelatinized starch. Incidentally, Buonocore *et al.* [52] described their method as suitable for purification of any amylase interacting with the albumin inhibitors from wheat. Similar results were also reported by Huper and Rauenbusch [53].

The influence of several parameters (time, order of addition of reactants, temperature, ionic strength) on the inhibition reaction has been studied. For the 0.19–*T. molitor* amylase system [51] maximal inhibition was obtained after 10 min preincubation at pH 5.0 and 37°, and for the 0.19–chicken pancreatic amylase system [31] after 30 min preincubation at pH 5.8 and 30°. Maximal activities of the inhibitor purified by O'Donnel and McGeeney with human salivary and human pancreatic α-amylases were observed after a 30 min preincubation at pH in the range 6.4–8.0 and 5.8–7.0, respectively [35]. Several authors [28, 30, 34, 35] have stressed the importance of the sequence of addition of reactants (inhibitor, enzyme, starch) for the extent of the inhibition. In some cases [30, 35] such an importance has been attributed to the tendency of the inhibitor to bind with polysaccharides. However, it is not entirely clear whether a significant binding of the inhibitor with starch actually occurs under the conditions of the inhibition assay; it

is possible that the effects observed are rather due to the fact that the inhibition reaction is much slower than starch hydrolysis.

The inhibition kinetics of a number of inhibitor–amylase systems have been studied with classical methods. In all the cases (salivary amylase–Kneen's inhibitor [29], chicken pancreatic amylase–0.19, or –inhibitor I, or –inhibitor II [31], and *T. molitor* amylase–0.19 [51]) a typical uncompetitive inhibition has been observed. These data, however, are poorly informative in terms of reaction mechanisms, because under the experimental conditions chosen the inhibitor concentration was not far in excess in comparison to the enzyme concentration, as it is generally assumed in the classical kinetic treatments of enzyme inhibition. Moreover, the concentration of wheat albumin inhibitors might also be significantly reduced during kinetic treatments in view of the reported affinity of these inhibitors for starch [30, 35].

The kinetic data available and the impossibility of obtaining 100% inhibition of the enzyme at any inhibitor concentration tested are consistent with an active enzyme–inhibitor complex. The formation of a *T. molitor*–0.19 complex was shown by polyacrylamide gel electrophoresis and the complex was isolated by filtering an enzyme–inhibitor mixture through a Sephadex G-100 column [51]. Preliminary characterization studies of the complex indicated a 1:1 molar stoichiometric combination ratio [51]. Among several highly purified α-amylases studied, *T. molitor* amylase was the only inhibited by both 0.19 and 0.28. The inhibitory activities of these two albumins were additive thus suggesting independent binding sites of the two albumins with the insect amylase. Further support to this standpoint came from the observation that maltose interferred less effectively with the binding of 0.28 to the amylase than with the binding of 0.19.

Nutritional significance

Applebaum [54] showed that the addition of a crude protein extract from wheat bran to a synthetic diet adversely affected development and greatly increased mortality of *T. molitor* larvae. This author attributed such effects to the *in vivo* inhibition of the insect amylase by wheat protein inhibitors. Since these effects were not observed when wheat β-amylase (which is not inhibited by the inhibitors) was present in the diet, it was concluded that wheat β-amylase actually acts in the larval intestine and substitutes for the insect intestinal amylase. It should be considered that wheat α-amylase inhibitors and wheat β-amylase might have been stabilized in the larval intestine by the inhibitors of trypsin and of insect protease which are well known in the wheat kernel [36, 55–60]. The hypothesis that albumin inhibitors naturally occurring in wheat might provide a measure of insect resistance is also supported by the results of Silano *et al.* [46] who found that most of the insects that attack wheat grain and flour seem to have very high amylase activities and amylases inhibited by wheat albumins, whereas insect species that do not normally feed on wheat had relatively low amylase activities and amylases resistant to inhibition by wheat albumins.

The effect of α-amylase inhibitors during rat feeding has been studied to some extent by Lang *et al.* [61] Rats were kept on a caloric-deficient starch-free diet. Addition of starch to this basal diet caused an increase in daily weight gain, and measurement of fecal carbohydrate content indicated 100% availability of the starch. Inclusion into the starch-enriched diet of wheat α-amylase inhibitors (2, 4 and 8% of the diet) caused a large decrease in starch availability. When the inhibitors were inactivated by autoclaving, starch availability was more similar to that of the control. When sucrose replaced starch in the diet, there was no change in fecal carbohydrate content or in daily weight gain in the presence of the inhibitors. Consequently it was concluded that the biological activity of the inhibitors was due to their ability to interfere with starch metabolism.

Puls and Keup [62] have studied the influence of an amylase inhibitor preparation from wheat on blood glucose and insulin levels in human volunteers, rats and dogs. They showed that hyperglycaemia and hyperinsulinaemia resulting from raw starch loading could be reduced dose-dependently by addition of the inhibitor to the starch load. As similar effects were observed in diabetic and obese patients, these authors suggested that amylase inhibitors from wheat could possibly be deliberately employed as therapeutic agents to reduce postprandial hyperglycaemia and hyperinsulinaemia in patients suffering from diabetes mellitus, obesity, hyperlipoproteinaemia and related diseases. However, when cooked starch was given instead of raw starch, the smoothing effect on hyperglycaemia was markedly reduced and much higher doses of the inhibitors were necessary to obtain comparable effects. The suggestion was made that this effect is due to the higher affinity of amylase for gelatinized starch as compared to raw starch and it was suggested that an inhibitor with greater affinity for α-amylase might be more advantageous for therapeutic use.

The effects induced in chickens by continuous intake of a starch-rich diet added with a purified albumin preparation from wheat flour were studied by Macri *et al.* [63]. Albumin amylase inhibitors were extracted from wheat flour, precipitated with ammonium sulphate, and enclosed into cellulose-coated microgranules resistant to the peptic action in the chicken gizzard. Continuous intake of gastro-resistant wheat albumins since 1 day of age, at 0.4% level of the diet, significantly depressed chicken growth rate, whereas native wheat albumins, at 0.8% level of the diet, did not show such an effect. After 4 weeks of treatment, treated chickens showed a growth rate identical to that of control chickens thus showing that an adaptation to the presence of wheat albumins in the diet had occurred. Treated chickens also showed pancreas hypertrophy and a number of histological changes at the pancreas level indicating that degenerative phenomena were in progress. Moreover, the production of pancreatic amylase of treated chickens was markedly increased. In relation to possible therapeutic uses of wheat albumin inhibitors, the changes observed at the pancreas level as well as the chicken adaptation to the continuous intake of wheat albumins cast doubt upon the desirability of using oral administration of amylase inhibitors to control postprandial hyperglycaemia in man. In any case these data show that gastro-resistant preparations can be successfully used to increase *in vivo* effectiveness of wheat amylase inhibitors.

All these data show that, in spite of their sensitivity to pepsin [24, 30], protein α-amylase inhibitors from wheat, when supplied in large amounts [61, 62], can overcome gastric digestion in man and other mammals. The percentage of the amylase inhibitors ingested which

Table 4. α-Amylase inhibitors from *Triticum* and *Aegilops* genera

MW of inhibitor fraction	Activity towards: Human salivary α-amylase	Activity towards: *T. molitor* α-amylase	*Triticum* and *Aegilops* species in which the inhibitor fraction has been found
12000	–	+	*T. aestivum, T. timopheevi, T. turgidum, Ae. longissima, Ae. bicornis, Ae. umbellulata, Ae. caudata, Ae. squarrosa.*
12000	+	+	*Ae. speltoides.*
24000	+	+	*T. aestivum, T. timopheevi, T. turgidum, T. urartu, Ae. longissima. Ae. bicornis, Ae. mutica, Ae. caudata, Ae. squarrosa.*
44000	+	+	*Ae. speltoides.*
60000	+	+	*T. aestivum, T. timopheevi, T. turgidum, Ae. longissima, Ae. umbellulata, Ae. caudata.*

is not destroyed by gastric digestion presumably depends upon the composition of the diet, the amount of food ingested and the physiology of the individuals. Therefore, it is not easy to establish whether the true caloric value of α-amylase inhibitor-containing foodstuffs will be lower than that expected on the basis of their starch content. Protein α-amylase inhibitors may represent as much as 1% of wheat flour [36, 38] and, because of their thermostability, they persist through bread baking being found in large amounts in the center of loaves [25, 64]. A number of wheat-based human breakfast cereals contain α-amylase inhibitory activity [17], and this might also be the case for other wheat foods such as paste products, cakes and others.

Therefore, in view of their possible action *in vivo*, when maximal nutritional value of the foodstuff is required, the presence of amylase inhibitors in foodstuffs is to be considered undesirable and special attention has to be required to ensure their destruction during food processing. In particular, the opportunity to prevent the presence of active amylase inhibitors in the diet of infants and patients affected by impaired proteolysis should be accurately considered.

Strumeyer [12] has recently suggested that amylase inhibitors may be responsible for the sensitivity to wheat flour in coeliac disease (gluten-induced enteropathy). Individuals with this disorder exhibit a generalized malabsorption including impaired ability to metabolize starch and absorb fats [65]. The nutrients are excreted and the patients suffer from vitamin deficiency and malnutrition. The role of α-gliadin as a toxic factor in the pathogenesis of coeliac disease was first suggested by Hekkens *et al.* [66, 67] and confirmed by Kendall *et al.* [68]. Strumeyer and Fisher [69] claimed that a gliadin fraction chemically and electrophoretically identical to the toxic factor isolated by Hekkens *et al.* [66, 67] is an effective pancreatic α-amylase inhibitor. These authors speculated [12, 42] that pancreatic amylase deficiency might be the primary lesion in coeliac disease and that the toxic role of α-gliadin might depend upon its activity towards α-amylase. The Strumeyer's hypothesis on the etiology of coeliac disease is not in agreement with the generally accepted view that malabsorption of the starch and the other nutrients is not the primary lesion in coeliac disease, but rather the consequence of the subtotal mucosal atrophy which is a typical symptom of this disorder [65]. Moreover, as already mentioned elsewhere in this paper, the amylase inhibitory activity of α-gliadin has been questioned [27] and preliminary experiments by Auricchio *et al.* [70] suggested that wheat albumins containing very active inhibitors of mammalian amylases cause no reaction in coeliac disease.

Phylogenesis

Protein inhibitors extracted with water from seeds of a number of *Triticum* and genetically related *Aegilops* species were characterized according to their apparent MWs, electrophoretic mobilities and specificities in inhibiting α-amylases from human saliva and *T. molitor* larvae [37, 71]. Several amylase inhibitor fractions with different MWs were found in the seven *Aegilops* species tested (Table 4). But *Triticum urartu* which contained only one inhibitor fraction (MW 24000) active towards both the mammalian and insect amylases, no detectable amylase inhibitory activity was found in the extracts obtained from the diploid wheats tested (Table 4). The presence of the inhibitor fraction with MW 24000 in *T. urartu* was attributed [71] to some transfer of amylase inhibitor-coding genes from diploid *Aegilops* species to diploid *Triticum* species. The correspondence of the MWs of the inhibitor fractions obtained from different diploid *Aegilops* species suggests that these fractions all derive from common ancestral genes. Further investigations are needed to show whether, in agreement to that observed in *T. aestivum* [38], also in the *Aegilops* species the inhibitor fractions with higher MW's are composed of subunits with MW 12000. If this is the case, the appearance or disappearance of an inhibitor peak might even be the result of any mutation capable of affecting polymerization properties of the basic peptide units of the amylase inhibitors. Therefore, in the most simple hypothesis, the five inhibitor types of Table 4 might derive from a very limited number of ancestral genes coding for peptide units with MW 12000 and the distribution of the inhibitor fractions in diploid *Aegilops* species might indicate a duplication of these ancestral genes followed by divergence of duplicated genes through mutation.

Tetraploid *Triticum* species (*turgidum* and *timopheevi*) exhibited amylase inhibitor patterns of equal or higher complexity than diploid *Aegilops* and *Triticum* species, suggesting that different *Aegilops* diploid species could have contributed inhibitor-coding genes to tetraploid

wheats. Vittozzi and Silano [71] suggested that such a mixed contribution might have occurred through repeated hybridizations among different *Triticum* x *Aegilops* amphiploids, in agreement with the hypothesis of the polyphiletic origin of polyploid wheats [72]. Both from qualitative and quantitative standpoints, amylase protein inhibitors from *Triticum aestivum* were the summation of those from *T. turgidum plus* the ones from *Aegilops squarrosa*. These results indicated a significant homology between the amylase inhibitor-coding genes of the wheat D genome and those of the *Aegilops* D genome and confirmed that *Ae. squarrosa* is the donor of the D genome to *T. aestivum* [37].

Coding genes

The location of genes controlling the synthesis of 7 wheat albumin fractions including 0.19 and, presumably, other components of the 0.19 family has been investigated by Cubadda [73]. Albumin fractions were extracted from the kernel of a number of nulli-tetrasomic lines of Chinese Spring with 0.13 M magnesium sulphate, precipitated with ammonium sulphate between 0.8 and 1.2 M, and analyzed by polyacrylamide gel electrophoresis [73]. Only one albumin fraction is controlled by chromosome 3A, two are coded by chromosome 3B and the remaining 4 fractions including 0.19 by chromosome 3D. Genes coding for 5 albumin components, extracted with 70% ethanol from flour and separated by combined electrofocusing and electrophoresis, were studied by Aragoncillo *et al.* [74]. Coding gene for one of these components (named component 5 and probably corresponding to 0.19) was located in chromosome arm 3Dβ and coding genes for the other 4 components were in 3BS. In conclusion, although Bozzini *et al.* [75] assigned 0.19 to chromosome 4D, it seems likely that albumins are mainly controlled by homologous chromosome groups 3, whereas genes coding for gliadins are located in homologous chromosome groups 1 and 6 [76]. These findings are in agreement with the presence of 0.19 in *Aegilops squarrosa* [37] and with the absence of this albumin inhibitor in *durum* wheats which have a tetraploid character and lack the D genome. Incidentally, because of such a property, 0.19 has been used as a marker for the presence of *bread* (hexaploid) wheat in *durum* (tetraploid) wheat food products [77, 78].

Role in the kernel

The heterogeneity pattern of albumin α-amylase inhibitors from wheat kernel is typical of storage proteins whose biological role essentially is that of being broken down to peptides and amino acids upon germination of the seed to provide a source of nitrogen for the proteins being synthesized by the developing embryo [79, 80]. Such a tendency towards multiplicity is usually considered the expression of a high tolerance for mutations deriving from a not crucial relationship between structure and function. It might also be due to the allopolyploid character of many wheats and perhaps to the triploid nature of the endosperm in angiosperms.

Albumin inhibitors are located in the endosperm and appear to be closely associated with starch [18, 24–27]. Accordingly to Barlow *et al.* [81] and Simmonds *et al.* [82] albumins strongly adhere to starch granules in the endosperm and make a significant contribution to endosperm hardness playing the role of cementing substances between starch granules and storage proteins (mainly gliadins) Although wheat albumins surrounding starch granules in the seed have not been tested for α-amylase inhibitory activity, they exhibited electrophoretic mobilities and differential staining with aniline blue black [83] identical to albumin amylase inhibitors. Moreover some purified inhibitors showed a tendency to associate with polysaccharides [30, 35]. The speculation can be made that, although inactive towards wheat α-amylase *in vitro*, albumin amylase inhibitors affect, *during maturation* of the kernel, starch digestion by preventing the access of amylase to starch granules. As the area surrounding starch granules, that is filled of albumins and other water-soluble substances, is capable of rapid swelling on hydration [81], it seems confirmed the hypothesis of Wall [84] that *during germination* wheat albumins might serve as hydrocols in promoting water absorption. Thus, upon germination, hydrated albumins get detached from starch granules which become available to α-amylase. In the meantime, hydrated albumins are broken down by proteases, behaving as an easily digestible protein pool useful for the earliest development of the embryonic plant.

Although not essential for plant survival, albumin amylase inhibitors, mostly acquired by polyploid wheats through hybridization of diploid *Triticum* and *Aegilops*

Table 5. Essential amino acid compositions (g/16g of N) of different food proteins

Amino acid	Egg	Milk	Meat	Wheat kernel				
				Total	Albumin	Globulin	Gliadin	Glutenin
Lys	7.2	8.7	10.1	2.6	4.3	7.7	0.7	2.5
His	2.4	2.6	3.4	2.1	2.7	2.6	2.1	1.9
Arg	6.5	4.2	6.9	4.3	6.4	9.2	3.0	4.4
Thr	5.3	4.7	5.1	2.9	3.9	4.4	2.6	3.3
$\frac{1}{2}$Cys	2.7	1.0	1.3	2.3	3.4	3.7	2.3	1.7
Val	8.8	7.0	7.0	4.0	5.9	5.4	3.5	3.2
Met	3.8	3.2	2.6	1.8	2.2	1.6	1.9	1.6
Ile	5.7	7.5	5.4	4.2	3.7	3.4	4.0	3.8
Leu	8.8	11.0	8.1	6.8	7.4	7.3	6.8	7.2
Tyr	2.9	—	—	3.3	4.0	3.6	3.2	4.0
Phe	5.7	4.4	4.4	5.0	4.0	3.6	5.7	4.6
Trp	1.3	1.5	1.1	1.3	1.6	–	0.9	1.8

species [71], might be considered as one of the advantages acquired by wheat through its evolution. Albumin inhibitors, in fact, might have given a measure of insect resistance to polyploid wheats and, therefore, contributed to their natural selection.

Wheat improvement through breeding

Although for a long time it has been believed that wheat endosperm albumins were mainly enzymes [85], it is now well established that the bulk of wheat albumins consists of a few amylase isoinhibitors families very likely phylogenetically related and coded by a small number of parental genes. Therefore, selection through breeding of wheat varieties with higher contents of albumins appears to be the most rational approach to nutritional improvement of wheat. For such a breeding strategy, it can be very helpful, as a screening test, to use the amylase inhibition assay that is rapid and easy to perform even on a half seed. The selection of wheats with higher albumin contents is very desirable as the nutritional inadequancy of wheat mainly depends upon the low content of lysine-rich proteins. In fact, storage proteins (gliadins and glutenins) which make up as much as 80% of total protein of the kernel are very poor in lysine, whereas albumins and globulins which have well balanced amino acid compositions similar to those of milk, meat and egg proteins (Table 5) only account for the remaining 20%. When evaluating lysine content of storage proteins, it should be taken into account that the level of lysine in whole gliadin shown in Table 5 is probably increased by the presence of some albumin and globulin contaminants [86]; similarly the lysine content of glutenins is probably increased by the presence of membrane proteins [87]. We fully agree with Kasarda *et al.* [87] that the low levels of lysine in storage proteins make it unlikely that a substantial improvement in the lysine of wheat can be achieved by genetic manipulation of these components. Moreover, in our opinion, it is definitively questionable that wheat varieties with high-lysine storage proteins might have desirable technological (dough and baking) properties. In fact, the insertion of a high number of lysine residues in the primary structures of gliadins and glutenins would probably affect conformations of these proteins and, as a consequence, interfere with the highly specific cooperative aggregation phenomena on which dough development is based. In conclusion, it seems more sensible to focus breeding efforts on the selection of wheat varieties with increased levels of albumins (and possibly globulins) at the expense of storage proteins. Such a standpoint is also supported by the results obtained up to now with selected varieties of other cereal species whose high-lysine character has been related to a higher amount of water-soluble proteins. Finally, it should be considered that wheat varieties with higher contents of albumin amylase inhibitors might also offer a higher resistance to insects. It has to be stressed once more that, in view of the possible action of amylase inhibitors *in vivo*, amylase inhibitory activity should be destroyed during processing of foods obtained with wheat varieties containing high albumin amounts.

REFERENCES

1. Hemberg, T. and Larsson, I. (1961) *Physiol. Plantarum* **14**, 861.
2. Hemberg, T. (1967) *Acta Chem. Scand.* **21**, 1665.
3. Hemberg, T. (1975) *Physiol. Plantarum* **31**, 11.
4. Niwa, T., Inouye, S., Tsuruoka, T., Koaze, T. and Niida, T. (1970) *Agr. Biol. Chem.* **34**, 966.
5. Veda, S. and Koba, Y. (1973) *Agr. Biol. Chem.* **37**, 2025.
6. Murao, S. and Ohyama, K. (1975) *Agr. Biol. Chem.* **39**, 2271.
7. Frommer, W., Puls, W., Schäfer, D. and Schmidt, D. (1972) *German Offen.* 2 064 092.
8. Miller, B. S. and Kneen, E. (1947) *Arch. Biochem.* **15**, 251.
9. Strumeyer, D. H. and Malin, M. J. (1969) *Biochim. Biophys. Acta* **184**, 643.
10. Chrzaszcz, T. and Janicki, J. (1933) *Biochem. Z.* **260**, 354.
11. Chrzaszcz, T. and Janicki, J. (1934) *Biochem. J.* **28**, 296.
12. Strumeyer, D. H. (1972) *Nutr. Rep. Intern.* **5**, 45.
13. Elliot, B. B. and Leopold, A. C. (1953) *Physiol. Plantarum* **6**, 65.
14. Marshall, J. J. and Lauda, C. M. (1975) *J. Biol. Chem.* **250**, 8030.
15. Rao, M. N., Shurpalekar, K. S. and Sundaravalli, E. O. (1970) *Ind. J. Biochem.* **7**, 241.
16. Mattoo, A. K. and Modi, V. V. (1970) *Enzymologia* **39**, 237.
17. Marshall, J. J. (1975) *ACS Symposium series* **16**, 244.
18. Saunders, R. M. (1975) *Cereal Fd World* **20**, 282.
19. Bernfeld, P. (1955) *Methods in Enzymology* Vol. 1 (Colowick, S. P. and Kaplan, N. O. eds.), pp. 149–158. Academic Press, New York.
20. Nelson, N. (1944) *J. Biol. Chem.* **153**, 375.
21. Hopkins, R. M. and Bird, R. (1954) *Biochem. J.* **56**, 86.
22. Fossum, K. and Whitaker, J. R. (1974) *J. Nutr.* **104**, 930.
23. Vittozzi, L., Morisi, G. and Silano, V. (1976) *J. Sci. Fd Agric.* **27**, 449.
24. Kneen, E. and Sandstedt, R. M. (1946) *Arch. Biochem.* **9**, 235.
25. Kneen, E. and Sandstedt, R. M. (1943) *J. Am. Chem. Soc.* **65**, 1247.
26. Sandstedt, R. M. and Beckord, O. C. (1946) *Cereal Chem.* **23**, 548.
27. Pace, W., Parlamenti, R., Rab, A., Silano, V. and Vittozzi, L. (1977) *Cereal. Chem.* submitted.
28. Militzer, W., Ikeda, C. and Kneen, E. (1946) *Arch. Biochem.* **9**, 309.
29. Militzer, W., Ikeda, C. and Kneen, E. (1946) *Arch. Biochem.* **9**, 321.
30. Shainkin, R. and Birk, Y. (1970) *Biochim. Biophys, Acta* **221**, 502.
31. Saunders, R. M. and Lang, J. A. (1973) *Phytochemistry* **12**, 1237.
32. Sodini, G., Silano, V., De Agazio, M., Pocchiari, F., Tentori, L. and Vivaldi, G. (1970) *Phytochemistry* **9**, 1167.
33. Silano, V., Pocchiari, F. and Kasarda, D. D. (1973) *Biochim. Biophys. Acta* **317**, 139.
34. Petrucci, T., Rab, A., Tomasi, M. and Silano, V. (1976) *Biochim. Biophys. Acta* **420**, 288.
35. O'Donnel, M. D. and McGeeney, K. F. (1976) *Biochim. Biophys. Acta* **422**, 159.
36. Petrucci, T., Tomasi, M., Cantagalli, P. and Silano, V. (1974) *Phytochemistry* **13**, 2487.
37. Bedetti, C., Bozzini, A., Silano, V. and Vittozzi, L. (1974) *Biochim. Biophys. Acta* **362**, 299.
38. Deponte, R., Parlamenti, R., Petrucci, T., Silano, V. and Tomasi, M. (1976) *Cereal Chem.*, **53**, 805.
39. Fish, W. W. and Abbot, D. C. (1969) *J. Sci. Fd Agric.* **20**, 723.
40. Cantagalli, P., Di Giorgio, G., Morisi, G., Pocchiari, F. and Silano, V. (1971) *J. Sci. Fd Agric.* **22**, 256.
41. Redman, D. G. (1975) *Biochem. J.* **149**, 725.
42. Redman, D. G. (1976) *Biochem. J.* **155**, 193.
43. Strumeyer, D. H. and Fischer, B. R. (1973) *Fed. Proc.* **32**, 624.
44. Huebner, F. R. and Rothfus, J. A. (1968) *Cereal Chem.* **45**, 242.
45. Kasarda, D. D., Bernardin, J. E. and Gaffield, W. (1968) *Biochemistry* **7**, 3950.
46. Silano, V., Furia, M., Gianfreda, L., Macri, A., Palescandolo, R., Rab, A., Scardi, V., Stella, E. and Valfrè, F. (1975) *Biochim. Biophys. Acta* **391**, 170.

47. Gertzman, G. B. R. (1975) *Diss. Abs. Int.* **36**, 2058.
48. Simpson, F. J. and McCoy, E. (1953) *Appl. Microbiol.* **1**, 228.
49. Appelbaum, S. W., Harpaz, I. and Bondi, A. (1964) *Comp. Biochem. Physiol.* **13**, 107.
50. Appelbaum, S. W. and Konijn, A. M. (1965) *J. Nutr.* **85**, 275.
51. Buonocore, V., Pace, W., Petrucci, T., Poerio, E., Silano, V. and Tomasi, M. *FEBS Letters* **67**, 202.
52. Buonocore, V., Poerio, E. Gramenzi, F. and Silano, V. (1975) *J. Chromatog.* **114**, 109.
53. Huper, F. and Rauenbusch, E. (1974) *German. Offen.* 2 309 280.
54. Appelbaum, S. W. (1964) *J. Insect Physiol.* **10**, 897.
55. Shymala, G. and Lyman, R. Z. (1964) *Can. J. Biochem.* **42**, 1825.
56. Hochstrasser, K. and Werle, E. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 249.
57. Mikola, J. and Kirsi, M. (1972) *Acta Chem. Scand.* **26**, 787.
58. Camues, M. C. and Laporte, J. C. (1973) *Ann. Technol. Agric.* **22**, 111.
59. Appelbaum, S. W. and Konijn, A. M. (1966) *J. Insect Physiol.* **12**, 665.
60. Apelbaum, S. W. and Konijn, A. M. (1967) *J. Stored Prod. Res.* **2**, 323.
61. Lang, J. A., Chang-Hum, L. E., Reyess, P. S. and Briggs, G. M. (1974) *Fed. Proc.* **33**, 718.
62. Puls, W. and Keup, N. (1973) *Diabetologia* **9**, 97.
63. Macri, A., Valfrè, F., Parlamenti, R. and Silano, V. (1977) *Poultry Sci.*, in press.
64. Bessho, H. and Kurosawa, S. (1967) *Eiyo to Shokuryo* **20**, 317.
65. Kasarda, D. D. (1975) *Protein Nutritional Quality of Foods and Feeds* (Friedman, M., ed.), Vol. 2. Dekker, New York.
66. Hekkens, W. Th. J. M., Haex, A. J., Willighagen, R. G. J. (1970) *Coeliac Disease* (Booth, C. C. and Dowling, R. H. eds.), pp. 11–19. Churchill Livingstone, Edinburgh.
67. Hekkens, W. Th. J. M., Van der Aarsen, C. J., Gilliams, J. P., Lems-Van Kan, Ph. and Boumafrolich, G. (1974) *Coeliac Disease* (Hekkens, W. Th. J. M. and Pena, A. S. eds), pp. 39–45. Stenfert Kroese, Leiden.
68. Kendall, M. J., Schneider, R., Cox, D. S. and Hawkins, C. F. (1972) *Lancet* **11**, 1065.
69. Strumeyer, D. H. and Fisher, B. R. (1973) *Fed. Proc.* 2310.
70. Auricchio, S., De Vizia, B., Carta-De Angelis, L. and Silano, V. (1974) *Lancet* **1**, 98.
71. Vittozzi, L. and Silano, V. (1976) *Theoret. Appl. Genetics*, **48**, 279.
72. Sarkar, P. and Stebbins, G. L. (1956) *Am. J. Botany* **43**, 297.
73. Cubadda, R. (1973) in *Genetics and Breeding of durum Wheat* (Scarascia Mugnozza, G. T. ed.), pp. 653–659. Biblioteca della Facoltà di Agraria, Bari.
74. Aragoncillo, C., Rodriguez Loperena, M. A., Corboners, P. and Garcia-Olmedo, F. (1975) *Theoret. Appl. Genetics* **45**, 322.
75. Bozzini, A., Cantagalli, P. and Piazzi, S. E. (1971) *European Wheat Ancuploid Newslett.* **3**, 16.
76. Wrigley, C. W. and Shepherd, K. W. (1973) *Ann. N.Y. Acad. Sci.* **209**, 154.
77. Piazzi, S. E., Riparbelli, G., Sordi, S., Cantaglali, P., Pocchiari, F. and Silano, V. (1972) *Cereal Chem.* **49**, 72.
78. Silano, V. (1975) *Bull. Lab. Chim. Prov.* **26**, 338.
79. Varner, J. E. (1965) *Plant Biochemistry* (Bonner, J. and Varner, J. E. eds), p. 763–792. Academic Press, New York.
80. Kasarda, D. D., Nimmo, C. C. and Kohler, G. O. (1971) *Wheat Chemistry and Technology* (Pomeranz, Y. ed.), 2nd ed., pp. 230–242. Am. Ass. Cereal Chem., St. Paul, MN.
81. Barlow, K. K., Buttrose, M. S., Simmonds, D. H. and Vesk, M. (1973) *Cereal Chem.* **50**, 443.
82. Simmonds, D. H., Barlow, K. K. and Wrigley, C. W. (1973) *Cereal Chem.* **50**, 553.
83. Minetti, M., Petrucci, T., Cattaneo, S., Pocchiari, F. and Silano, V. (1973) *Cereal Chem.* **50**, 198.
84. Wall, J. S. (1964) *Symposium on Food Proteins and their Reactions* (Schultz, M. W. and Angelmier, A. F. eds), pp. 315–319. Avi, Westport, CT.
85. Shukla, T. P. (1975) *CRC Crit. Rev. Food Sci. Nutr.* **6**, 1.
86. Kasarda, D. D., Bernardin, J. E. and Nimmo, C. C. (1976) *Advances in Cereal Science and Technology* (Pomerani, Y. ed.) pp. 158–236. AACC, St. Paul, Minnesota.
87. Simmonds, D. H. (1972) *Cereal Chem.* **49**, 324.