Biochimica et Biophysica Acta, 391 (1975) 170—178

BBA 67491

INHIBITION OF AMYLASES FROM DIFFERENT ORIGINS BY ALBUMINS FROM THE WHEAT KERNEL

V. SILANO[1], M. FURIA[2], L. GIANFREDA[2], A. MACRI[3], R. PALESCANDOLO[2], A. RAB[1], V. SCARDI[2], E. STELLA[4] and F. VALFRE[3]

[1] *Laboratori di Chimica Biologica,* [3] *Laboratori di Veterinaria and* [4] *Laboratori di Parassitologia, Istituto Superiore di Sanità, Roma and* [2] *Istituto di Fisiologia Generale, Università di Napoli and Stazione Zoologica di Napoli (Aquarium), Napoli (Italy)*

(Received December 24th, 1974)

Summary

The amylase activity of water extracts from 18 insect species, from 23 marine species and from 17 different species of birds and mammals was determined quantitatively. The inhibition of amylase in these extracts by three albumin fractions from the mature wheat kernel, which had been separated according to their molecular weights (60 000, 24 000 and 12 500 D), was determined as well. The inhibition activity of the three albumin fractions toward amylases extracted from a number of cereal species or from immature and germinating wheat kernel was also tested. The extracts from insects that are destructive of wheat grain and stored wheat products showed much higher amylase activities as compared to the other insect species that do not attack wheat and wheat products. 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 resistent. Susceptible amylases, inhibited by any of the three 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 three albumin fractions, were present in a few avain and mammalian species including man. Resistent 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, wheat α-amylase was inhibited by the albumin fractions from the mature kernel. The 12 500 dalton albumin fraction was the most effective in inhibiting insect amylases, but it was inactive toward avian and mammalian amylases. The 24 000 dalton albumin fraction was the most effective in inhibiting amylases from marine avian and mammalian species and inhibited as much as 33 amylases over 66 different amylases tested. It is suggested that protein inhibitors of amylase contributed to natural selection of polyploid wheats by giving some insect resistence to such wheats, even though

some insect species were able to overcome this biochemical defense to a large degree by producing higher amylase activities.

Introduction

A wheat protein fraction that inhibited α-amylases from human saliva, hog pancreas and *Bacillus subtilis* was first described by Kneen and Sandstedt [1] in 1943 (see also: Kneen and Sandstedt [2] and Militzer et al. [3,4]). Further studies showed that this inhibitor was also active toward several amylases from *Streptomyces* [5] and toward some insect amylases including those from *Tenebrio molitor* L. [6], *Prodenia litura* F. [7] and *Tribolium castaneum* Herbst [8]. Moreover, this inhibitor was inactive toward α-amylases from wheat, barley and sorghum grains as well as toward barley β-amylase [1]. More recently, however, Shainkin and Birk [9] isolated two protein inhibitors from the wheat kernel with different inhibition patterns toward amylases from different origins thus providing the first indication that the inhibitor of Kneen and Sandstedt was heterogeneous. Since then, it has been established [9—12] clearly that the wheat kernel contains many protein components capable of inhibiting insect and mammalian α-amylases (referred to hereafter simply as amylases). Petrucci et al. [12] divided the wheat albumin inhibitors into three main families that differed in molecular weight (60 000, 24 000 and 12 500) and in their physico-chemical properties. In each inhibitor family several active components were found that differed slightly in their electrophoretic mobilities in a Tris/glycine buffer (pH 8.5), but all the components of the family had similar specificities with regard to inhibition of different amylases.

Because the more recent work described above has demonstrated different specificities for some of the components present in the mixture, we have separated the wheat amylase inhibitors according to their molecular weight and used the three inhibitor fraction of Petrucci et al. [12] to compare inhibition effects on amylases from a number of different insect, marine, avian, mammalian and cereal species. With this study we also intended to give some contribution to elucidate the significance of these naturally occurring protein inhibitors that make up about 2/3 of the whole albumin of the wheat kernel [12].

Experimental methods

Extraction of amylase

Whole adult insects or samples of pancreas from avian or mammalian species, which had been kept frozen at −20° C, were warmed to room temperature and then homogenized with water (1 : 100 w/w) in a small Potter homogenizer at 0° C. Samples of digestive glands from marine species were homogenized in smaller volumes of water (1 : 10, w/w). The suspension was centrifuged at 40 000 × *g* for 20 min and the clear supernatant was used for amylase activity and amylase inhibition assays as described below. *Tenebrio molitor* L. larval amylase was prepared according to Applebaum et al. [13]. Human pancreatic amylase was whole pancreatic juice supplied by S. Auricchio, II clinica Pediatrica, University of Naples. Human saliva amylase was freeze-dried crude

saliva. The amylase extraction from mature cereal grains was carried out on 5 g of finely ground grains with 18 mM $CaCl_2$ according to the procedure described by Perten [14]. This procedure was also used for the extraction of amylase from the wheat kernel at various stages of maturity and germination. The soft winter wheat (variety Strampelli) sampled at 5 and 12 days from fertilization was kindly supplied by A. Bozzini, Laboratorio di Applicazioni in Agricoltura, C.N.E.N., S. Maria in Galeria, Rome. The intact heads of wheat plants were excised and stored in a deep freeze until analysis. Growing wheat was sampled at 5 and 12 days from fertilization because at such early stages of development no inhibition activity of human saliva amylase could be detected by Sandstedt and Beckord [15] in the wheat kernel. By direct measurement carried out according to Bedetti et al. [16], we confirmed the absence of amylase inhibitors in the 5-day-sampled wheat, whereas a very small inhibition activity (equal to about 1/50 of that of the mature kernel) was found in the 12-day-sampled wheat. Wheat germination was carried out for 3 days under the conditions described by Kruger [17] to allow the full set of germination amylases being formed. Then roots, coleoptyle and their attachments to germinated seeds were excised and the remaining grain was submitted to amylase extraction by following the Perten's procedure [14].

Extraction and fractionation of wheat albumins

The 12 500, 24 000 and 60 000 dalton albumin fractions from the wheat kernel were prepared by submitting to gel filtration the whole albumin fraction obtained by ammonium sulphate salting out of the 0.15 M NaCl extract from whole ground grain of soft winter wheat (variety Mentana) according to Petrucci et al. [12].

Activity and inhibition of amylase

Tests of the activity of amylases from insect and marine species as well as their inhibition by wheat albumin fractions were carried out in a 0.03 M acetate/barbiturate buffer (pH 5.4) containing 0.44 M NaCl and 0.001 M $CaCl_2$. The pH 5.4 was chosen because some insect amylases [8,13] showed a maximum of activity at this pH and it was not very far from the maximum of activity of some amylases from marine species [18]. Tests involving the avian and mammalian pancreatic amylases were performed in a similar buffer adjusted to pH 7.6 that was very close to the physiological one. Wheat albumins in amounts ranging between 0.1 and 2.0 μg were dissolved in 0.4 ml of buffer and then 0.5 ml of enzyme solution (suitably diluted to give, in the absence of inhibitor, a 70% hydrolysis of the starch present) was added. This solution was held at 28°C for 30 min and then 0.1 ml of a solution containing 10 mg/ml of starch (Merck, Germany) was added. The mixed solution was incubated at 37°C (avian and mammalian amylases) or at 28°C (amylases from insect and marine species) for 10 min. Following this, the reaction was stopped by adding 0.5 ml of 1 M HCl and the undigested starch was determined by adding 1 ml of an I_2/KI solution (1.2 and 1.8 mM, respectively) and measuring the change in absorbance at 620 nm. Controls without inhibitors were included to determine amylase activity of each preparation, which was expressed as Amylase Unit (A.U.) where one A.U. was the amount of enzyme that gave 50% hydrolysis of

the added starch under our experimental conditions. Amylase activity of extracts from cereal grains was determined in an acetate buffer (pH 4.7) at 30° C with limit dextrin as substrate by following the method of the International Association for Cereal Chemistry as described by Perten [14]. For inhibition tests, wheat albumins were used in amounts ranging from 1 to 100 μg under the experimental conditions above described.

Results

Amylases from insect species

Amylase activities of water extracts from whole adult insects that belonged to 17 different species and the quantitative inhibitions of these amylases by three different albumin fractions from the whole wheat kernel that had been separated according to their molecular weights of 60 000, 24 000 and 12 500 are given in Table I. We found higher amylase activities in insects that are destructive of wheat grain or stored wheat products [19] (samples 1—6, 10—12) and lower amylase activities in insects that do not usually attack wheat grain or wheat flour products (samples 7—9, 13—15) [20]. Two insects (samples 16 and 17), which presumably do not ingest significant amounts of starch, showed no detectable amylase activity. With regard to the inhibitory effects of the wheat albumins, we found that, with only one exception (sample 5), the amylases from insects that eat wheat grain or wheat flour products were all inhibited by small amounts of the three albumin fractions tested, whereas significantly higher albumin concentrations were needed to inhibit equivalent amounts of amylase from *Chrysomela decemlineata* Say and *Anacridium aegyptium* L. and none of the other insect amylases tested showed any sensitivity to the wheat albumins. Inhibitions of amylase from *Tenebrio molitor* L. larvae by the three albumin fractions were identical to those found for the amylase from adults as reported in Table I (sample 3). A low amylase activity (1 A.U./g of whole insect) not inhibited by any of the three wheat albumin fractions was found in *Philosamia cynthia* Drury (ailantus silk-moth) larvae.

Amylases from marine species

Amylase activities of water extracts from 23 marine species and the quantitative inhibitions of these amylases by the three albumin fractions from the wheat kernel are given in Table II. It appears that only five amylases from marine species (samples 12, 17, 20, 22 and 23) were not inhibited by any of the wheat albumin fractions and that as much as 13 amylases were inhibited by any of the three fractions. Table II also shows that the 24 000 dalton fraction was more effective in inhibiting amylases from marine species than the 60 000 and 12 500 dalton fractions.

Amylases from avian and mammalian species

Of the 17 different avian and mammalian species tested (Table III), amylases from only 6 species (samples 1—3, 9—12) were inhibited by the 24 000 or 60 000 dalton fraction, whereas the 12 500 dalton fraction showed no inhibition of any of the amylases from avian and mammalian species. We were unable to confirm the inhibition of hog pancreatic amylase described by

TABLE I

AMYLASE ACTIVITIES OF INSECT SPECIES (ADULTS) AND INHIBITION OF THESE ACTIVITIES BY WHEAT ALBUMIN FRACTIONS

Sample Number	Species		Amylase activity (A.U.*/g of insect)	Amount of wheat albumin that gives 30% inhibition of 1 Amylase Unit (ng)		
	Scientific name	Trivial name		60 000 Dalton fraction**	24 000 Dalton fraction***	12 500 Dalton fraction***
1	*Calandra granaria* L.	granary weevil	18 400	1 342	221	38
2	*Calandra oryzae* L.	rice weevil	12 400	1 217	117	37
3	*Tenebrio molitor* L.	yellow mealworm	6 100	1 098	200	66
4	*Tribolium confusum* Duv.	confused flour beetle	7 000	1 311	86	98
5	*Oryzaephilus surinamensis* L.	sawtoothed grain beetle	7 800	1 617	329	1 562
6	*Rhizopertha dominica* F.	lesser grain borer	320	No inhibition	No inhibition	297
7	*Chrysomela decemlineata* Say	colorado potato beetle	13	Not tested	523	1 028
8	*Cerambyx cerdo* L.	longhorned borer	2	Not tested	No inhibition	No inhibition
9	*Acanthoscelides obsoletus* Say	bean weevil	1	Not tested	No inhibition	No inhibition
10	*Periplaneta americana* L.	american cockroach	160	1 312	141	246
11	*Blattella germanica* L.	german cockroach	135	1 277	13	5
12	*Sitotroga cerealella* Oliv.	angoumois grain moth	26	Not tested	100	60
13	*Galleria mellonella* L.	greater wax moth	23	Not tested	No inhibition	No inhibition
14	*Ephestia kuehniella* Zell.	mediterranean flour moth	9	Not tested	No inhibition	No inhibition
15	*Anacridium aegyptium* L.	egyptian grasshopper	5	Not tested	1 111	590
16	*Triatoma infestans* Klug.	reduviid bug	No activity	—	—	—
17	*Anax imperator* Leach	empress dragon-fly	No activity	—	—	—

* One Amylase Unit (A.U.) is the amount of enzyme that gives 50% hydrolysis of the added starch under our experimental conditions.

** Tested at a maximum concentration in the incubation mixture of 2μg/ml.

*** Tested at a maximum concentration in the incubation mixture of 1 μg/ml.

TABLE II

AMYLASE ACTIVITIES OF MARINE SPECIES (ADULTS) AND INHIBITION OF THESE ACTIVITIES BY WHEAT ALBUMIN FRACTIONS

Sample Number	Species		Amylase activity (A.U.*/g of digestive gland)	Amount of wheat albumin that gives 30% inhibition of 1 Amylase Unit (ng)		
	Scientific name	Trivial name		60 000 Dalton fraction**	24 000 Dalton fraction***	12 500 Dalton fraction***
1	*Loligo vulgaris* Lam.	a variety of squid	20	314	80	237
2	*Illex coindeti* Verany	a variety of squid	20	No inhibition	157	561
3	*Allotheutis subulata* Lam.	a variety of squid	19	428	127	94
4	*Sepia officinalis* L.	a variety of cuttlefish	45	318	25	48
5	*Sepia elegans* D'Orbigny	a variety of cuttlefish	37	99	14	44
6	*Sepiola rondeleti* Leach	a variety of cuttlefish	39	180	15	27
7	*Octopus vulgaris* Lam.	a variety of octopus	63	513	12	73
8	*Eledone moschata* Lam.	a variety of octopus	98	489	6	59
9	*Eledone aldrovandii* Rafinesque	a variety of octopus	93	384	4	45
10	*Pleurobranchaea meckelii*	nudibranch	47	190	34	153
11	*Tethys leporina* L.	sea hare	23	520	124	133
12	*Umbraculum mediterraneum* Lam.	mollusk	16	No inhibition	No inhibition	No inhibition
13	*Cardium tuberculatum* L.	red nosed cockle	74	707	21	61
14	*Mactra corallina* L.	radiated through shell	39	313	53	95
15	*Cytherea chione* L.	cock	28	539	16	129
16	*Donax trunculus* L.	wedge shell	36	No inhibition	No inhibition	203
17	*Venus verrucosa* L.	wart venus	17	No inhibition	No inhibition	No inhibition
18	*Natica hebraea* Martyn	snail	64	321	20	No inhibition
19	*Murex trunculus* L.	murex	19	1 500	90	No inhibition
20	*Leander serratus* Penn.	prawn	65	No inhibition	No inhibition	No inhibition
21	*Maja verrucosa* M. Edwards	a variety of crab	62	No inhibition	31	No inhibition
22	*Scorpaena ustulata* Rafinesque	a variety of scorpionfish	27	No inhibition	No inhibition	No inhibition
23	*Mugil auratus* Risso	golden grey mullet	52	No inhibition	No inhibition	No inhibition

* One Amylase Unit is the amount of enzyme that gives 50% hydrolysis of the added starch under our experimental conditions.

** Tested at a maximum concentration in the incubation mixture of 2 μg/ml.

*** Tested at a maximum concentration in the incubation mixture of 1 μg/ml.

TABLE III

AMYLASE ACTIVITIES OF AVIAN AND MAMMALIAN SPECIES (ADULTS) AND INHIBITION OF THESE ACTIVITIES BY WHEAT ALBUMIN FRACTIONS

Sample Number	Species		Amylase activity (A.U.*/g of pancreas)	Amount of wheat albumin that gives 30% inhibition of 1 Amylase Unit (ng)		
	Scientific name	Trivial name		60 000 Dalton fraction**	24 000 Dalton fraction***	12 500 Dalton fraction***
1	*Gallus gallus* L.	chicken	73	913	388	No inhibition
2	*Meleagris g. gallopavo* L.	turkey	109	159	No inhibition	No inhibition
3	*Coturnix c. coturnix* L.	quail	151	1 912	547	No inhibition
4	*Alauda a. arvensis* L.	skylark	1	No inhibition	No inhibition	No inhibition
5	*Emberiza c. calandra* L.	bunting	1	No inhibition	No inhibition	No inhibition
6	*Fringilla c. coelebs* L.	chaffinch	38	No inhibition	No inhibition	No inhibition
7	*Passer Italiae Vieill.*	sparrow	156	No inhibition	No inhibition	No inhibition
8	*Turdus p. philomelus* Brehm	thrush	1	No inhibition	No inhibition	No inhibition
9	*Homo sapiens*	man (pancreatic juice)	—	No inhibition	1 067	No inhibition
10	*Homo sapiens*	man (saliva)	—	1 219	441	No inhibition
11	*Canis familiaris* L.	dog	9	1 249	409	No inhibition
12	*Felis domesticus*	cat	1	No inhibition	570	No inhibition
13	*Bos taurus* L.	ox	13	No inhibition	No inhibition	No inhibition
14	*Cavia cobaya*	guinea pig	38	No inhibition	No inhibition	No inhibition
15	*Equus caballus* L.	horse	13	No inhibition	No inhibition	No inhibition
16	*Macaca rhesus* Show	monkey	107	No inhibition	No inhibition	No inhibition
17	*Oryctolagus cuniculus* L.	rabbit	31	No inhibition	No inhibition	No inhibition
18	*Sus scrofa domesticus*	pig	15	No inhibition	No inhibition	No inhibition

* One Amylase Unit (A.U.) is the amount of enzyme that gives 50% hydrolysis of the added starch under our experimental conditions.

** Tested at a maximum concentration in the incubation mixture of 2 μg/ml.

*** Tested at a maximum concentration in the incubation mixture of 1 μg/ml.

Kneen and Sandstedt [2] although we did note that when tested at high concentrations both the 24 000 and 12 500 dalton fractions exhibited a low inhibition of hog amylase equivalent to about 1/50 of that found for inhibition of *Tenebrio molitor* L. amylase (Table I, sample 3). This result could possibly be due to small amounts of impurities with different specificities of inhibition.

Amylases from cereal species

We tested inhibition activity of the three albumin fractions toward amylases extracted from the kernel of several varieties of diploid (*Triticum monococcum*), tetraploid (*Triticum durum*) and hexaploid (*Triticum aestivum*) wheats. No inhibition activity could be detected toward any of the wheat amylases even when wheat albumins were tested at concentrations as high as 100 μg/ml. The albumin fractions were also inactive toward amylases extracted from immature kernels sampled at 5 and 12 days from fertilization or from 3-day-germinated seeds. As expected, the amylase activity of immature and germinated kernel was much higher than that of the mature kernel. In particular, the amylase activities of the kernels sampled at 5 and 12 days from fertilization were respectively 825- and 500-fold higher than that of the mature kernel, whereas the amylase activity of the 3-day-germinated seed was 1250-fold higher. Finally, we could not detect any inhibition activity of the three albumin fractions toward amylases extracted from mature grains of a number of other cereal species including *Hordeum vulgare*(barley), *Zea mais* (corn), *Secale cereale* (rye), *Oryza sativa* (rice) and *Panicum milaceum* (millet).

Discussion

On the basis of the effectiveness with which the three wheat albumin fractions of Petrucci et al. [12] inhibit their activities, we can divide the amylase preparations tested into three groups: susceptible, inhibited by any of the three albumin fractions, partially susceptible, inhibited by only one or two fractions, and resistent, not inhibited by any of the three fractions. Susceptible amylases were found mainly in insects that attack wheat and stored wheat products as well as in some marine species (mainly *Cephalopoda*). Partially susceptibel amylases were found in a few avian, mammalian and marine species. Resistent amylases were largely distributed in cereal, avian and mammalian species as well as in insect species that do not attack wheat grain or wheat products. Although valid interspecies comparisons of amylases are difficult because amylase activity and action patterns are easily affected by experimental conditions, it appears that amylase protein inhibitors from the wheat kernel show a quite unexpected picture of relationships among amylases from very different origins.

In our opinion, the fact that at no stage of the kernel development wheat α-amylase could be inhibited by the albumin fractions extracted from the mature kernel, together with the related results reported by other authors [1,9,15], conclusively rule out the possibility that these protein inhibitors play some role in regulating α-amylase activity of the wheat kernel. Actually, our results suggest that the significance of these naturally occurring protein inhibitors is to provide a measure of insect resistence to the wheat kernel. A

clear correlation exists between insect feeding behaviour and both amylase activity of the species and the susceptibility of the amylase to inhibition by the wheat albumin fractions. High amylase activities and strong inhibition by wheat albumins were found in those insect species that normally attack wheat grain or wheat products, whereas those insect species that do not normally eat wheat had relatively low amylase activities and amylases resistent to inhibition by the wheat albumins. Since Applebaum [6] showed that wheat albumins inhibit insect amylase not only in vitro, but also in vivo after being ingested by the insect, it seems likely that the higher amylase activities in those insect species that attack wheat have resulted from an adaptive mechanism developed to overcome the presence in wheat of highly-active amylase inhibitors. From this standpoint, since amylase protein inhibitors are absent in diploid *Triticum* species, but present in tetraploid and hexaploid *Triticum* species [16], protein inhibitors of insect amylases could be considered as one of the advantages acquired by wheat through its evolution.

Acknowledgements

We thank Dr D.D. Kasarda for his stimulating advice.

References

1 Kneen, E. and Sandstedt, R.M. (1943) J. Am. Chem. Soc. 65, 1247
2 Kneen, E. and Sandstedt, R.M. (1946) Arch. Biochem. 9, 235—249
3 Militzer, W., Ikeda, C. and Kneen, E. (1946) Arch. Biochem. 9, 309—320
4 Militzer, W., Ikeda, C. and Kneen, E. (1946) Arch. Biochem. 9, 321—329
5 Simpson, J. and Mac Coy, E. (1954) in Industrial Fermentation (Leland Underkoffer R.R. Hickey), Vol. II, 228—235
6 Applebaum, S.W. (1964) J. Ins. Physiol. 10, 897—906
7 Applebaum, S.W., Harpaz, I. and Bondi, A. (1964) Comp. Biochem. Physiol. 13, 107—111
8 Applebaum, S.W. and Konijn, A.M. (1965) J. Nutr. 85, 275—281
9 Shainkin, R. and Birk, Y. (1970) Biochim. Biophys. Acta 221, 502—513
10 Silano, V., Pocchiari, F. and Kasarda, D.D. (1973) Biochim. Biophys. Acta 317, 139—148
11 Saunders, R.M. and Lang, J.A. (1973) Phytochemistry 12, 1237—1241
12 Petrucci, T., Tomasi, M., Cantagalli, P. and Silano, V. (1974) Phytochemistry 13, 2487—2495
13 Applebaum, S.W., Jankovic, M. and Birk, Y. (1961) J. Ins. Physiol. 7, 100—108
14 Perten, H. (1966) Cereal Chem. 43, 336—342
15 Sandstedt, R.M. and Beckord, O.C. (1946) Cereal Chem. 23, 548—559
16 Bedetti, C., Bozzini, A., Silano, V. and Vittozzi, L. (1974) Biochim. Biophys. Acta 362, 299—307
17 Kruger, J.E. (1972) Cereal Chem. 49, 391—398
18 Bidder, A.M. (1966) in Physiology of Mollusca (Wilbur, K.M. and Yonge, L.M. eds), Vol. II, pp. 97—124
19 Dal Monte, G. (1972) Molini d'Italia 11, 413—424
20 Grandi, G. (1951) Introduzione allo studio della entomologia (Ed. Agricole, Bologna), Vol. II, pp. 70 and 193