Phytochemistry, 1974, Vol. 13, pp. 2487 to 2495. Pergamon Press. Printed in England.

COMPARISON OF WHEAT ALBUMIN INHIBITORS OF α-AMYLASE AND TRYPSIN

TAMARA PETRUCCI, MAURIZIO TOMASI, PIERO CANTAGALLI* and VITTORIO SILANO

Laboratori di Chimica Biologica, Istituto Superiore di Sanità, Viale Regina Elena, 299, 00161, Rome, Italy

(*Received* 16 *December* 1973)

Key Word Index—*Triticum aestivum*; Gramineae; wheat; amylase inhibitors; trypsin inhibitors; albumins; disc-electrophoresis; gel filtration; protein families.

Abstract—Wheat albumins were extracted from whole wheat flour with 150 mM sodium chloride solution and precipitated between 0·4 and 1·8 M ammonium sulphate. The albumin precipitate was separated by gel filtration on Sephadex G100 into five peaks. Three peaks (II, III, and IV), whose MWs were 60000, 24000 and 12500 daltons respectively, were active toward several insect α-amylases, whereas only peak III inhibited human saliva and pancreatic α-amylases. Peaks III and IV also inhibited trypsin. In each active peak, we found several α-amylase inhibitors slightly different in their electrophoretic mobilities in a Tris–glycine buffer system (pH 8·5), whereas only one major trypsin inhibitor was present in peaks III and IV. In contrast to α-amylase inhibitors that were all anodic, trypsin inhibitors migrated to the cathode under our experimental conditions. From a quantitative standpoint, wheat albumins that inhibit trypsin are negligible, whereas about 2/3 of the total albumin inhibits amylases from different origins. All inhibitor components of peak III were active toward both insect and mammalian α-amylases. Moreover, they reversibly dissociated in the presence of 6 M guanidine hydrochloride giving two similar subunits.

INTRODUCTION

A WHEAT protein inhibitor of human saliva and pancreatic α-amylases was first described by Kneen *et al.*[1–4] Applebaum *et al.*[5–7] found that this inhibitor also inhibited several insect amylases including those from *Tenebrio molitor* L., *Prodenia litura* F. and *Tribolium castaneum* Hbst. The preparations used in this earlier work probably contained a mixture of proteins. More recently, Shainkin and Birk[8] isolated from wheat two protein inhibitors (named AmI_1 and AmI_2) that showed different specificities toward α-amylases from different origins. AmI_1 was only active against insect α-amylases, whereas AmI_2 also inhibited human saliva α-amylase. After the investigations carried out by Silano *et al.*[9] and by Saunders and Lang,[10] it became evident that in wheat seed there are multiple molecular forms of proteins capable of inhibiting insect and mammalian α-amylases. All these inhibitors have an albumin nature, but gliadins which behave as inhibitors of several α-amylases

* Laboratorio Chimico Provinciale, Siena, Italy.

[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.
[3] MILITZER, W., IKEDA, C. and KNEEN, E. (1946) *Arch. Biochem.* **9**, 309.
[4] MILITZER, W., IKEDA, C. and KNEEN, E. (1946) *Arch. Biochem.* **9**, 321.
[5] APPLEBAUM, S. W. (1964) *J. Insect Physiol.* **10**, 897.
[6] APPLEBAUM, S. W., HARPAZ, I. and BONDI, A. (1964) *Comp. Biochem. Physiol.* **13**, 107.
[7] APPLEBAUM, S. W. and KONIJN, A. M. (1965) *J. Nutrit.* **85**, 275.
[8] SHAINKIN, R. and BIRK, Y. (1970) *Biochim. Biophys. Acta* **221**, 502.
[9] SILANO, V., POCCHIARI, F. and KASARDA, D. D. (1973) *Biochim. Biophys. Acta* **317**, 139.
[10] SAUNDERS, R. M. and LANG, J. A. (1973) *Phytochemistry* **12**, 1237.

seem[11] to be present in wheat as well. α-Amylase protein inhibitors occur in wheat seed as well as in flour and in baked wheat flour goods.[12] They not only inhibit α-amylase *in vitro*, but also *in vivo*, as was shown in men and other mammals by Puls and Keup[13] and in insect larvae by Applebaum.[5] A possible role of α-amylase inhibitors as triggers of the symptoms of coeliac disease (gluten-induced enteropathy) by lowering an already deficient pancreatic amylase activity has also been suggested.[11]

In view of possible uses in nutrition and therapy we made a systematic study of protein α-amylase inhibitors from wheat to evaluate their quantitative importance as compared to other wheat proteins and to determine whether they belong to the albumin or to the gliadin protein class. Also, since protein trypsin inhibitors[14–17] from wheat seem to represent[18] a certain percentage of water-soluble proteins to which α-amylase inhibitors also belong, we compared trypsin inhibitors with amylase inhibitors in order to investigate possible correlations between these two protein inhibitor classes.

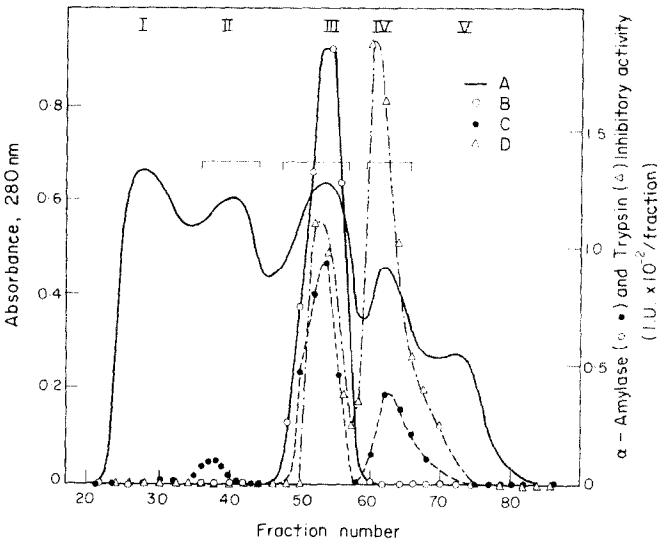

FIG. 1. GEL FILTRATION ON SEPHADEX G100 OF WHEAT ALBUMIN INHIBITORS OF α-AMYLASE AND TRYPSIN. (1 g of albumin in 100 ml of phosphate buffer, pH 7·4 (137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4 and 15-mM NaN_3) applied to a 4·5 × 110 cm column of Sephadex G100. Flow rate was 60 ml/hr. Fractions (20 ml) were collected and 10–100 μl samples were used for amylase and trypsin inhibition tests).

RESULTS

Extraction and precipitation

A 0·15 M sodium chloride solution was quite effective in extracting α-amylase and trypsin protein inhibitors from whole wheat flour (18×10^3 i.u./g). However, exhaustive extraction was not achieved even with five consecutive treatments of the flour with this sol-

[11] STRUMEYER, D. H. (1972) *Nutr. Reports Int.* **5**, 45.
[12] BESSHO, H. and KUROSAWA, S. (1967) *Eiyo To Shokuryo* **20**, 317.
[13] PULS, W. and KEUP, U. (1973) *Diabetologia* **9**, 97.
[14] SHYMALA, G., KENNEDY, B. M. and LYMAN, R. L. (1961) *Nature* **192**, 360.
[15] LAPORTE, P. and TREMOLIERES, I. (1962) *C.R. Sci. Soc. Biol.* **156**, 1261.
[16] SHYMALA, G. and LYMAN, R. L. (1964) *Can. J. Biochem.* **42**, 1825.
[17] HOCHSTRASSER, K. and WERLE, E. (1969) *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 249.
[18] MIKOLA, J. and KIRSI, M. (1972) *Acta Chem. Scand.* **26**, 787.

vent. By using 65% (v/v) aqueous ethanol instead of the salt solution, we observed no significant improvement of the extraction of residual α-amylase inhibitors. No trypsin inhibitory activity was detectable in the ethanol extracts. The ethanol solvent did not affect activity of trypsin inhibitors extracted with the salt solution.

We obtained an almost quantitative precipitation of the inhibitors by salting out between 0·4 and 1·8 M ammonium sulphate according to the procedure described by Pence and Elder[19] to obtain a typical wheat albumin preparation. Recoveries of α-amylase and trypsin inhibitors in the albumin precipitate were 76 and 60% respectively. Protein yield was about 0·3% of seed weight which is close to the theoretical value for wheat albumins.[20] Precipitations performed in narrower ranges of ammonium sulphate concentrations resulted in much lower inhibitor recoveries.

Gel filtration and specific inhibitory activity

By submitting the albumin precipitate to gel filtration on a Sephadex G100 column, we obtained five peaks of absorbance (Fig. 1a). Peaks I and V appeared strongly reduced when proteins were extracted, under identical experimental conditions, from flour instead of whole wheat flour. Three peaks (II, III and IV) were active toward TmL amylase* (Fig. 1c), whereas only peak III inhibited HS amylase (Fig. 1b). Peak III was equally active toward human and chicken pancreatic α-amylases as well as toward several insect amylases including those from *Blatella germanica* L. and *Tribolium confusum* Duv. Peak IV only inhibited the insect α-amylases. Both peaks III and IV exhibited a very low inhibitory activity toward pig pancreatic α-amylase equal to about 1/50 of that displayed toward the other mentioned amylases.

According to the observations of Mikola and Kirsi[18] who submitted a salt extract of whole wheat to gel filtration on a Sephadex G75 column, two peaks (III and IV) showed inhibitory activity toward trypsin (Fig. 1d).

Fractions corresponding to central cuts of each active peak of Fig. 1 (indicated by bars) were pooled, dialyzed and freeze-dried. In the course of the paper we will refer to these lyophilised samples as peaks II, III and IV, using these symbols in a slightly different meaning from that we have used up to now. Protein recoveries in peaks II, III and IV were 27, 24 and 6% (w/w applied) respectively. Recoveries of TmL amylase inhibitory activity in these three peaks were 5·0, 41·8 and 32·4%. In peaks III and IV we recovered 24 and 57% respectively of the trypsin inhibitory activity loaded on the column. Such high recoveries of proteins and inhibitors showed no significant irreversible binding of protein inhibitors to the Sephadex gel under our experimental conditions.

Specific activity measurements indicated (Table 1) a 3-fold purification of HS amylase inhibitors and 2-fold purification of TmL amylase inhibitors over the ammonium sulphate precipitate in peak III. Purification factors of TmL amylase and trypsin inhibitors in peak IV were 5·1 and 6·2 respectively, whereas specific inhibitory activity of peak II toward TmL amylase was 1/5 of that exhibited by the unfractionated ammonium sulphate precipitate.

As shown in Table 1, the highly purified inhibitors described by Sodini *et al.* (0·19),[21,9,10] Shainkin and Birk (AmI_3)[8] and Saunders and Lang (I)[10] exhibited inhibition patterns very

* Abbreviations: TmL amylase, *Tenebrio molitor* L. α-amylase; HS amylase, human saliva α-amylase.

[19] Pence, J. W. and Elder, A. H. (1953) *Cereal Chem.* **30**, 275.
[20] Pence, J. W. and Nimmo, C. C. (1964) *Bakers Dig.* **38**, 38.
[21] Sodini, G., Silano, V., de Agazio, M., Pocchiari, F., Tentori, L. and Vivaldi, G. (1970) *Phytochemistry* **9**, 1167.

similar to that of peak III both from qualitative and quantitative standpoints. Moreover, the AmI_1 inhibitor purified by Shainkin and Birk[8] and the inhibitors of the 0·28 family[9] appeared strictly related to peak IV. On the contrary, specific inhibitory activities of peak III and IV toward trypsin were much lower than those shown by the purified inhibitors described by Shymala and Lyman (WWTI)[16] and by Hochstrasser and Werle (Peaks I and II).[17]

TABLE 1. SUMMARY OF SPECIFIC INHIBITORY ACTIVITIES TOWARD AMYLASE AND TRYPSIN OF PEAKS II, III AND IV AND OF THE PROTEIN INHIBITORS PURIFIED FROM WHEAT SEED BY DIFFERENT AUTHORS

Sample	Specific inhibitory activity (I.U./μg protein) Human saliva α-amylase	*Tenebrio molitor* L. α-amylase	Trypsin
Unfractionated albumin precipitate	1·3	1·0	0·4
Peak II	—	0·2	—
Peak III	3·9	2·0	0·4
Peak IV	—	5·1	2·5
Purified amylase inhibitors*:			
AmI_1 (Shainkin and Birk, 1970)	—	5·5	—
0·28 (Silano *et al.*, 1973)	—	7·0	—
0·19 (Sodini *et al.*, 1970)	4·0	4·0	—
AmI_2 (Shainkin and Birk, 1970)	3·3	3·3	—
Inhibitor I (Saunders and Lang, 1973)	4·5	Not determined	—
Purified trypsin inhibitors*:			
WWTI (Shymala and Lyman, 1964)	—	—	6·9
Peak I (Hochstrasser and Werle, 1969)	—	—	29·0
Peak II (Hochstrasser and Werle, 1969)	—	—	41·0

* Values were all recalculated as inhibition units defined in the text.

Polyacrylamide gel electrophoresis and electrofocusing

Both anodic (Fig. 2c and i) and cathodic (Fig. 2f and k) protein bands were found in polyacrylamide gel electrophoretic patterns of peaks III and IV in a Tris–glycine buffer system (pH 8·5). Since to obtain electrophoretic patterns of comparable intensity, we loaded 100 μg of proteins for the anodic and 1500 μg for the cathodic runs, it appears that cathodic fractions represent quite a low percentage (about 5%) of peaks III and IV. Only anodic fractions were found in peak II (Fig. 2a). All protein bands were blue–black, thus indicating an albumin nature.[22] By testing α-amylase and trypsin inhibitory activity of protein fractions eluted from the gel at the end of the electrophoretic analysis, we showed that all the main anodic fractions of peak III inhibit both HS amylase (Fig. 2d) and TmL amylase (Fig. 2e). Similarly, the main anodic protein fractions from peak IV (Fig. 2h) as well as those from peak II (Fig. 2b) were found active toward TmL amylase. The slowest amylase inhibitor of peak III had a mobility of 0·19 relative to bromophenol blue (= 1), identical to that of the 0·19 inhibitor under identical experimental conditions.[23] The mobilities of the two main fractions of peak IV were 0·28 and 0·39, in good correspondence with the mobilities reported for the two main components of the 0·28 family.[9] Typically, trypsin inhibitors were all cathodic at pH 8·5. Two inhibitor fractions were found in peak III (Fig. 2g) and only one in the peak IV (Fig. 2j). Since the slower trypsin inhibitor from

[22] MINETTI, M., PETRUCCI, T., CATTANEO, S., POCCHIARI, F. and SILANO, V. (1973) *Cereal Chem.* **50**, 198.

peak III had an electrophoretic mobility identical to that of the inhibitor from peak IV, the presence of this inhibitor in peak III might be a contamination by peak IV. A significant trypsin inhibitory activity found at the very top of the gel both for peak III and IV suggested, however, that other trypsin inhibitors with very low mobility (not shown in Fig. 2) could be present.

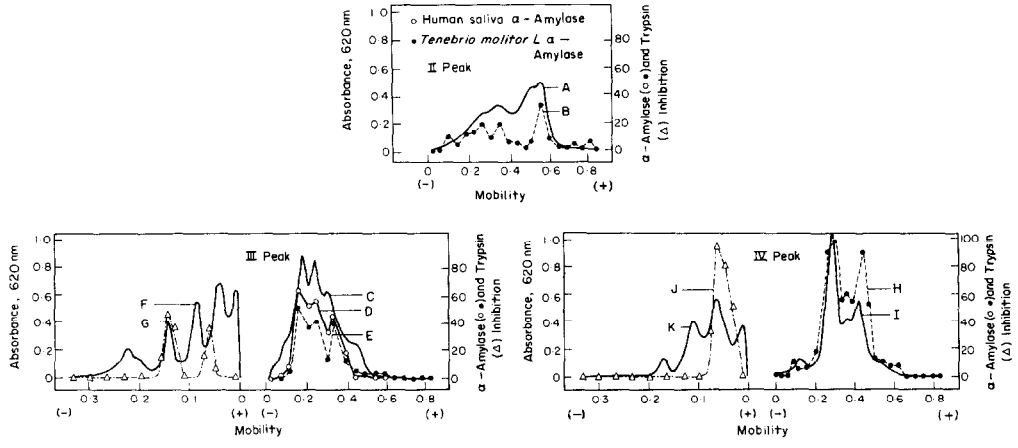

FIG. 2. COMPARISON OF PROTEIN, AMYLASE INHIBITOR, AND TRYPSIN INHIBITOR ELECTROPHORETIC PATTERNS OF PEAKS II, III AND IV.

(Disc-electrophoresis on polyacrylamide gel was carried out in a 0·05 M Tris–0·38 M glycine buffer, pH 8·5. Protein patterns were obtained by staining the gel with Aniline blue black in the conventional way. Amylase and trypsin inhibitor patterns were obtained by slicing the gel and eluting proteins with buffer. The eluate 10–200 μl was used for amylase inhibition test and 800 μl for trypsin inhibition test).

Four inhibitor fractions (pI 7·0, 6·3, 5·8 and 5·0) active toward both HS amylase and TmL amylase and two main fractions (pI 6·2 and 5·2) only active toward TmL amylase were found by submitting to polyacrylamide gel electrofocusing (pH range 3–10) peaks III and IV respectively.

Molecular weight and dissociation by guanidine hydrochloride

Apparent MWs of peaks II, III and IV, determined by gel filtration according to Andrews,[23] were 60000, 24000 and 12500 daltons respectively. The sedimentation patterns of the three peaks showed the presence of a single homogeneous peak with an apparently homogeneous boundary. Sedimentation coefficients ($S^{\circ}_{w,20}$) were 4·05 (peak II), 2·60 (peak III) and 1·62 (peak IV). The MWs of the purified inhibitors AmI_2, I and 0·19 were all close to the MW found for peak III, whereas those of the AmI_1 and of 0·28 family inhibitors were comparable to the molecular weight of peak IV. Finally, the MWs of the two trypsin inhibitors isolated from wheat by Hochstrasser and Werle,[17] as calculated by gel filtration, resulted 17000 (Peak I) and 12000 (Peak II) and, therefore, not very different from our peaks III and IV.

In the presence of 6 M guanidine hydrochloride, peak III split into two peaks (III_a and III_b) different in their MWs. MW of peak III_a (representative of about 95% of peak III) was 10000 daltons. This value is very close to that determined for peak IV under identical

[23] ANDREWS, P. (1964) *Biochem. J.* **91**, 222.

experimental conditions as well as in the absence of the dissociating agent. Peak III_b exhibited a MW identical to that of the undissociated peak III. These results are consistent with those previously obtained[9] with the purified 0·19 whose MW in the presence of 6 M guanidine hydrochloride, as determined by the sedimentation equilibrium method, was 12500 daltons and equal to about 1/2 of the MW determined in the absence of the dissociating agent. The purified 0·19 submitted to gel electrophoresis in a buffer containing sodium dodecyl sulphate showed two bands of similar intensities and MWs (only 1000 dalton difference) in contrast to the single band seen in non dissociating solvents.[9] Interesting enough, two subunits were also found by Hochstrasser and Werle[17] in the trypsin inhibitor denominated peak I.

Reversibility of guanidine hydrochloride dissociation effect

We showed the full reversibility of guanidine hydrochloride dissociation effect on peak III by pooling fractions corresponding to peak III_a and filtering them through a Sephadex G50 column to remove the dissociating agent. After this treatment, peak III_a showed a MW of 22000 daltons and α-amylase and trypsin inhibitory activities identical to those of the untreated peak III. Also protein and amylase inhibitor electrophoretic patterns of peak III_a, after removal of guanidine hydrochloride, appeared identical to those already described for peak III in Fig. 2 c and d).

Amino acid composition

Amino acid compositions of peaks II, III and IV and those of the purified amylase inhibitors reported in Table 1 are compared in Table 2. The similarity of the compositions of 0·19, AmI_2, Inhibitor I and peak III as well as that of the compositions of AmI_1, 0·28 and peak IV is evident when they are compared on a common basis (μmol of each amino acid/total μmol obtained in each analysis). Amino acid compositions of peaks II, III and IV, although clearly not identical, were not greatly different. They can all be considered typical of wheat albumins.[24]

DISCUSSION

The data reported strongly indicate that a relevant part of α-amylase as well as trypsin inhibitors have a typical albumin nature. However, an α-amylase inhibitor pool with an extraction behaviour suggestive of a gliadin nature, and possibly related to Strumeyer's α-amylase gliadin inhibitors,[11] might be present in wheat seed. Trypsin albumin inhibitors differ from amylase albumin inhibitors in their more basic character. From a quantitative standpoint, wheat albumins that inhibit trypsin are negligible, whereas an albumin comprising two thirds of the total inhibits TmL amylase and one third is also active toward human saliva and pancreatic α-amylases. In spite of the limits of the method used, we have been able to differentiate seven major albumin components that inhibit α-amylase, whereas only three trypsin albumin inhibitors were found. There is little doubt that further studies focused on amylase or trypsin inhibitors present in smaller amounts in wheat seed might show a much higher heterogeneity of these two inhibitor pools.

By means of gel filtration on Sephadex G100, we separated TmL amylase albumin inhibitors into three heterogeneous groups whose MWs were 60000, 24000 and 12500 daltons. Specific inhibitory activities of these three groups toward TmL amylase, calculated from values reported in Table 1 and expressed as i.u./nmol instead of i.u./μg, were 12, 48 and

[24] Feillet, P. (1967) *Ann. Technol. Agric.* **16**, 135.

64 respectively. These values are much closer than those reported in Table 1 and suggest that, as far as amylase inhibitors are concerned, the gel filtration of Fig. 1 should rather be considered a partition according to the MW of a mixture of inhibitors with comparable activities than a real purification step.

TABLE 2. SUMMARY OF AMINO ACID COMPOSITIONS* OF PEAKS II, III AND IV AND OF THE AMYLASE INHIBITORS PURIFIED FROM WHEAT SEED BY DIFFERENT AUTHORS

Amino acid	Amino acid composition (μmol %) Peak II	Peak IV	AmI_1 inhibitor (Shainkin and Birk, 1970)	0·28 Inhibitor (Silano *et al.*, 1973)
Lys	3·6	4·6	5·2	5·0
His	2·9	trace	trace	trace
Arg	4·7	6·0	6·5	6·4
Asp	8·4	8·1	7·8	7·5
Thr†	4·4	3·7	1·9	2·7
Ser†	6·1	7·1	7·2	7·3
Glu	12·8	12·3	8·5	11·4
Pro	7·4	8·6	9·0	7·6
Gly	9·3	9·9	9·1	7·8
Ala	7·6	7·6	9·1	7·8
½ Cys	4·3	6·5	5·7	8·2
Val‡	7·0	10·9	12·2	10·8
Met	2·2	2·0	2·7	2·6
Ileu‡	3·8	2·1	2·2	1·6
Leu	8·0	6·6	8·0	8·2
Tyr	3·4	3·1	3·6	3·3
Phe	3·6	trace	trace	trace
	Peak III	0·19 Inhibitor (Sodini *et al.*, 1970)	AmI_2 Inhibitor (Shainkin and Birk, 1970)	Inhibitor I (Saunders and Lang, 1973)
Lys	3·2	2·6	2·5	2·8
His	2·0	1·6	1·4	1·4
Arg	5·3	5·6	6·6	5·4
Asp	6·1	5·9	6·1	6·4
Thr†	4·3	2·9	2·7	2·8
Ser†	5·5	6·3	5·4	6·7
Glu	12·5	12·8	10·7	11·6
Pro	8·4	7·3	8·2	7·7
Gly	8·5	8·4	8·4	10·4
Ala	12·2	13·3	13·4	14·9
½ Cys	5·1	6·1	6·9	3·3
Val‡	7·2	7·4	7·2	8·4
Met	2·2	2·0	2·3	1·7
Ileu‡	3·0	2·4	2·3	2·5
Leu	7·4	8·3	8·2	8·4
Tyr	3·5	3·3	4·2	3·6
Phe	1·1	1·6	1·8	1·6

* Values were all recalculated as percentages.
† Extrapolated to zero time, assuming first-order kinetics of destruction.
‡ Average values from 48 and 72 hr hydrolysates only.

As far as we know this is the first report on 60000 dalton albumin inhibitors from wheat, whereas the 11000 dalton inhibitors are, beyond any doubt, coincident with the components of the 0·28 albumin family[9] and, therefore, also related to the AmI_1 inhibitor of Shainkin and Birk.[8] We have also confirmed the data of Saunders and Lang[10] on the presence in wheat seed of several 24000 dalton inhibitors. Since the amino acid composition

and specific inhibitory activities toward amylase of the 24000 dalton inhibitor pool were found almost identical to those reported for the 0·19 component. it appears that all the other inhibitor components quantitatively representative of this pool must be very similar to the 0·19 inhibitor. The amylase inhibitors of MW 24000 daltons dissociated in the presence of 6 M guanidine hydrochloride. This behaviour was identical to that of purified 0·19, which is an associating system of two similar subunits.[9] Removal of guanidine hydrochloride caused amylase inhibitors to associate again in such way that we were unable to differentiate electrophoretic inhibition patterns of treated and untreated inhibitors. At this moment we have no explanation for such a highly specific reassociation.

Our results provide considerable evidence for the close relationship of the α-amylase inhibitor components of MW 24000 daltons and justify referring to these components as a family of proteins. From the electrophoretic mobility of the main component. we will refer to this albumin group as the 0·19 family. From the comparison of the properties and compositions reported for the AmI_2 inhibitor of Shainkin and Birk[8] and for the inhibitor I of Saunders and Lang,[10] it appears that these two inhibitors are most likely to be considered components of this albumin family. The components of the 0·19 family represent about 1/3 of the total albumin pool. that is about 0·1% of seed weight. They not only inhibit insect α-amylases. but also α-amylases from mammals and birds and are, therefore. less specific and of more relevant nutritional interest as compared to the other inhibitors only active toward insect amylases.

In view of the considerable similarity between components of the 0·19 and 0·28 families in their activities on insect amylases. we speculate that subunits of the 0·19 family components may be related to the albumin components of the 0·28 family. In support of this proposal, it may be noted that MWs of the subunits of the 0·19 family components fall within the range of MWs obtained for the 0·28 family and that the amino acid compositions of the two albumin families appear quite similar. Moreover, Shainkin and Birk[8] by means of CNBr degradation of the AmI_2 inhibitor obtained a protein fragment whose inhibition pattern was identical to that of the AmI_1 inhibitor. If fingerprinting and sequencing studies on the subunits of the 0·19 family components and on the components of the 0·28 family confirm this hypothesis, a monophyletic origin of such a consistent part of wheat albumins might be inferred.

EXPERIMENTAL

Protein extraction and purification. Finely ground whole wheat flour (1 kg), pure variety Mentana, was extracted at room temp. for 3 hr in a shaker with 1·5 l. of a 150 mM NaCl soln. After extraction, the suspension was centrifuged for 20 min at 20000 *g*. The clear supernatant was used as starting material for preparation of albumin inhibitors. Further extractions of the residue were carried out, under identical experimental conditions. by mechanical suspension of the sediment in 1·5 l. of the salt soln or of other solvents specified in the paper. The albumins were separated from the first salt extract by salting out between 0·4 and 1·8 M $(NH_4)_2SO_4$. The ppt obtained was collected by centrifuging at 45000 *g*. dissolved in H_2O, dialysed for 48 hr against cold H_2O and freeze-dried.

Gel filtration. About 1 g of albumins was dissolved in 100 ml of a pH 7·4 phosphate buffer (137 mM NaCl, 3 mM KCl, 1 mM KH_2PO_4, 8 mM Na_2HPO_4 and 15 mM NaN_3), applied on a column (110 × 4·5 cm) of Sephadex G100 (Pharmacia, Uppsala, Sweden) and eluted with the same phosphate buffer at a rate of 60 ml/hr. The absorbance of the eluate at 280 nm was monitored and recorded continuously. Fractions (20 ml) were collected and tested for amylase and trypsin inhibitory activities as described below. Fractions corresponding to central cuts of each active peak were pooled, dialysed for 48 hr at 4° and freeze-dried. Gel filtration on Sephadex G100 was also used to determine MWs of amylase and trypsin inhibitors in the presence and in the absence of 6 M guanidine hydrochloride as described below. Removal of guanidine hydrochloride from protein samples was achieved by filtering the sample through a Sephadex G50 (type coarse) column (2·5 × 25 cm).

Polyacrylamide gel electrophoresis and electrofocusing. Anodic and cathodic disc electrophoretic runs were carried out in a 0·05 M Tris–0·38 M glycine buffer (pH 8·5) as previously described.[25] 100–300 μg of proteins were used for anodic and 1500 μg for cathodic runs. Bromophenol blue mobility in the system was assumed to be 1. Electrofocusing fractionations were performed according to Cantagalli *et al.*[26] on 100 μg protein samples. Amylase and trypsin inhibitor patterns were determined by slicing the gel at the end of electrophoresis and focusing. Each slice, 0·3-cm thick was eluted for 18 hr at 4° in 1 ml of buffer. 10–200 μl of the eluate was used for amylase inhibition assay and 800 μl for trypsin inhibition test as described below.

MW. The MW of amylase and trypsin inhibitors were determined by gel filtration on Sephadex G100 according to the method of Andrews.[23] A 0·8 × 110 cm column was used with a flow rate of 6 ml/hr. The gel column was calibrated with the following proteins: ribonuclease (MW 13700) and chymotrypsinogen (MW 25700) from Sigma Chemical Company (St. Louis, U.S.A.), bovine serum albumin (MW 69000) from Armour Pharmaceutical Company (Chicago, U.S.A.). Blue Dextran was from Pharmacia (Uppsala, Sweden). Gel filtrations on Sephadex G100 in the presence of 6 M guanidine hydrochloride were performed on a 1·5 × 71 cm column at a flow rate of 10·4 ml/hr. Standard proteins used to calibrate the column were chymotrypsinogen (MW 25700), ribonuclease (MW 13700) and insulin (MW 5800) from Sigma Chemical Company (St. Louis, U.S.A.). Sedimentation studies were made in a Spinco model E Ultracentrifuge, with schlieren optics, under the following conditions: rotor speed 60000 rpm, temp. 4–6°, protein concentrations were between 0·5–1·0%. Sedimentation velocity was measured in the conventional way.[27–28].

Amino acid analysis. The determination of the amino acid composition of the albumins studied was carried out according to the chromatographic technique of Spackman *et al.*[29] Duplicate samples (2 ml HCl/mg of protein) were hydrolysed at 110° under N_2 for 24, 48 and 72 hr. The analyses were performed automatically by means of a Technicon AA Analyser (Technicon Instrument Corporation, Ardsley, N.Y., U.S.A.) using a 140 × 0·6 cm column, filled with Cromo-beds resins (type A), according to the Technicon standard method.

Amylase and trypsin inhibition. The inhibitory effect of our proteins was determined on α-amylase from several sources. *Tenebrio molitor* L. larval midgut α-amylase was prepared according to Applebaum *et al.*,[30] whereas all other insect amylase preparations were crude extracts obtained homogenizing the whole insect in a barbiturate buffer (10 mM sodium barbiturate, 10 mM sodium acetate, 150 mM NaCl, 0·3 mM $CaCl_2$, adjusted to pH 5·4 with 1 N HCl). Chick and hog pancreatic α-amylases were extracted by homogenizing pancreas in the barbiturate buffer adjusted to pH 7·6. Human pancreatic amylase was whole pancreatic juice supplied by S. Auricchio, II Clinica Pediatrica (University of Naples), diluted 10 × with the pH 7·6 barbiturate buffer, and human saliva α-amylase was freeze-dried crude saliva. Amylase inhibition tests based upon starch hydrolysis were performed according to the procedure described by Silano *et al.*[9] The barbiturate buffer was used for inhibition assay of all the amylases tested. The buffer pH, however, was different according to the origin of the amylase. It was 5·4 for insect amylases, 7·0 for human saliva amylase and 7·6 for chick, hog and human pancreatic amylases. All incubations lasted 5 min. The inhibition unit (I.U.) represents the protein amount that gives 30% inhibition of the amylase amount that produces 1 mg of maltose in 5 min under our experimental conditions.

Trypsin inhibition tests were based upon the hydrolysis of benzoil-DL-arginine-*p*-nitroanilide (DL-BAPA) and carried out according to Erlanger *et al.*[31] About 6 μg of trypsin (Merck Darmstadt, Germany) dissolved in 0·1 ml H_2O were added to 0·9 ml of Tris–HCl buffer (50 mM Tris, 20 mM $CaCl_2$, pH 8·2) containing the inhibitor. The mixture was held at room temp. for 30 min and then 25 μl of DL-BAPA solution (43·5 mg/ml dimethylsulfoxide) was added. After additional 15 min at 25°, addition of 0·3 ml of 30% HOAc terminated the reaction and the quantity of *p*-nitroaniline was estimated spectrophotometrically at 410 nm. Controls without inhibitors were also included. The trypsin inhibition unit (I.U.) represents the amount of protein that gives 30% inhibition of 0, 2 μg of trypsin.

Protein measurements. Just before each experiment, fresh solns were prepared from weighed portions of the lyophilized fractions. After centrifugation, the protein concentration of the clear supernatant was determined spectrophotometrically according to Waddell.[32]

Acknowledgements—We wish to thank Miss R. Di Salvio for skilful assistance in preparative gel filtration, Mr. F. Gramenzi for competent technical assistance, Mr. F. Malatesta for drawing work and Mr. A. Farina for photographic work.

[25] MINETTI, M., PETRUCCI, T., POCCHIARI, F., SILANO, V. and AVELLA, R. (1971) *J. Sci. Food Agr.* **22,** 72.
[26] CANTAGALLI, P., GIORGIO, G., MORISI, G., POCCHIARI, F. and SILANO, V. (1971) *J. Sci. Food Agr.* **22,** 256.
[27] ALEXANDER, P. and BLOCK, R. J. (1961) *Analytical Methods in Protein Chemistry*, Vol. 3, Pergamon Press, Oxford.
[28] SCHACHMAN, M. K. (1959) *Ultracentrifugation in Biochemistry*, Academic Press, New York.
[29] SPACKMANN, D. M., STEIN, W. H. and MOORE, S. (1958) *Analyt. Chem.* **30,** 1190.
[30] APPLEBAUM, S. W., JANKOVIC, M. and BIRK, Y. (1961) *J. Insect. Physiol.* **7,** 100.
[31] ERLANGER, B. F., KOKOWSKY, N. and COHEN, W. (1961) *Arch. Biochem. Biophys.* **95,** 271.
[32] WADDELL, W. J. (1956) *J. Lab. Clin. Med.* **48,** 311.