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AFFINITY COLUMN PURIFICATION OF AMYLASES ON PROTEIN INHIBITORS FROM WHEAT KERNEL

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SUMMARY

An affinity column was devised for the purification of a large number of amylases inhibited by the albumin from wheat kernel. The procedure involved linking the protein inhibitors from wheat to Sepharose and then specifically eluting the amylase adsorbed to the gel with a high concentration of maltose. By this procedure, the amylases from *Tenebrio molitor* L. (yellow mealworm) larvae and chicken pancreas were purified to homogeneity with good yields for the first time, as shown by both alkaline and acidic electrophoresis. Human saliva α-amylase, purified by the same procedure, showed specific activity and electrophoretic patterns similar to those obtained by other workers with different techniques.

INTRODUCTION

A protein fraction from wheat kernel that inhibits α-amylase was first described in 1943[1], but only recently has it been shown[2] that a large part of wheat albumins displays inhibitory activity towards a number of amylases from very different origins. Wheat albumin inhibitors were separated into three groups that differed in their apparent molecular weights (60,000, 24,000 and 12,500) and in other physico-chemical properties[2,3]. In the 24,000- and 12,500-dalton albumin fractions, several related protein components, which were slightly different in their electrophoretic mobilities but with identical amylase inhibition specificity, were observed[2,4,5]. The 12,500-dalton albumin fraction was the most active in inhibiting amylases from insect species, such as yellow mealworm (*Tenebrio molitor* L.), granary weevil and sawtoothed grain beetle, that attack wheat and stored wheat products. This fraction also inhibited a number of amylases from marine species (octopus, squid, cockle), but was inactive toward avian and mammalian amylases[6]. With a few minor exceptions, the 24,000-dalton albumin fraction inhibited all of the amylases that were susceptible to the ac-

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tion of the 12,500-dalton fraction and, in addition, was very active toward several avian (chicken, turkey, quail) and mammalian (man, cat, dog) amylases[6]. Some microbial amylases from *Streptomyces* and from *Bacillus subtilis* mutants inhibited by wheat albumins have also been described[1,7]. Amylase inhibition by wheat proteins has been detected not only *in vitro*, but also in man and in insect larvae[8,9]. Of all the amylases inhibited, only those from human saliva and pancreas have been purified so far[10-12], and we therefore considered it worthwhile to exploit the interaction among amylases and wheat albumins in order to develop a rapid affinity column procedure on agarose-bound albumin inhibitors for the purification of amylases.

EXPERIMENTAL

Whole albumin fraction (Alb*) was obtained by salting-out with ammonium sulphate of a saline extract of wheat flour according to Petrucci *et al.*[2]. Alb 12 and Alb 24 were prepared by gel filtration of Alb[2]. The Alb, Alb 12 and Alb 24 fractions were covalently bound to Sepharose 2B (Pharmacia, Uppsala, Sweden) by a technique described elsewhere[13]. For Sepharose activation, 250 mg of cyanogen bromide were used per millilitre of settled gel. Coupling was carried out at pH 6.6 in 0.1 *M* sodium phosphate buffer solution by using 5 mg of inhibitors per millilitre of settled gel. Coupling to Affi-Gel 10 (Bio-Rad Labs., Richmond, Calif., U.S.A.) was performed according to the manufacturer's instructions; 50 mg of inhibitor, dissolved in 30 ml of 0.1 *M* sodium phosphate buffer solution, pH 7.0, were used per gram of dry gel. In order to measure the efficiency of the coupling reactions, 0.25 ml of packed gel were hydrolyzed in 6 *N* hydrochloric acid at 120° for 20 h and processed on an amino acid analyzer.

T. molitor L. α-amylase was extracted by homogenizing in a Waring blender 40 g of insect larvae with 100 ml of 0.02 *M* sodium acetate buffer solution, pH 5.4, that was 0.1 *M* in sodium chloride. The homogenate was centrifuged for 30 min at 20,000 *g*. The supernatant was filtered through cotton-wool and submitted to salting-out with ammonium sulphate of concentration between 1.8 and 4.2 *M*. The precipitate, collected by centrifuging at 20,000 *g* for 30 min, was re-dissolved and extensively dialyzed against the acetate buffer. This precipitation step was mainly intended to reduce adsorption of contaminating material on the affinity column; omission of this step did not affect the purity of the final product. About 2000 α-amylase units were loaded on to a column (9.0 $\times$ 1.0 cm) of Sepharose–Alb 12 gel equilibrated with the acetate buffer. The column was washed at a flow-rate of 15 ml/h until no absorbance at 280 nm was detectable in the effluent. The enzyme was then eluted from the column with the same buffer that was 0.5 *M* in maltose. The fractions containing the enzyme were pooled and loaded on to a Sephadex G-25 fine column (50 $\times$ 2.5 cm) equilibrated with a 10-fold dilution of the acetate buffer. The fractions containing the enzymatic activity were pooled and freeze-dried. The whole procedure was carried out at 4°.

The starting material for the purification of human saliva α-amylase was crude saliva. Freeze-dried saliva (100 mg) from several donors was dissolved in 100 ml of 0.02 *M* sodium barbital buffer, pH 7.0, that was 0.1 *M* in sodium chloride. The suspension was clarified by centrifuging at 40,000 *g* for 20 min and the clear supernatant,

* The abbreviations used are: Alb = whole albumin preparation; Alb 12 = albumin fraction of molecular weight 12,500; Alb 24 = albumin fraction of molecular weight 24,000.

containing about 10,000 α-amylase units, was loaded on to a Sepharose–Alb 24 column (9.0 × 1.0 cm) equilibrated with the barbital buffer. The affinity chromatography was run at a flow-rate of 30 ml/h.

Chicken amylase was extracted by homogenizing samples of pancreas with the barbital buffer (1:10, w/v) in a Potter homogenizer at 0°. The suspension was centrifuged at 40,000 *g* for 20 min and the clear supernatant containing about 200 amylase units was passed through a small column (2.5 × 0.5 cm) of Sepharose–Alb gel. The subsequent purification steps for human saliva and chicken pancreas amylases were identical with those for *T. molitor* α-amylase.

Assay of amylase activity was carried out in the acetate buffer for the insect species and in the barbital buffer for the human and avian species. The amylase activity was measured by following the production of new reducing ends with the Nelson method as modified by Robyt and Whelan[14]. One amylase unit was defined as the amount of enzyme that produced 1 μmole of maltose in 1 min at 37° under our experimental conditions. For routine assays and amylase determinations in the presence of maltose, the iodine-staining method was used[14].

Disc gel electrophoresis was carried out in 0.05 *M* Tris–0.383 *M* glycine buffer, pH 8.5, as described by Davis[15]. In order to locate on the gel the protein bands that exhibited amylase activity, 0.1% of gelatinized starch was included in the acrylamide gel. The amylase zymogram was developed by incubating the gel at the end of the electrophoresis in the appropriate buffer for 20 min and staining the gel with the iodine-staining reagent used for the amylolytic assay. Slab gel electrophoresis was carried out in 0.35 *M* β-alanine–0.14 *M* acetic acid buffer, pH 4.3, under the experimental conditions described by Mimmo *et al.*[16].

Protein concentration was determined by using the method of Lowry *et al.*[17].

RESULTS AND DISCUSSION

As Alb 12 was the most active albumin fraction from wheat kernel in inhibiting insect amylases, it was chosen for the preparation of a Sepharose–inhibitor affinity gel to be used for the purification of α-amylase from *T. molitor* L. larvae. As shown by the amino acid analysis, Alb 12 was effectively bound to Sepharose by the procedure of Cuatrecasas and Anfinsen[13]; 1 ml of the packed gel contained about 4.5 mg of albumin inhibitor. The Sepharose–Alb 12 gel exhibited high affinity and selective binding for *T. molitor* α-amylase. When about 2,000 units of α-amylase, obtained by salting-out a crude extract from insect larvae (Table I), were passed through a short column of the affinity gel at a flow-rate of 15 ml/h, over 94% of the protein and no amylase activity were recovered in the effluent. By doubling the flow-rate during the column loading, a 20% reduction in the amylase units retained was observed. This result indicated that, at least under our experimental conditions, the effectiveness of α-amylase retention by the column was dependent upon the equilibration time between the enzyme and the insolubilized inhibitor. This was in agreement with the results reported by other workers[1,5] who, in a homogeneous phase, obtained an increase in inhibitory activity (and presumably in the enzyme–inhibitor complex formation) by increasing the incubation time between amylase and inhibitor in the absence of starch. After the unadsorbed material was washed out from the affinity column, several attempts were made to elute the retained α-amylase. Solutions of high ionic strength

TABLE I

PURIFICATION OF *Tenebrio molitor* L. AND HUMAN SALIVA α-AMYLASES

Amylase	*Total protein (mg)*	*Amylase activity (units)*	*Specific activity (units/mg)*	*Relative purification*	*Recovery (%)*
T. molitor amylase					
Crude extract	177	1,926	10.9	1	100.0
Ammonium sulphate precipitate	71	1,695	23.9	2	88.0
Affinity chromatography*, gel filtration and freeze-drying	1.5	1,259	859.3	75	65.4
Salivary amylase					
Crude saliva	26.5	10,000	377	1	100.0
Affinity chromatography*, gel filtration and freeze-drying	3.2	5,780	1806	5	57.8

* Amylase activity was not evaluated in this step because of maltose inhibition.

(up to 2) or low pH (down to 4) were ineffective, whereas the α-amylase was eluted as a sharp peak on passage through the acetate buffer that was 0.5 *M* in maltose or contained 1% of gelatinized starch, thus showing, for the first time, the reversibility of wheat albumin inhibition. Maltose was preferred to starch because of the difficulty of removing the starch from the eluted enzyme. The maltose was separated from the eluted enzyme by chromatography on a Sephadex G-25 column equilibrated with 10-fold diluted acetate buffer. The enzymatic activity was eluted well ahead of the maltose and the active fractions were pooled and freeze-dried. The overall recovery of enzyme through the two chromatographic steps was 74% with a 37-fold increase in the specific activity (Table I). When maltose concentrations of less than 0.5 *M* were used for the α-amylase elution, much lower α-amylase recoveries were obtained. The recovery of α-amylase decreased by about 15% when the flow-rate was doubled during elution. This finding showed that not only the formation of the enzyme–inhibitor complex, but also the dissociation of this complex by maltose, was a time-dependent process. *T. molitor* α-amylase purified with the affinity procedure summarized in Table I gave a single band when analyzed by both anionic (Fig. 1) and cationic polyacrylamide gel electrophoresis. This band, as shown by the zymogram obtained for the alkaline acrylamide gels containing 0.1% of gelatinized starch, exhibited amylase activity; only one amylase band with identical mobility was also found in the crude extract from *T. molitor* larvae.

As human saliva α-amylase, which is not inhibited by Alb 12, was not retained on the Sepharose–Alb 12 column, the purification of this α-amylase was achieved by chromatographing crude human saliva on a column of Sepharose–Alb 24 gel. About 10,000 α-amylase units were retained by the column when the enzyme loading was carried out at a flow-rate of 30 ml/h. The best result in the α-amylase desorption was obtained by eluting with the barbital buffer that was 0.5 *M* in maltose at the same flow-rate. As already described for the *T. molitor* L. α-amylase, increasing the flow-rate during α-amylase loading or elution adversely affected the recovery of the pure enzyme. With a single-step affinity purification, we obtained a 5-fold increase in the α-amylase specific activity with a 58% recovery of the amount loaded on the affinity column (Table I). This result appears to be comparable with those obtained by other

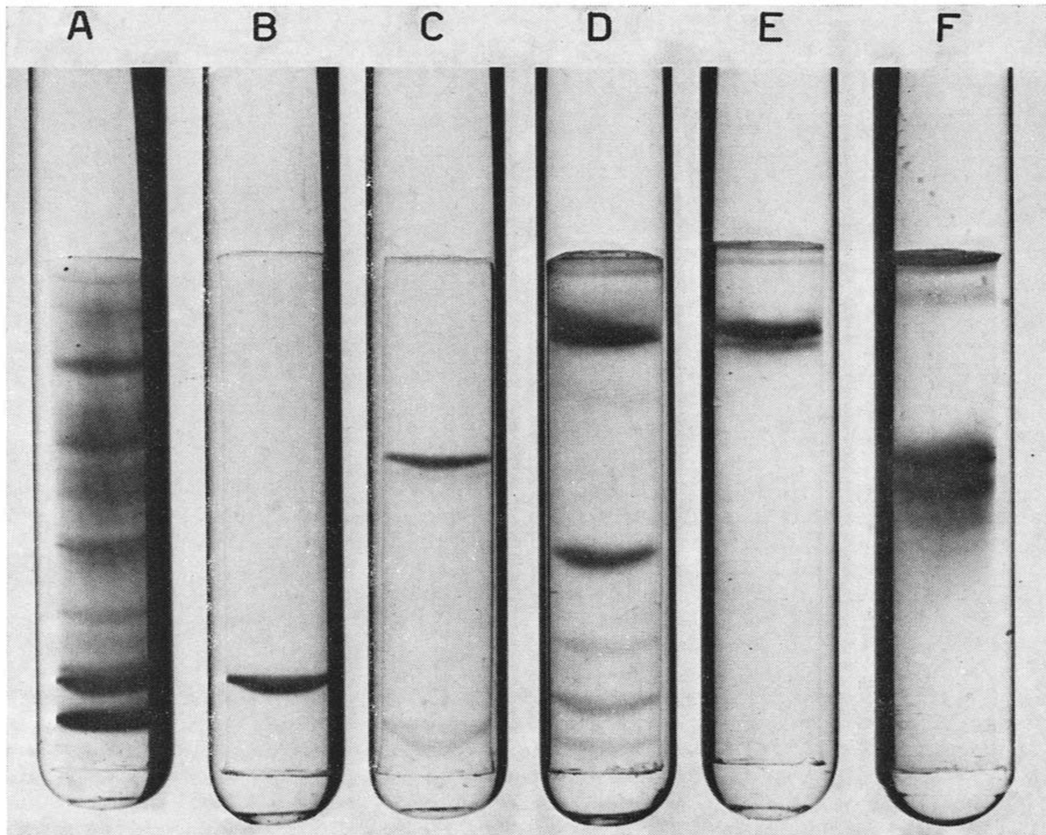

Fig. 1. Polyacrylamide gel electrophoresis of crude and purified amylases. The electrophoresis was carried out in 0.05 *M* Tris–0.383 *M* glycine buffer, pH 8.5. The anode is at the bottom. Gels A and B, *T. molitor* α-amylase after ammonium sulphate fractionation and after affinity chromatography, respectively; gel C, chicken pancreas amylase purified by affinity chromatography; gel D, crude human saliva; gels E and F, 60- and 120-min runs, respectively, of human saliva α-amylase purified by affinity chromatography.

workers with longer procedures[10–12]. The polyacrylamide gel electrophoresis pattern of the purified α-amylase was similar to that obtained by other workers[10] (Fig. 1).

Comparable results in the purification of these α-amylases were obtained by using, instead of the Sepharose–Alb 12 or Sepharose–Alb 24 gels, gels prepared by coupling Alb to Sepharose or to Affi-Gel 10. However, these two Alb gels exhibited a lower amylase retention efficiency.

The affinity column procedure was also used for the purification of chicken pancreas α-amylase. When about 200 amylase units from crude pancreatic extracts were filtered through a Sepharose–Alb column, 96% of the protein and no amylase activity were recovered in the effluent, whereas with 0.5 *M* maltose in the barbital buffer, about 90% of the enzyme was eluted. The amylase obtained showed two enzymatically active bands on anionic (Fig. 1) and cationic polyacrylamide gel electrophoresis.

The above Sepharose-inhibitor gels have been found to be very effective for the rapid purification of three amylases representative of the many from insect, mammalian and avian species that are inhibited by wheat albumins. Preliminary (unpublished) experiments have shown that octopus amylase can also be purified with such gels. Therefore, the general suitability of the affinity method described in this paper for the purification of any amylase that is able to interact with wheat albumins could be inferred.

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