INTERACTION OF *TENEBRIO MOLITOR* L. α-AMYLASE WITH A WHEAT FLOUR PROTEIN INHIBITOR

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1. Introduction

It is well known [1,2] that crude preparations of larval *Tenebrio molitor* L. (yellow mealworm) α-amylase (1,4-α-D-glucan glucano-hydrolase, EC 3.2.1.1) are effectively inhibited by a number of highly purified albumins from wheat flour. The best known of these protein inhibitors has a mol. wt. 24 000 and consists of two similar subunits which are dissociated by chemical agents such as dodecyl sulphate or guanidine [2]. This inhibitor, coded 0.19 according to its gel electrophoretic mobility relative to bromophenol blue [3] and referred to here as 0.19 albumin, inhibits, in addition to the amylase from *T. molitor*, a large number of amylases of different origin [4]. The influence of several enzymatic and physico-chemical treatments on the activity of 0.19 [4] and other wheat albumin inhibitors [1] of *T. molitor* α-amylase has been studied.

Recently, the α-amylase from *T. molitor* larvae has been purified by one-step chromatography on a Sepharose–albumin inhibitor column and extensively characterized [5,6]. The enzyme has a single polypeptide chain with mol. wt. 68 000 and exhibits several properties similar to those of mammalian α-amylases [6].

This paper deals with the influence of a number of physical and chemical parameters on the enzyme-inhibitor reaction and provides conclusive evidence of the formation of an enzyme–inhibitor complex. A preliminary characterization of such a complex is also reported.

2. Experimental

T. molitor α-amylase was obtained in a homogeneous form from insect larvae by following the method of Buonocore et al. [5] and the 0.19 albumin was purified from wheat flour as already described [3].

Amylase activity was determined by the Nelson's colorimetric method [7] in 50 mM sodium cacodylate-HCl buffer, pH 5.8, containing 10 mM NaCl and 0.1 mM $CaCl_2$, as already described [6]. Standard inhibition assay was carried out by preincubating the amylase with 0.19 albumin for 10 min at 37°C in 20 mM sodium cacodylate-HCl buffer, pH 5.0, containing 10 mM NaCl and 0.1 mM $CaCl_2$. Amylase assay in the presence of mono- and oligo-saccharides was carried out with the iodine-staining method [8].

Protein concentration was determined by the Lowry method as modified by Hartree [9] using bovine serum albumin as a standard, or spectrophotometrically as described by Waddel [10].

Polyacrylamide gel electrophoresis was carried out in 50 mM Tris–383 mM glycine buffer, pH 8.5, as described by Davis [11]. Protein elution from the gel was carried out by slicing the gel at the end of the

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electrophoretic run; each slice, 0.3 cm thick, was eluted for 24 h at 20°C in 0.5 ml of 50 mM sodium cacodylate-HCl buffer, pH 5.8, containing 10 mM NaCl and 0.1 mM $CaCl_2$. Polyacrylamide gel electrophoresis in sodium dodecyl sulphate was carried out as described by Laemmli [12].

Gel filtration was performed on a Sephadex G-100 column (1.5 × 90 cm) equilibrated with the cacodylate buffer (pH 5.0). Elution was carried out with the same buffer at a flow rate of 9 ml/h. Each fraction (1.5 ml) was tested for absorbance at 280 nm and amylase and inhibitor activity. The fractions containing the enzyme–inhibitor complex were pooled and ultrafiltered through a Diaflo UM-10 membrane.

Sedimentation-equilibrium runs were performed at 12°C in a Beckman E analytical ultracentrifuge for either 48 h at 22 000 rev/min or 72 h at 15 000 rev/min.

Ultraviolet measurements were carried out with a Cary 15 spectrophotometer and circular dichroism measurements with a Cary 60 spectropolarimeter equipped with a 6002 CD accessory.

3. Results and discussion

3.1. *Inhibition of amylase*

Inhibition of *T. molitor* α-amylase was tested at several concentrations of inhibitor using different pre-incubation times before the addition of starch, as well as without pre-incubation. The inhibition rapidly increased in the first few minutes and reached a maximum within 10 min of pre-incubation. Further increases in the pre-incubation time up to 3 h did not affect the inhibitory activity. The pattern of the α-amylase residual activity after treatment, under standard conditions, with different amounts of inhibitor is shown in fig.1a. The inhibitory activity increased linearly with the pre-incubation temperature up to 40°C (fig.2a). The optimum pH for the inhibition reaction was 5.0 (fig.2b) which is slightly lower than the optimum pH for the enzymatic activity and almost intermediate between the isoelectric points of the enzyme and inhibitor [3,6].

The inhibition of *T. molitor* amylase by 0.19 was studied according to the Michaelis-Menten kinetics under optimal conditions in the inhibitor concentration range which gave a linear response to inhibitor. A family of parallel lines was obtained with both the graphical methods of Lineweaver-Burk and Dixon. Assuming an uncompetitive inhibition, an apparent K_i of 3×10^{-7} M was calculated from the Dixon plot. This value is very close to that obtained for the chicken pancreatic amylase-0.19 albumin system by Saunders and Lang [13] whose data also were in agreement with an uncompetitive inhibition mechanism. An uncompetitive inhibition was also reported by Militzer et al. [14] in their early work on the inhibition of human salivary amylase by a crude protein inhibitor from wheat flour. All these data,

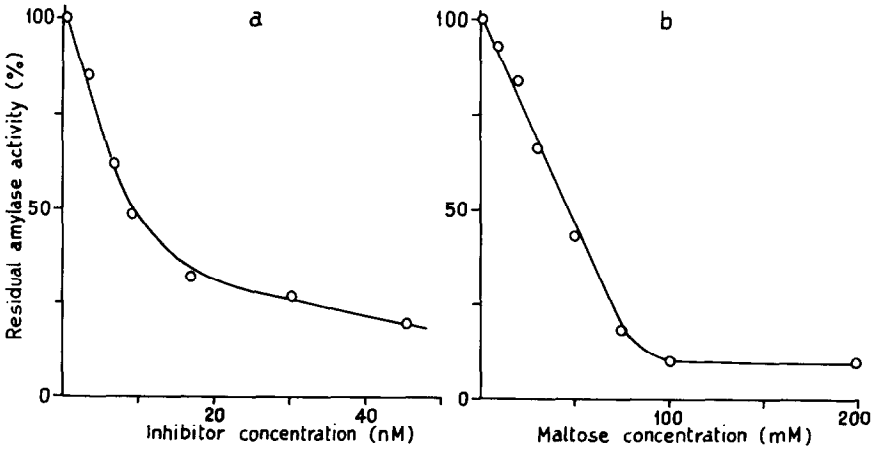

Fig.1. Inhibition of *T. molitor* amylase by (a) 0.19 albumin or (b) maltose. *T. molitor* amylase (1.4 nM) was tested under standard conditions with the inhibitor concentrations indicated.

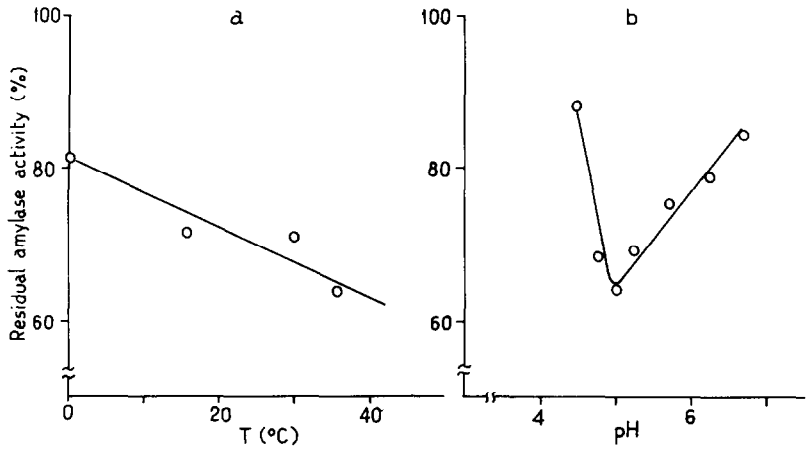

Fig.2. Effect of (a) temperature and (b) pH on the amylase inhibitory activity of 0.19 albumin. (a) *T. molitor* amylase (1.4 nm) was pre-incubated with inhibitor (7 nM) for 10 min at pH 5.0 at the temperatures indicated, then starch was added. (b) *T. molitor* amylase (1.4 nM) was pre-incubated with inhibitor (7 nM) for 10 min at 37°C in 20 mM sodium cacodylate-HCl buffers of the pH values indicated, then starch was added.

however, give little information in terms of the mechanism of inhibition because, under the experimental conditions used, the inhibitor concentration is not greatly in excess in comparison with the enzyme concentration, as is generally assumed in the classical kinetic treatments of enzyme inhibition. A further complication in the interpretation of these data come from the possibility that part of the substrate is actually bound with the inhibitor [15]. The kinetic data reported might indicate that the enzyme–inhibitor complex is capable of hydrolyzing starch. Such a possibility is also supported by the fact that complete inhibition of the enzyme could not be obtained at any inhibitor concentration tested (fig.1a).

Maltose inhibited the insect amylase (fig.1b) more effectively than pig pancreatic [16] and human salivary [4], α-amylases. From a comparison of fig.1a and 1b, it appears that 0.19 albumin was, on a molar basis, about 2×10^6 times more effective than maltose in inhibiting the insect amylase. Methyl α-D-glucoside exhibited an inhibitory activity equal to about 3% of that of maltose; these findings are in good agreement with the results of Thoma et al. [17] who showed that the methyl α-D-glucoside can act as a competitive inhibitor of the α-amylase from *Bacillus amyloliquefaciens*. Maltotriose and maltotetraose showed an amylase inhibitory activity slightly higher than that of maltose; such a higher activity might be related to their being slowly reacting substrates [18]. D-Glucose and other monosaccharides (D-galactose, D-mannose, D-xylose, L-arabinose, L-sorbose and *N*-acetyl-D-glucosamine) did not show any amylase inhibitory activity even when tested at concentrations as high as 500 mM. The inhibitory activities of 0.19 albumin and maltose towards *T. molitor* amylase were not additive, suggesting that the two inhibitors bind to two distinct, but interacting, sites on the enzyme. As averaged from 8 experiments, the percent inhibition observed after treatment with 6.4 nM 0.19 albumin and 10 mM maltose was 43 ± 3, whereas the percent inhibitions observed with 0.19 albumin or maltose alone were 35 ± 2 and 31 ± 2, respectively. These findings are in agreement with those obtained with human salivary α-amylase [4] and with previous evidence [5] concerning the ability of maltose to reduce the affinity of the amylase for the albumin inhibitor. At the moment, it cannot be established whether maltose causes such an effect by evoking local structural changes or by stabilizing an amylase conformation less favorable for the binding of 0.19 albumin.

3.2. *Amylase–inhibitor complex*

When *T. molitor* amylase and 0.19 albumin were mixed a major new band appeared on gel electrophoresis (fig.3b). The mobility of the new band was

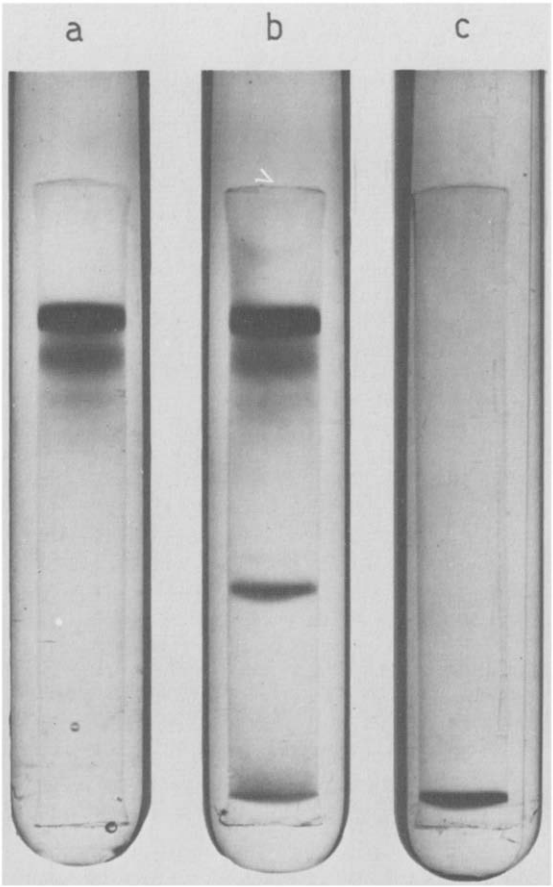

Fig.3. Polyacrylamide gel electrophoretic patterns of (a) 0.19 albumin, (b) a mixture of 0.19 albumin and *T. molitor* amylase, and (c) *T. molitor* amylase. *T. molitor* amylase and 0.19 albumin were incubated for 1 h at room temperature and then submitted to electrophoresis at pH 8.5. Proteins migrated to the anode (bottom).

intermediate between those of the inhibitor (fig.3a) and of the enzyme (fig.3c). By testing the protein fractions eluted from the gel, at the end of the electrophoretic run, we found in the new band a significant fraction of the amylase activity loaded on the gel. After thermal inactivation of the enzyme, we also detected, a significant amylase inhibitory activity in the band. Moreover, when this protein fraction was submitted to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, two bands corresponding to the enzyme and the inhibitor were clearly evident. It appears that the new band corresponds to the amylase–0.19 albumin complex. Further evidence of the formation of the complex was obtained by submitting an inhibitor–enzyme mixture to gel filtration through a Sephadex G-100 column. Although different in their mol. wts., the enzyme and the inhibitor exhibited an identical retention volume on this column because the amylase was retarded by the Sephadex matrix. When an inhibitor–enzyme mixture, containing 8 nmol of amylase and 10 nmol of 0.19 albumin, was filtered through the Sephadex column, two peaks were obtained (fig.4). As shown by polyacrylamide gel electrophoresis both in the presence and absence of sodium dodecyl sulphate (fig.4, insert), peak I, which was eluted at the position of bovine serum albumin, consisted of the enzyme–inhibitor complex, whereas peak II only contained 0.19 albumin. As

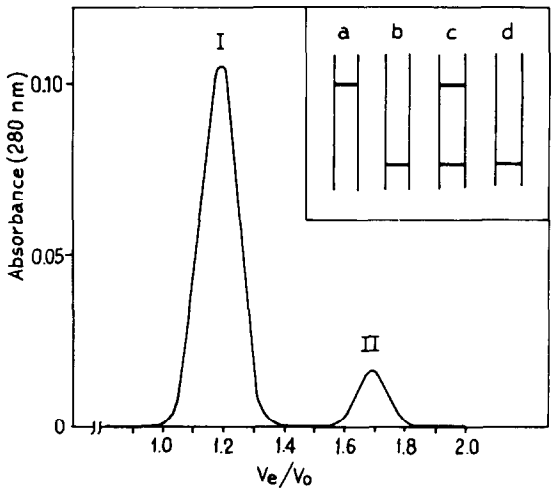

Fig.4. Elution profile of a mixture of *T. molitor* amylase and 0.19 albumin from Sephadex G-100. *T. molitor* amylase (8 nmol) and 0.19 albumin (10 nmol) were incubated for 1 h at room temperature and then loaded on a Sephadex G-100 column (1.5 × 90 cm). Retention volume (Ve/Vo) for reference proteins were: bovine serum albumin, 1.2; chymotrypsinogen, 1.7; cytochrome *c*, 1.95. Ve = elution volume; Vo = elution volume of blue dextran 2000. (Insert) Polyacrylamide gel electrophoresis in sodium dodecyl sulphate of (a) *T. molitor* amylase; (b) 0.19 albumin; (c) peak I; (d) peak II. The scheme shows the relative mobility of the bands towards the anode (bottom). When 0.19 albumin was loaded in high concentrations on such gels the two closely moving subunits, which are formed upon dissociation of the inhibitor in sodium dodecyl sulphate, were evident [2]; with very faint bands of inhibitor, as in the case of gels (c) and (d), the separation was not clearly detectable.

about 4 nmoles of 0.19 albumin were recovered in peak II, it was calculated that 8 nmol of amylase had reacted with 6 nmol of inhibitor.

The ultraviolet spectrum of the enzyme–inhibitor complex eluted as peak I (fig.4) is typical of a protein with a maximum at 278 nm. The extinction coefficient at this wavelength is 8.8 and therefore much closer to that of the enzyme [6] than to that of the inhibitor [2]. The circular dichroism spectrum of the complex in the aromatic region was closely related to that of the enzyme as well [6]. The sedimentation pattern of the complex showed a strong tendency of the complex to precipitate under the ultracentrifugal field. Such a tendency increased with the centrifugation time as shown by submitting the same sample to successive centrifugations. Under such conditions, no exact estimation of the complex mol. wt. was possible and a large spectrum of molecular weights was observed; the minimum mol. wt. calculated for the complex was 95 000. These data show that *T. molitor* amylase and 0.19 albumin form a relatively stable complex which, under the experimental conditons chosen, consists of 1 mole of enzyme for each mole of inhibitor. The 1:1 molar stoichiometric ratio is in good agreement with the ultracentrifugal properties of the enzyme–inhibitor complex. However, further studies are needed to show whether such a combination ratio is found under all experimental conditions.

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