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THE INHIBITION OF HUMAN SALIVARY α-AMYLASE BY TYPE II α-AMYLASE INHIBITOR FROM *TRITICUM AESTIVUM* IS COMPETITIVE, SLOW AND TIGHT-BINDING

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A kinetic analysis of the inhibition of human salivary α-amylase (EC 3.2.1.1) by wheat seed (*Triticum aestivum*) type II α-amylase inhibitor revealed the inhibition was slow and tight-binding. The inhibition was competitive with an inhibition binding constant of the α-amylase inhibitor for α-amylase of 0.29 nM. The K_M of α-amylase for soluble starch (calculated per mole of α–1,4 linked maltose residues) was 5.87 mM.

KEY WORDS: Human salivary α-amylase; *Triticum aestivum* type II α-amylase inhibitor; Competitive, slow, tight-binding inhibition

INTRODUCTION

Three different proteins in the 20–25,000 M_r range have been isolated from wheat seed (*Triticum aestivum*) that have much greater specificity for human salivary α-amylase (EC 3.2.1.1) than pancreatic α-amylase.[1-4] Although not rigorously quantified, the type II inhibitor (M_r, 21,400) appears to be more than 6 times a more effective inhibitor of human salivary α-amylase than type I (M_r, 22,500) whereas type I has a greater specificity for human salivary α-amylase relative to human pancreatic α-amylase by about the same factor. On the other hand, type II is more than an order of magnitude more specific for human salivary α-amylase relative to human pancreatic α-amylase than type III (M_r, 25,000).[3] Although the mode of inhibition of human salivary α-amylase (M_r, 98,000) by any of these three inhibitors has not been defined, it has been noted that the inhibition is strong and that the order of addition of reaction components is important.[2,4] Because these observations mirror characteristics of other more defined hydrolase : protein hydrolase inhibitor systems[5–8] including the inhibition of porcine pancreatic α-amylase by a red kidney bean α-amylase inhibitor,[8] we were prompted to test the question as to whether or not the

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mode of inhibition operating in the human salivary α-amylase : wheat germ type II α-amylase inhibitor system is also slow and/or tight-binding.

In instances of slow binding inhibition there is a discernible lag period before inhibition is manifested. In tight-binding inhibition the effective concentrations of inhibitor are in the same order as that of the enzymes they inhibit, thus, tight-binding systems cannot be analyzed using classical Michaelis-Menten inhibition kinetics.[2,5,7–11] In fact, Goldstein[12] pointed out in 1944 that in instances where the ratio of [enzyme]: K_i is greater than 0.001 the Michaelis-Menten assumptions do not hold.

We report here experiments demonstrating that the mode of inhibition of the human salivary α-amylase: wheat germ type II α-amylase inhibitor system is of the competitive, slow, tight-binding type (K_i, 0.29 nM). This is in contrast to the porcine pancreatic α-amylase : kidney bean α-amylase inhibitor system which, although slow and tight-binding, is non-competitive (K_i, 30 pM).[8]

MATERIALS AND METHODS

Human salivary α-amylase (Type IX-A), Type II α-amylase inhibitor from wheat germ (*Triticum aestivum*), maltose (Grade I), and soluble starch (ACS reagent grade) were obtained from the Sigma Chemical Company, St. Louis, MO, USA. The activity of α-amylase was measured using the method of Bernfield[13] in which the reducing groups of maltose units liberated from starch are measured by the production of a chromophore (maximum absorbance at 540 nm) that results from reduction of 3,5-dinitrosalicylic acid (Worthington Biochemical Corp., Freehold NJ, USA).

Solutions (1%) of 3,5-dinitrosalicylic acid were prepared weekly as follows. First, 1.0 g 3,5-dinitrosalicylic acid was dissolved in 50 ml water. To this 30 g sodium potassium tartrate were slowly added while stirring. Next, 20 ml of 2.0 M NaOH were added and the mixture was then diluted to 100 ml with water.

A standard curve for detection of reducing equivalents was prepared using a variety of maltose concentrations (0.58–5.84 μmol ml^{-1}) all in 1.0 ml. To each tube 1.0 ml of the 3,5-dinitrosalicylic acid reagent (above) was added, the resulting mixtures covered with marbles and then heated in a boiling water bath for 5 min. After cooling to 25°C, 10.0 ml of water was added to each tube and after mixing, absorbances at 540 nm were determined versus a water blank. Using the slope of this linear standard curve, the μmol of reducing equivalents produced when soluble starch was incubated with α-amylase could be estimated by dividing the A_{540} values by 0.172. Several concentrations of maltose were routinely assayed each time enzymatic assays were performed as assay controls.

Soluble starch solutions for enzymatic assays were prepared as follows. 2 g of starch were suspended in a final volume of 100 ml 20 mM sodium phosphate buffer (pH 6.9, 6 mM NaCl) and gently boiled. After cooling to 25°C, the volume was readjusted to 100 ml with water. Prior to assay, the stock starch solutions were incubated at 25°C for 4–5 min. Because molar concentrations of soluble starch solution cannot be determined, the concentration of α-amylase's hydrolytic substrate, α-maltose residues,[14] was calculated assuming the starch was composed of 100% α–1,4

linked glucan units. Thus, the starch stock solution translated to 58.6 μmol maltose residues ml^{-1}.

The standard starch solutions were used in enzymatic assays (all done at 25°C) in varying amounts depending on the particular experiment. For example, to determine both the linearity of the assay with respect to time and velocity with respect to α-amylase concentration, matrix-type experiments were performed using saturating amounts of starch (29.3 mM maltose residues; K_M, 5.87 mM). For such an experiment 0.5 ml of starch was added to each tube including the non-enzymatic controls. Next 0.25 ml water was added and then reactions were begun in 0.5 min intervals by addition of 0.25 ml of various concentrations of α-amylase (2.9–8.6 nM final concentrations). After incubating for different times (0.5–7.0 min), the reactions were stopped by addition of 1.0 ml of the 3,5-dinitrosalicylic acid reagent (44 mM in 0.4 M NaOH). Plots of velocity (μmol reducing equivalent produced min^{-1}) as a function of α-amylase concentration were linear for 5.0 min at all α-amylase concentrations and for 7.0 min up to 6.45 nM.

When reaction mixtures contained soluble starch, α-amylase inhibitor and α-amylase, they were also in a final volume of 1.0 ml that was 10 mM in sodium phosphate buffer, pH 6.9 and 3 mM NaCl and were done at 25°C.

A unit of α-amylase activity is defined as the amount of α-amylase necessary to release one μmol of reducing equivalent min^{-1} at 25°C and pH 6.9.

RESULTS

Slow Binding

Wheat germ type II α-amylase inhibitor was determined to be a slow-binding inhibitor of human salivary α-amylase by carrying out two types of preincubation experiments. First, starch and α-amylase inhibitor were preincubated for 15 min and then the reaction was started with the addition of α-amylase. An initial steady-state velocity changed to a slower steady-state velocity after 2–3 min. Next, amylase and the inhibitor were preincubated (also for 15 min) after which the reaction was initiated by the addition of soluble starch. After a 2–3 min lag a steady rate of reaction was observed that was essentially identical to the above steady-state velocity. Both results indicated that a slow type of binding had occurred between inhibitor and enzyme.[15]

Tight Binding

Preliminary experiments showed that the molar concentration of α-amylase inhibitor necessary to effectively inhibit α-amylase was of the same order of concentration as α-amylase, a result suggesting that the inhibition was tight-binding. An experiment was then performed to measure the steady state velocities obtained using a range of inhibitor or concentrations at each concentration of α-amylase. As shown by Figure 1, this experiment generated a series of curves with approximately linear and curved segments. At low inhibitor concentrations the linear regions had slopes

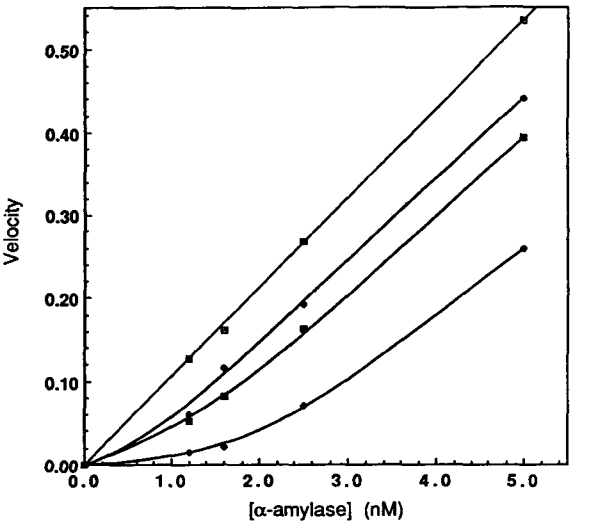

FIGURE 1 Tight binding between α-amylase and α-amylase inhibitor. □, no inhibitor; ♦, 1.7 nM inhibitor; ■, 2.5 nM inhibitor; ◇, 5.3 nM inhibitor. In the presence of 10.5 nM inhibitor no activity was observed. The line for 'no inhibitor' was "best fit"; the other lines were approximated. Velocities are μmol reducing equivalents produced min^{-1}.

roughly parallel to that of the uninhibited case. (At greater inhibitor concentrations the linear segments are not apparent because the concentration of free inhibitor is significant).[11] By replotting the data of Figure 1 as well as other data not shown (where the inhibitor concentration relative to enzyme concentration was sufficient for complete inhibition) as depicted in Figure 2, it is apparent that inhibition of α-amylase by α-amylase inhibitor is sigmoidal with 50% inhibition at equal molar concentrations of enzyme and inhibitor and that inhibition was essentially complete when the [α-amylase inhibitor] : [α-amylase] was 5:1. This does not represent a stoichiometric ratio, but rather a ratio which represents the steady-state situation of the various equilibria involved in the overall system.

Determination of the type of tight-binding inhibition

To determine the type of inhibition and the K_i of the inhibitor for α-amylase, velocities were determined at several substrate concentrations in the absence and presence of varied amounts of α-amylase inhibitor, each at a fixed concentration of α-amylase. The data generated by this experiment were then analyzed as described by Dixon.[9] Figure 3 is for a single concentration of substrate (14.6 mM) and is representative of those obtained at all other concentrations of substrate.

A "$K_{average}$" (K_{ave}) was thus obtained for each concentration of starch. A plot of these K_{ave}'s versus substrate concentration produced a straight line with ascending slope that is characteristic of competitive tight-binding inhibition (Figure 4). The ordinate

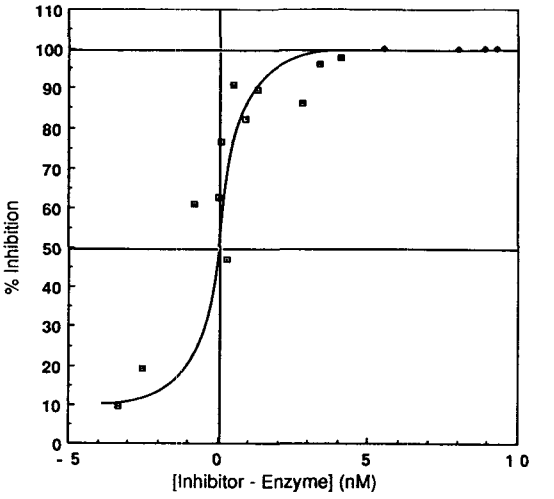

FIGURE 2 Inhibition of α-amylase by α-amylase inhibitor. Data from Figure 1 and other data not shown in Figure 1 are plotted to show the relationship between the concentration of free α-amylase and the extent of inhibition. The abscissa shows the nM excess of inhibitor to enzyme.

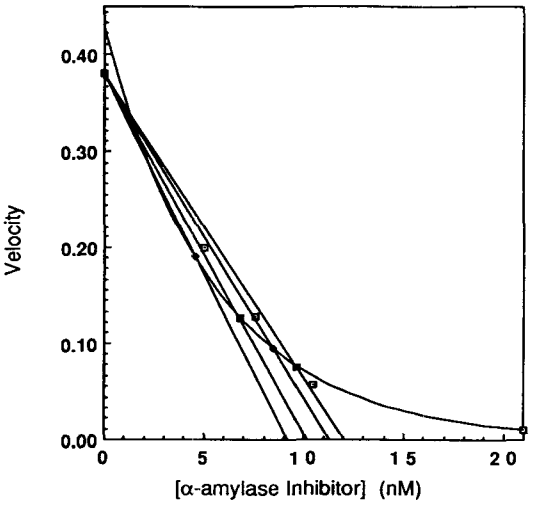

FIGURE 3 A plot at one substrate concentration (14.6 mM α–1,4 maltose residues) showing how velocity changed with inhibitor concentration after steady-state between α-amylase inhibitor and α-amylase was established. Following a 15 min preincubation of α-amylase and α-amylase inhibitor, reactions (2.5 min) were begun by addition of substrate. The curved line represents a curve generated by the "exponential" fit of empirical velocity measurements at various concentrations of inhibitor and in the absence of inhibitor (v_O, the ordinate value of 0.381 μmol min^{-1}. Points on the curve were selected at $\frac{v_o}{2}$, $\frac{v_o}{3}$, $\frac{v_o}{4}$, and $\frac{v_o}{5}$ ■ and straight lines were generated from v_o through these points to the abscissa. The distance values between the straight lines' intersections with the abscissa were determined and averaged to obtain the K_{ave} at the particular concentration of substrate; in the case illustrated, 0.97 nM.

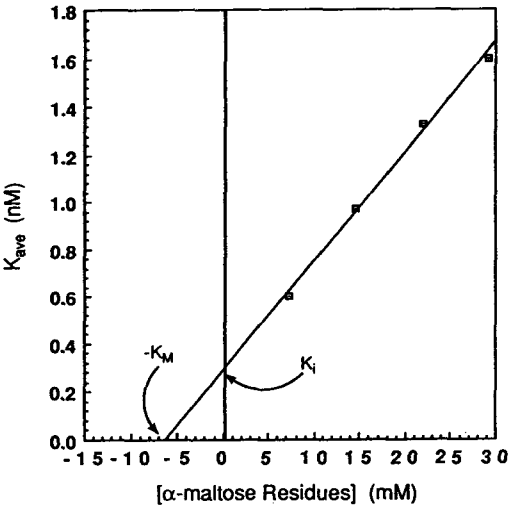

FIGURE 4 Determination of wheat germ, type II α-amylase inhibitor's inhibition binding constant (K_i) for human salivary α-amylase (0.29 nM), α-amylases K_M for soluble starch (as calculated for α–1,4 maltose residues, 6.20 mM) and the mode of inhibition (competitive). K_{ave} values for each concentration of substrate were obtained as explained in the legend to Figure 3. The line is "best fit".

intercept is K_i (0.29 nM) and the abscissa intercept yields a value for $-K_M$ (6.20 mM). This K_M was in good agreement with that calculated from a Lineweaver-Burk plot of the uninhibited system (5.54 mM). The K_M's found by these two different kinetic methods were averaged to obtain the K_M we report here; 5.87 mM α-maltose units in the soluble starch.

DISCUSSION

Analysis of the mode of inhibition of human salivary α-amylase by wheat seed type II α-amylase inhibitor was performed following procedures developed by Goldstein,[12] Morrison,[15,16] Cha,[17,18] and Dixon.[9,10]

Formation of the α-amylase: α-amylase inhibitor complex was found to be slow by first preincubating substrate and inhibitor and initiating reaction by addition of α-amylase. As significant amounts of α-amylase became complexed with α-amylase inhibitor, the initial steady-state composed of free enzyme and productive enzyme : substrate complex was perturbed and a new steady-state situation of free enzyme, productive enzyme : substrate complex, and non-productive enzyme: inhibitor complex was established. The length of time necessary to establish this latter, slower steady-state velocity was in the order of 2–3 minutes. When enzyme and inhibitor were preincubated for 15 minutes preceding addition of substrate, a lag of about 2–3 minutes was noted prior to production of product. This lag was

consistent with establishment of a steady-state among free enzyme, enzyme: inhibitor and enzyme : substrate, with the latter leading to product. Therefore, both binding (first trial) and release (second trial) of α-amylase by α-amylase inhibitor is slow, i.e., in the order of 2–3 minutes. It might be relevant to note that steady-state production of product in the typical uninhibited enzymatic reaction is in the order of msec.[19]

Tight-binding should be suspected if an inhibitor is effective at about the same concentration as that of the enzyme. Tight-binding can be confirmed or rejected by observing the effect of covarying enzyme and inhibitor under conditions of saturating substrate.[11] A plot of the data produced by a tight-binding inhibitor produces a series of curves each having linear and curved regions. The curved regions can be extended to the 0:0 coordinate. Extrapolation of the linear portions, actually asymptotes, to the abscissa yields information about the moles of inhibitor bound per mole of enzyme.[11]

Tight-binding can be further classified as to its mode: i.e., whether it is non-competitive, competitive, etc. Non-competitive and competitive can be distinguished by determining the K_{ave} values obtained by geometric measurements of data produced by covarying substrate and inhibitor at a fixed enzyme concentration. If the same K_{ave} is obtained at different substrate concentrations, the inhibition is non-competitive, but if K_{ave} increases with increasing substrate concentration, the inhibition is competitive. In the latter instance the line extended to the abscissa yields $-K_M$.[9]

The dimensionless ratio of K_M : K_i is another indicator of tight-binding if values over 100 are seen.[15] In our case the K_M : K_i ratio, 5.87 mM : 0.29 nM, yielded a value of 20.2×10^6. Finally, the [enzyme] : K_i ratio of our system was in the 28 to 103 range; clearly much greater than the 0.001 value arrived at by Goldstein[12] as the upper limit for application of Michaelis-Menten assumptions and kinetic analyses.

Both the human salivary α-amylase : wheat germ type II α-amylase inhibitor system studied here and the porcine pancreatic α-amylase : kidney bean α-amylase inhibitor system studied by Wilcox and Whitaker[8] are slow and tight-binding. However in contrast to the latter system, which follows a non-competitive inhibitory mechanism, the one studied here demonstrated competitive inhibition.

There have been too many reports distinguishing between salivary and pancreatic α-amylases and various categories of α-amylase inhibitors to review here. (See references 3, 4 and 8 for some of these). Thus, it was not surprising that the type of inhibition reported here (competitive) for the human salivary α-amylase : wheat germ type II α-amylase inhibitor system differed from the porcine pancreatic α-amylase : kidney bean α-amylase inhibitor system studied by Wilcox and Whitaker.[8] While the difference between non-competitive and competitive inhibition has some mechanistic implications the significance of this difference between the two systems is largely speculative at this point. The only other difference between these two systems, that perhaps should be mentioned, is in the substrates used for the kinetic analyses : *p*-nitrophenyl α-D-maltoside[8] as opposed to soluble starch.

Similar to the α-amylase : α-amylase inhibitor system reported on here and the system studied by Wilcox and Whitaker,[8] some proteinases and ribonucleases have been shown to be inhibited by slow, tight-binding proteinaceous inhibitors.[5–7] In all these cases the proteinaceous inhibitors seem to have evolved to serve two different types of functions : regulatory and defensive.

At least two different mammalian ribonucleases are inhibited by mammalian ribonuclease inhibitor : pancreatic ribonuclease A, a secretory (extracellular) enzyme and latent alkaline ribonuclease, an intracellular enzyme.[7] Although the ribonuclease A system can be considered artificial in the sense that there is no evidence of ribonuclease A ever encountering the inhibitor in normal cells, the latter system seems to be involved in intracellular regulation of RNA metabolism.[20,21]

In contrast to the mammalian ribonuclease system that exists within the same cell, it's assumed that the tight-binding mode of inhibition has evolved in the inter-kingdom proteinase : proteinase inhibitor systems because it confers some sort of selective advantage to plants whose seeds contain the inhibitor. That is, predators would be less likely to benefit from consumption of the seeds containing the inhibitors. The same sort of evolutionary logic could be applied to the α-amylase : α-amylase inhibitor system studied here.

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