Substrate−Inhibitor Interactions in the Kinetics of α-Amylase Inhibition by Ragi α-amylase/Trypsin Inhibitor (RATI) and Its Various N-Terminal Fragments†

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ABSTRACT: The ragi α-amylase/trypsin bifunctional inhibitor (RATI) from Indian finger millet, Ragi (*Eleucine coracana* Gaertneri), represents a new class of cereal inhibitor family. It exhibits a completely new motif of trypsin inhibitory site and is not found in any known trypsin inhibitor structures. The α-amylase inhibitory site resides at the N-terminal region. These two sites are independent of each other and the inhibitor forms a ternary (1:1:1) complex with trypsin and α-amylase. The trypsin inhibition follows a simple competitive inhibition obeying the canonical serine protease inhibitor mechanism. However, the α-amylase inhibition kinetics is a complex one if larger (≥7 glucose units) substrate is used. While a complete inhibition of trypsin activity can be achieved, the inhibition of amylase is not complete even at very high molar concentration. We have isolated the N-terminal fragment (10 amino acids long) by CNBr hydrolysis of RATI. This fragment shows a simple competitive inhibition of α-amylase activity. We have also synthesized various peptides homologous to the N-terminal sequence of RATI. These peptides also show a normal competitive inhibition of α-amylase with varying potencies. It has also been shown that RATI binds to the larger substrates of α-amylase. In light of these observations, we have reexamined the binding of proteinaceous inhibitors to α-amylase and its implications on the mechanism and kinetics of inhibition.

A large number of proteinaceous inhibitors of α-amylase (EC 3.2.1.1) have been characterized with respect to their molecular properties and inhibitory effects. The crystal structures of some of the inhibitors are known (*1*−*12*). The crystal structures of a few of the complexes of α-amylase with Tendamistat, a microbial inhibitor (*13*), RATI, a cereal inhibitor (*14*), barley α-amylase/subtilisin inhibitor (BASI) (*15*) and acarbose, an oligosaccharide analogue (*16*) are also known. A comparison of these structures shows that all the proteinaceous inhibitors bind to the enzyme at the catalytic site. Parallel studies on the substrate specificity and kinetics of amylase action have helped in the elucidation of some structural aspects of substrate binding sites. The kinetics of α-amylase inhibition by proteinaceous inhibitors does not obey a mechanism of simple competitive inhibition. In general, it appears that none of the proteinaceous inhibitors is capable of completely inhibiting the α-amylase, even at very high molar concentrations. Neither these observations nor the fact that the same inhibitors follow a simple competitive kinetics when a disaccharide substrate is used, could be explained till now. We have been working with the ragi (*Eleucine coracana* Gaertneri; Indian finger millet) bifunctional α-amylase/trypsin inhibitor (RATI). This inhibitor is the prototype of a cereal inhibitor superfamily. It has two independent sites for the cognate enzymes (*17*, *18*). It inhibits α-amylases from various sources. We have earlier determined the crystal structure of RATI (*19*) and its NMR structure has also been reported (*20*). It is a monomer of 122 amino acids with a pI of 10.3. The globular fold of RATI comprises of four α-helices with a simple "up and down" topology and two short antiparallel β-strands. It is a very stable molecule, resistant to urea, guanidine hydrochloride, and thermal denaturation (*18*). The trypsin inhibitory site is a canonical substrate-like conformational region while the N-terminal region is involved in the α-amylase inhibition. We report here the results of α-amylase inhibition by RATI, N-terminal decapeptide of RATI and synthetic peptides together with RATI-substrate interactions leading to the complex type of α-amylase inhibition which seems to have been generally observed with the proteinaceous inhibitors. The results of these studies are expected to be extended to other proteinacous inhibitors from other sources as well.

METHODS AND MATERIALS

Materials. Locally available ragi seeds were used. Zulkowsky starch (soluble starch with a mean length of 27 glucose units) was obtained from Merck, Darmstadt (Germany). Porcine pancreatic α-amylase (PPA), *p*-nitrophenyl-α-D-maltoside (NPM), and *N*-α-benzoyl-L-arginine-4-nitroanilide (L-BAPNA) were obtained from Sigma Chemical Co., St. Louis. The α-amylase paranitrophenyl (PNP) assay

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[1] Abbreviations: RATI, ragi α-amylase/trypsin inhibitor; NPM, *p*-nitrophenyl-α-D-maltoside; NPMH, *p*-nitrophenyl-α-D-maltoheptoside; PPA, porcine pancreatic α-amylase; CNBr, cyanogen bromide; FPLC, fast-protein liquid chromatography; TMA, *Tenebrio molitor* amylase (α-amylase from yellow meal worm); HBTU, [2-(1H-benzotriazol-1-yl]-1,1,3,3-tetramethyl uronium hexaflourophosphate.

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kit including *p*-nitrophenyl-α-D-maltoheptoside (NPMH) and α-glucosidase were purchased from Boehringer-Mannheim (Germany).

RATI Purification. The RATI was purified using the modified procedure of Gourinath et al. (*19*). The protein was extracted from ragi powder with 0.15 M NaCl and concentrated by precipitation at 55% saturated ammonium sulfate. The precipitate was dissolved and dialyzed against 2 mM sodium acetate, pH 5.0, containing 75 mM NaCl (buffer A). The inhibitor was adsorbed onto CM-Sephadex C-50 (20.0 × 2.6 cm) in buffer A and eluted using 150 mM NaCl in the same buffer. This fraction was concentrated by ultrafiltration and fractionated on Sephadex G-50 (100 × 2.6 cm). The active fractions were pooled and adsorbed on SP-Sepharose (Pharmacia, Uppsala, Sweden) equilibrated with buffer A. The column was eluted with a pH gradient using 5 mM NaH_2PO_4 and 2 mM Na_3PO_4 as the starting and limiting buffers. The active fractions were found homogeneous on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS−PAGE). The pI of the purified RATI was determined using isoelectric focusing cell (Rotofor, Bio-Rad, Hercules) in the presence of 5% glycerol.

α-Amylase Purification Using DEAE Cellulose Column. The amylase was purified according to Noelting and Bernfeld (*21*). A total of 0.5 g of PPA was dissolved in 50 mL of 70 mM sodium acetate buffer, pH 8.0, containing 1 mM benzamidine and 0.1 mM phenylmethanesulfonyl flouride (PMSF). This solution was cleared at 10000*g* and the α-amylase was precipitated with 45% saturation of ammonium sulfate. The precipitate was dissolved in 70 mM sodium acetate, pH 8.0, and fractionated between 50 and 70% acetone in cold. The two isoenzymes were separated by retardation chromatography on DEAE-Cellulose column that was equilibrated and eluted with the 50 mM sodium acetate buffer, pH 8.0, as described previously (*22*).

α-Amylase Inhibition Assay. (a) With NPM as Substrate. All assays were performed in 50 mM Pipes buffer, pH 7.0, 100 mM NaCl, and 1 mM $CaCl_2$ at 25°C. Isoform II of α-amylase was used for the inhibition assays. Assays with the disaccharide substrate NPM were performed at an α-amylase concentration of 0.4 μM, while the NPM concentrations were varied between 1 and 6 mM. RATI concentrations were varied from 0 to 0.2 μM, while the concentrations of peptide inhibitors were varied from 0 to 0.7 μM. Inhibitor/peptide and α-amylase were incubated for 24 h. The assay was carried by addition of 72 μL NPM stock solution in 0.6 mL reaction mixture. Product formation was followed by the increase in absorbance at 405 nm in a double beam Spectrophotometer (Shimadzu, model UV−Vis-160 A). Similar kinetic pattern was observed when the preincubation step was omitted.

(b) With NPMH as Substrate. A linked enzyme assay with α-glucosidase as second enzyme was applied when the heptasaccharide substrate NPMH was used. α-amylase (0.18 nM) was incubated with varying concentrations of RATI (0.0−0.2 μM)/peptide inhibitors (0.0−0.7 μM) in 1 mL of buffer (50 mM Pipes, pH 7.0, 100 mM NaCl, and 1 mM $CaCl_2$) for 24 h. The NPMH concentrations were varied between 0.2 and 10 mM. The reaction was started by the addition of 40 μL of a solution containing NPMH and α-glucosidase (125 units/mL) in buffer. The interaction of RATI with α-glucosidase was examined and it was found that RATI has no inhibitory effect on α-glucosidase (data not shown).

(c) With Starch as Substrate. Assays with Zulkowsky starch were performed in buffer (50 mM Pipes, pH 7.0, 100 mM NaCl, and 1 mM $CaCl_2$) with final concentration of 1.8 nM α-amylase. RATI (0.0−0.2 μM)/peptide inhibitors (0.0−0.7 μM) were incubated for 24 h in 4 mL of buffer and the reaction was started by addition of 1 mL of substrate solution in buffer to give a final concentration of 0.1−0.8 mM. Samples of 0.5 mL were removed after different incubation times, mixed with 0.5 mL 1% (w/v) dinitrosalicylic acid in 0.4 M NaOH, heated at 100 °C for 5 min and then cooled on ice. Product formation was detected by the increase in the absorbance at 546 nm. The kinetic experiments were repeated seven times.

Substrate−Inhibitor Interactions. RATI (0.0−0.2 μM) was incubated with varying concentrations (0.1−0.8 mM) of Zulkowsky starch for 10 min in 0.5 mL of 50 mM Pipes buffer, pH 7.0, 100 mM NaCl and 1 mM $CaCl_2$. The starch was precipitated by ethanol at 40% v/v concentration. The RATI present in the supernatant was measured using trypsin inhibitory assay with *N*-α-benzoyl-L-arginine-4-nitroanilide (L-BAPNA) as a substrate in the same buffer. To the precipitate formed, α-amylase was added directly to see whether the bound inhibitor could still inhibit the enzyme.

Purification of the Cleaved Fragments of RATI. Cyanogen bromide (CNBr) cleavage of RATI was carried out (*23*). A total of 5 mg of the CNBr cleaved fragments was purified by gel filtration on Superdex Peptide column (1 × 30 cm, Pharmacia, Uppsala, Sweden) using an FPLC system. Formic acid (10%) was used as solvent system at a flow rate of 0.1 mL/min.

Synthesis of Peptides. The following peptides were synthesized by solid-phase method in automatic peptide synthesizer (Rainin PS3 model Protein Technologies): (1) NH_2-Ser-Val-Gly-Thr-Ser-Cys-Ile-OH (P7), (2) NH_2-Ser-Val-Gly-Thr-Ser-Cys-Ile-Pro-Gly-OH (P9), (3) NH_2-Ser-Val-Gly-Thr-Ser-Cys-Ile-Pro-Gly-Met-Ala-OH (P11), (4) NH_2-Ala-Val-Gly-Thr-Ser-Cys-Ile-OH (Ala1-P7), and (5) Fmoc-Ser-Val-Gly-Thr-Ser-Cys-Ile-OH (Fmoc-P7). The peptides were synthesized using Fmoc Wang resins (Nova Biochem, Switzerland). The COOH group was activated by HBTU in *N*-methylmorpholine and reacted with the amino group. All these steps were repeated till the sequence is completed. Peptide-resin was washed with dimethylformamide, dichloromethane and ethanol and dried in a desiccator. Dried resin was stirred with triflouroacetic acid, anisole and water (90:5:5) for 2 h. Trifluoroaceticacid was removed by evaporation and peptide was precipitated from anhydrous ether. The precipitate was dissolved in 10% acetic acid and washed with ether and ethyl acetate. The peptides were purified by reversed-phase chromatography (RPC−FPLC) using C_{18} Silica column (1.6 × 10 cm). The elution gradient was run by varying methanol concentration from 0 to 100% at a flow rate of 2 mL/min. The purity was checked with 1H NMR and by doing N-terminal sequencing of these peptides.

RESULTS

α-Amylase Inhibition. The α-amylase inhibition studies were carried out with different substrates of varying length.

Table 1: K_I Values for RATI and Peptides with Different Substrates

inhibitor	NPM	NPMH	starch
none	$K_M = 6.0$ mM	$K_M = 6.0 \times 10^{-2}$ mM	$K_M = 0.2$ mM
units	nM	nM	nM
RATI	15 ± 2	varies with [S]	varies with [S]
P7	305 ± 5	310 ± 10	335 ± 5
P9	163 ± 3	140 ± 20	145 ± 10
P11	710 ± 30	675 ± 50	750 ± 25
Ala1-P7	1560 ± 40	1510 ± 30	1610 ± 40
Fmoc Ser1-P7	no activity	no activity	no activity

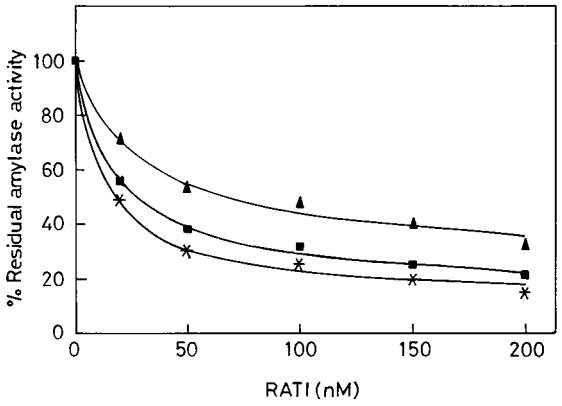


FIGURE 1: Inhibition of porcine pancreatic α-amylase by RATI at pH 7.0 and 25 °C assayed with NPMH (heptasaccharide). α-Amylase at 0.18nM was preincubated with RATI at indicated concentrations for 24 h. The reactions were carried out with different concentrations of substrate: 30 μM (*); 60 μM (×); 120 μM (▲). The solid lines correspond to nonlinear fit.

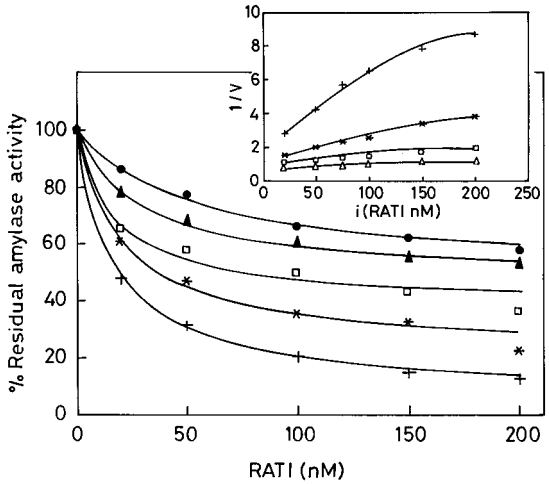


FIGURE 2: Inhibition of porcine pancreatic α-amylase by RATI at pH 7.0 and at 25 °C assayed with Zulkowsky starch (27mer). α-Amylase at 1.8 nM was preincubated with RATI at indicated concentrations (0.0−200 nM) for 24 h. The reactions were carried out with different concentrations of substrate: 0.1 mM (+); 0.2 mM (*); 0.4 mM (□); 0.8 mM (▲); 1 mM(●). The solid lines correspond to nonlinear fit. The inset shows the Dixon plot for the α-amylase inhibition by RATI.

RATI exhibits a purely competitive inhibition of α-amylase when NPM is used as a substrate with a K_I of 15 ± 2 nM (Table 1). With NPMH and Zulkowsky starch, the mode of inhibition is complex (Figures 1 and 2). In this case, the inhibition kinetics does not follow a simple competitive inhibition. The CNBr cleavage of RATI resulted in four fragments of molecular weights 1, 3, 3.5, and 6.5. Only the smallest fragment showed α-amylase inhibition activity. This corresponded to the N-terminal 10 amino acid residues of

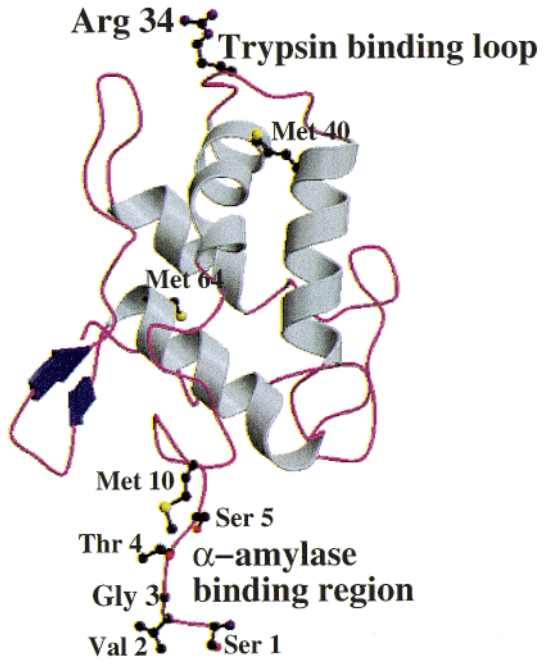


FIGURE 3: Structure of RATI, showing the positions of three methionine residues. The trypsin and α-amylase inhibitory sites are situated on the opposing sides of the molecule.

the RATI (Figure 3). The synthetic peptides contain the N-terminal 7, 9, and 11 residues. Additionally two peptides were synthesized: one contained Ala in place of the N-terminal Ser and another peptide contained Fmoc-Ser in place of Ser at the N-terminal. The α-amylase inhibition by the CNBr fragment of RATI and synthetic peptides followed a competitive inhibition. The K_I values of the peptides determined from the Lineweaver−Burk plots with different substrates are given in Table 1. The Ser1→Ala substitution greatly diminished the inhibitory effect of the peptide and blocking of NH_2 group by Fmoc moiety completely abolished the inhibitory activity.

Substrate Inhibitor Interactions. The final concentrations of the substrates and inhibitor are the same as used in the assay conditions. It is apparent that RATI binds to the substrate, in a manner proportional to the concentration of the substrate (Figure 4). When α-amylase was added to the substrate bound inhibitor, it did not have any inhibitory activity.

DISCUSSION

Since α-amylase isoforms are known to exist, these were separated and only isoform II was used in the present studies. Isoform II has twice the affinity of isoform I for RATI (data not shown). When NPM was used as substrate the inhibition was purely competitive. Using a measured K_M value of 6 mM for NPM and quick binding equilibria between the substrate and inhibitor to enzyme, a K_I value of 15 ± 2 nM for RATI was obtained. A completely different type of inhibition kinetics emerged when NPMH and Zulkowsky starch are used as substrates. The kinetic patterns were similar with both of these large substrates. The extent of inhibition varied with the substrate concentration. The observed inhibition was less than what should have been in the case of independent competitive binding. Figure 4 shows how much inhibitor is not available for any given concentration of

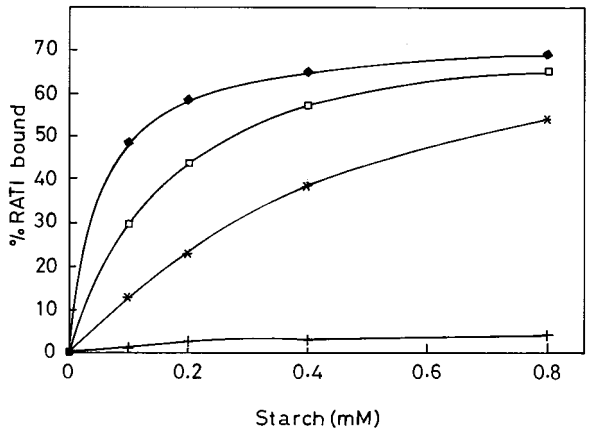
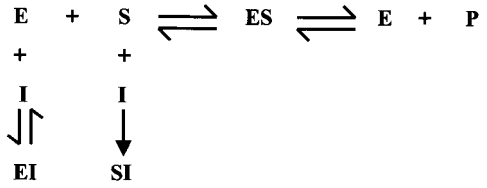


FIGURE 4: Binding of inhibitor with substrate. Constant amount of inhibitor was added to increasing concentration of starch in 50 mM Pipes buffer, pH 7.0, at 25 °C. After 10 min incubation, the starch was precipitated with ethanol. The unbound inhibitor in the supernatant was estimated by trypsin assay using L-BAPNA as substrate. The same reaction was performed with different concentrations of RATI: 20 nM (+); 50 nM (*); 100 nM (□); 200 nM (◆).

inhibitor concentration as the substrate concentration was varied. This was calculated assuming only a competitive mode of inhibition between α-amylase and RATI. The reasons for this assumption are 3-fold. First, RATI exhibits a competitive inhibition with NPM. Second, it was found from the crystallographic observations that RATI and other α-amylase inhibitors bind to the catalytic site of α-amylase only. Third, in all these complexes only one molecule of the inhibitor binds to α-amylase and the size exclusion chromatographic and kinetic experiments also establish a 1:1 stoichiometry for the amylase−inhibitor interactions (*16*−*18*, *24*). However, with all these studies on the interactions of α-amylase with its inhibitors, the following observations with regard to the α-amylase inhibition by RATI and other α-amylase inhibitors using starch as substrate, are not explained as yet (i) if only competitive inhibition was assumed, the observed inhibition was less than the predicted level, (ii) a complete inhibition was not achieved, even at high molar ratio, (iii) the type of inhibition was apparently not purely competitive, and (iv) a purely competitive and complete inhibition with NPM was achieved.

Our results on the substrate-inhibitor interactions establish that under the assay conditions, the RATI binds to the soluble substrate. Since cytochrome C, which is also a basic protein with a pI close to RATI, does not bind to the soluble starch, the interactions of RATI with substrate are not based predominantly on the positive charges of RATI and are not completely nonspecific in nature. It is known that nonpolar and H-bond interactions are involved in the binding of proteins to oligosaccharides and polysaccharides. While RATI is highly charged, it has 40% of its sequence comprised of nonpolar amino acids. It is possible that a combination of nonpolar and uncharged polar residues makes up a sugar binding region. Equally important is the loss of inhibitory activity upon binding, which is relevant to kinetics of amylase inhibition. The inhibitor molecules, which are bound to the substrate are not available for the inhibition of the enzyme. Thus, the inhibitor gets depleted upon binding with the substrate leading to less inhibition than what is expected. Since less inhibition is observed even with preincubation of enzyme and inhibitor, the added substrate shifts the binding equilibrium toward dissociation of enzyme and inhibitor. The concentration of the substrate is 3−4 orders of magnitude more than the inhibitor concentration in the assay conditions. Even if the interactions are weak, because of the high effective concentration of the substrate, the depletion of the inhibitor will be highly significant. Hence, a complete inhibition of α-amylase with proteinaceous inhibitors is generally not observed (*24*−*27*). It was expected that larger polysaccharide would be more efficient in binding with the inhibitor. The kinetic scheme of α-amylase inhibition using larger substrate will be as follows:



Since no inhibition was observed when SI was added to α-amylase, the binding site of RATI was not accessible to α-amylase. It implies that the complex formed between substrate (starch) and the RATI is a stable complex. It has already been reported that large substrates occupy the catalytic site and the surface site (a second sugar binding site) of α-amylase (*28*). The structural data on the complexes of α-amylases and their inhibitors have clearly shown that the inhibitor interactions do not cover the surface site (*13*−*15*). In view of these observations, it seems unlikely that a ternary complex, ESI is formed. This kind of impairment of the function of a proteinaceous inhibitor has not been reported so far in enzyme kinetics. Thus, in the absence of any evidence of the complex inhibition mode, the observed loss of inhibition should be explained by the substrate-inhibitor interactions. Since, it could be estimated as to how much inhibitor was lost by way of binding with the substrate, the amount of substrate bound to inhibitor was deducted from the added inhibitor amount. A Dixon plot of the data established a competitive mode of inhibition with starch as a substrate, when the corrected RATI concentrations were used (Figure 5). The calculated K_I value of 0.1 nM is comparable to other reported studies (*6*, *24*, *29*, *30*).

A small-sized substrate interacts weakly as compared to a larger-sized substrate therefore it was not surprising that the RATI was not precipitated by NPM. In the absence of any interactions, the RATI displayed a purely competitive mode of inhibition with NPM as a substrate. The N-terminal fragments of RATI interact with the catalytic region of α-amylase. The fact that RATI has more number of interactions with α-amylase than the N-terminal fragment, can be seen from the K_I values of the RATI and the peptides. The intact RATI has a K_I value of 15 ± 2 nM (NPM as substrate) whereas the peptides have 10−40 times more than this value. The nonamer peptide is more effective than the heptamer or elevenmer among the homologous synthetic peptides. The sequence -Cys6-Ile7-Pro8-Ala9- forms a type II β-turn in the intact RATI. This stable conformation might act as an anchor to place the first six amino acids appropriately in the binding region of α-amylase. In case of heptapeptide (P7), the β-turn structure is not available. On the other hand, in case of P11, the turn conformation may be available but since

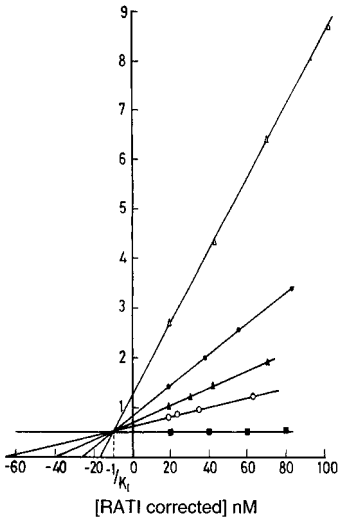


FIGURE 5: Dixon plot for amylase inhibition with corrected concentrations of RATI. Inhibition of PPA by RATI at pH 7.0 and 25 °C assayed with Zulkowsky starch (27mer). Amylase at 1.8 nM was preincubated with RATI at indicated concentrations for 24 h. The reactions were carried out with different concentrations of substrate: 0.1 mM (△); 0.2 mM (●); 0.4 mM (▲); 0.8 mM(○); [S] = 500K_M (■).

residues 10 and 11 do not contribute to interactions with α-amylase significantly they are not required for the inhibition thus making P11 as a poorer inhibitor of α-amylase than P9. Having all the possible interactions with α-amylase and a well-defined stable C-terminal conformation, P_9 shows more affinity to α-amylase. The critical role of the side chain of terminal residue is brought out in the Ser1→Ala1 substitution. The side chain of Ser1 is involved in the H-bonding with Nϵ2 of His299 and Oδ2 of Asp300 in the subsites 3 and 4 of the substrate binding region of the enzyme. The terminal nitrogen of Ser1 is involved in the H-bonding with Asp197 and Glu233. Derivatization of NH_2 group with Fmoc (in this study) or with 2,4,6-trinitrobenzene sulfonic acid (*17*) completely abolishes the α-amylase inhibitory activity. Since these peptides do not interact with substrates, they exhibit a competitive mode of inhibition with all substrates.

By quantifying the substrate-inhibitor binding, it has been attempted to explain the anomalous properties of kinetics of the α-amylase inhibition by proteinaceous inhibitors. Though, the protein−carbohydrate interactions are known to occur, this is the first report to show quantitatively the effects of these interactions in the α-amylase inhibition kinetics.

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