Sugars Intensify the Inhibitory Effect of Phenylboronic Acid on the Hydrolytic Activity of α-Chymotrypsin

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The inhibitory effect of phenylboronic acid which acts as a transition state analogue for certain peptidases is efficiently intensified by added saccharides: this finding enables us to control the enzyme activity with sugars.

Trigonal boron compounds contain a vacant 2p orbital which easily reacts as a Lewis acid with nucleophiles such as hydroxide, alkoxide or imidazole to give a tetrahedral boron adduct. This adduct formation also occurs in the active site of certain hydrolytic enzymes such as subtilisin and α-chymotrypsin and the active site serine (or histidine) is usually the fourth ligand of the tetrahedral structure.[1,2] Apparently, phenylboronic acid acts as a novel *transition state analogue* for these enzymes and the K_i values for phenylboronic acid are estimated to be 0.23–0.80 mmol dm^{-3} for subtilisin and 0.19 mmol dm^{-3} for α-chymotrypsin.[1,2] Meanwhile, it is known that boronic acids form cyclic esters with saccharides and the reaction occurs reversibly and rapidly at ambient temperature.

It has been demonstrated recently that boronic acids serve as a useful interface for the selective recognition of saccharides in water.[3–10] For example, saccharides in water can be spectrophotometrically detected by boronic acid-appended porphyrins[5] or fluorophores.[6,10] This type of saccharide detection is based on the idea that absorption and fluorescence spectra of porphyrins change sensitively in response to a shift in the aggregation–deaggregation equilibrium and the complexation of saccharides with the boronic acid moieties changes this equilibrium in favour of deaggregation because of the enhanced hydrophilicity of the complexed porphyrin. It occurred to us that phenylboronic acid working as an inhibitor in the enzyme active site should be withdrawn upon complexation with saccharides because of the enhanced hydrophilicity of the saccharide-complexed inhibitor. As a result, the enzyme activity should be regenerated. To test this intriguing hypothesis we investigated the influence of added saccharides on the inhibition ability of phenylboronic acid in α-chymotrypsin-catalysed hydrolysis of *N*-benzoyl-L-tyrosine-*p*-nitroanilide (Sub). Surprisingly, added saccharides efficiently intensified the inhibitory effect and in certain saccharides a distinct D/L discrimination in the inhibitory effect was observed.

α-Chymotrypsin was purchased from Sigma (Type II: M_r 25100). The hydrolytic reaction was carried out according to Kouzuma's method[11] (37 °C, standard pH 8.0 with 50 mmol dm^{-3} phosphate buffer, 0.3% v/v methanol plus 0.03% v/v dimethylsulfoxide) and the progress of the reaction was followed by monitoring the appearance of the absorption band at 410 nm (*p*-nitroaniline; P).

Plots of [P] *vs.* reaction time are illustrated in Fig. 1. The hydrolytic activity of α-chymotrypsin (6.08×10^{-7} mol dm^{-3}) was not affected by the addition of saccharides (7.69×10^{-2} mol dm^{-3}) whereas it was moderately inhibited by the addition of phenylboronic acid (6.31×10^{-3} mol dm^{-3}). When saccharides were added to the boronic acid-inhibited system, the rate of the hydrolytic reaction was further suppressed. The inhibition efficiency for monosaccharides is in the order of D-talose > D-fructose > D-glucose > D-mannose > D-galactose. This order is not necessarily consistent with the order of the association constant with phenylboronic acid (D-fructose > D-galactose > D-mannose > D-glucose).[6,9,10] Interestingly, the distinct D/L enantioselectivity in the inhibitory effect was observed for fructose and glucose: in both saccharides the inhibitory effect for D-isomers is larger than that for L-isomers (Fig. 1). A particularly large difference was observed for fructose: the inhibitory effect of L-fructose was even weaker than that of phenylboronic acid itself. The findings support the view that the enzyme active site *recognizes* the molecular structure of boronic acid–saccharide complexes. We here determined the K_i for phenylboronic acid in the absence and the presence of D-fructose. Dixon plots[12] showed that both systems feature a competitive inhibition, but the K_i for phenylboronic acid plus D-fructose (6.33×10^{-4} mol dm^{-3}) is much smaller than that for phenylboronic acid (1.86×10^{-3} mol dm^{-3}).

The pH dependence is shown in Fig. 2. In the absence of phenylboronic acid the maximum enzyme activity was observed at pH 7.5. In the presence of phenylboronic acid, on the other hand, the enzyme activity is suppressed and the maximum appeared at around pH 8.0. In the presence of both phenylboronic acid and D-fructose the enzyme activity is strongly suppressed at pH 4–9 and the maximum activity shifts to pH 8.5. How can we rationalize this strange pH dependence? The reaction processes involved in the present system are expressed as in Scheme 1.

As shown in Fig. 2, the inhibitory effect is scarcely seen above pH 9.5. This implies that $PhB^-(OH)_3$ and $[Ph(HO)B^-]$–sugar do not react with the active site serine (or histidine) in α-chymotrypsin (E–Nu; Nu denotes either serine or histidine acting as a nucleophile in the active site). On the other hand, it can react with $PhB(OH)_2$ and [PhB]–sugar to give the boron adducts. It is already known that the pK_a for [PhB]–sugar is lower by *ca.* 2.5 p*K* units than that for $PhB(OH)_2$: that is, [PhB]–sugar is more acidic as a Lewis acid than $PhB(OH)_2$.[6,9] Hence, the nucleophilic reaction between E–Nu and [PhB]–sugar occurs in preference to that between E–Nu and $PhB(OH)_2$. This difference causes the large inhibitory effect at pH 4–9. Above

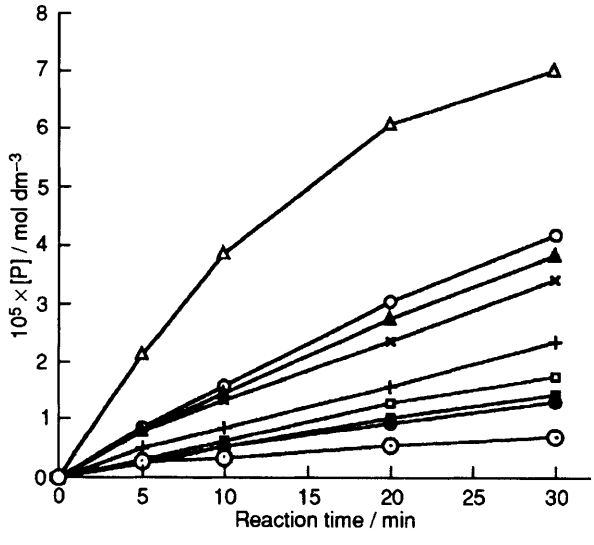


Fig. 1 Plots of [*p*-nitroaniline (P)] *vs.* reaction time: 37 °C, [α-chymotrypsin] = 6.08×10^{-7} mol dm^{-3}, [Sub] = 7.56×10^{-5} mol dm^{-3}, [phenylboronic acid] = 6.31×10^{-3} mol dm^{-3}, [saccharide] = 7.69×10^{-2} mol dm^{-3}: (△) control [in the absence of phenylboronic acid and in the presence of saccharides (7.69×10^{-2} mol dm^{-3})]; (○) L-fructose; (▲) no saccharide; (×) D-galactose; (+) D-mannose; (□) L-glucose; (■) D-glucose; (●) D-fructose, and (⊙) D-talose in the presence of phenylboronic acid

pH 9.5, on the other hand, [PhB]–sugar is totally converted to [Ph(HO)B$^-$]–sugar and cannot react with the nucleophile in the enzyme active site. This kinetic situation gives the maximum activity at pH 8.5.

To obtain further insights into the boron inhibitory effects we measured the ^{11}B NMR spectra (80.24 MHz, 25 °C).[13] Phenylboronic acid (1.87 mmol dm^{-3}) gave δ_B (quoted from external trimethyl borate in $CDCl_3$, linewidth 2–3) 13.8 at pH 6.5 for sp^2 boron and δ_B −11.8 at pH 10.3 for sp^3 boron. In the presence of D-fructose (76.9 mmol dm^{-3}) at pH 6.5 and δ_B appeared at −8.1, indicating that the boron is converted to sp^3 hybridization through complexation with D-fructose. In the presence of phenylboronic acid and α-chymotrypsin (0.76 mmol dm^{-3}) at pH 6.5, on the other hand, a new peak appeared at δ 3.3, which is assigned to the boron in a fast-exchange between the enzyme-bound sp^3 form and the unbound sp^2 form.[13] When D-fructose was added, other new peaks appeared at δ −4.7 and −9.6, both of which were not observed in the absence of D-fructose even at 2 °C. According to the references,[13] these peaks would be assigned to Ser-bound and His-bound, D-fructose-complexed sp^3 boron in a slow exchange. These results are consistent with the reactions shown in Scheme 1.

In conclusion, the present study established that the phenylboronic acid inhibition of the α-chymotrypsin activity is selectively intensified by the addition of D-monosaccharides. The synergic effect is attributed to the generation of a more acidic boron atom through self-complexation with D-monosaccharides, which efficiently reacts with the active site serine (or histidine) in α-chymotrypsin. The finding is directly applicable to the control of the α-chymotrypsin activity by physiologically non-toxic saccharides. We are currently investigating if this novel concept can be applied to di-, tri, oligo- and polysaccharides and to other nucleophilic enzymes such as subtilisin, papain, carboxypeptidase, *etc.*

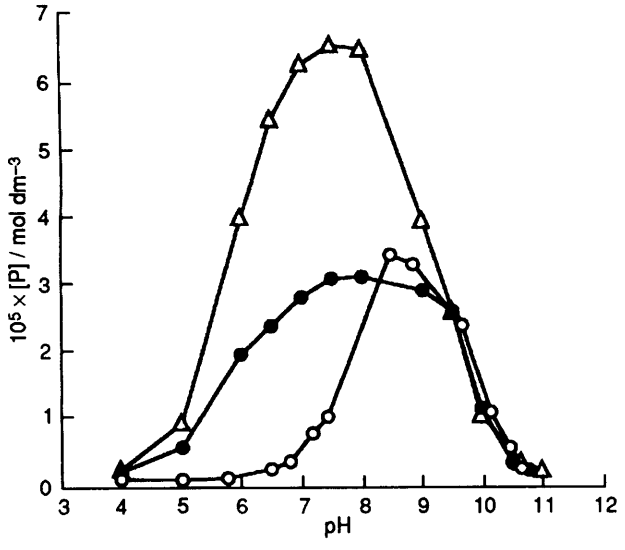
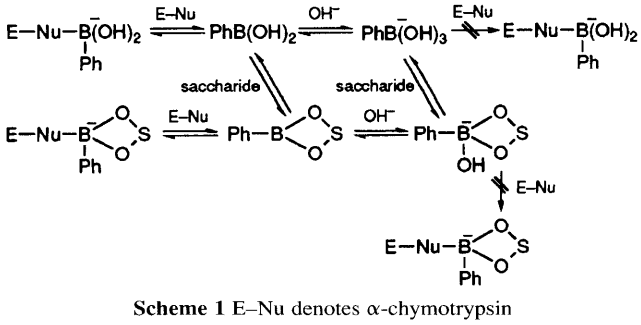


Fig. 2 Plots of [*p*-nitroaniline (P)] *vs.* pH: 37 °C, [α-chymotrypsin] = 6.08 × 10^{-7} mol dm^{-3}, [Sub] = 7.56 × 10^{-5} mol dm^{-3}, [phenylboronic acid] = 6.31 × 10^{-3} mol dm^{-3}, [saccharide] = 7.69 × 10^{-2} mol dm^{-3}: (△) control [in the absence of phenylboronic acid and in the presence of D-fructose (7.69 × 10^{-2} mol dm^{-3})]; (●) in the presence of phenylboronic acid, and (○) in the presence of phenylboronic acid and D-fructose



Scheme 1 E–Nu denotes α-chymotrypsin

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