Control of an α-Chymotrypsin Hydrolytic Activity by a Combination of Phenylboronic Acids and Polyols

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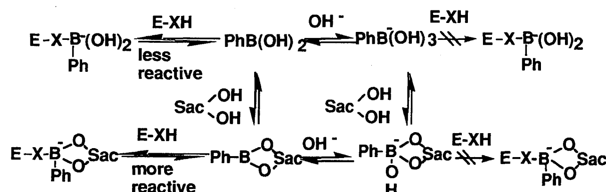
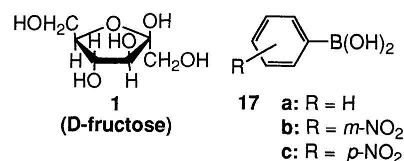
The hydrolytic activity of α-chymotrypsin is inhibited by phenylboronic acids. It was shown that the inhibitory effect is intensified by added diols but rather weakened (*i.e.*, the hydrolytic activity is regenerated) by tripodal additives. Hence, the activity can be controlled in a range of 0-100%. This is a novel method to control the activity of the nucleophilic hydrolytic enzymes.

It is known that phenylboronic acids ($PhB(OH)_2$) are efficient inhibitors which act as a transition-state analogue for certain hydrolytic enzymes (*e.g.*, α-chymotrypsin, subtilisin, *etc.*; E-XH).[1-5] In the active sites of these enzymes phenylboronic acids form covalently-linked adducts ($PhB^-(OH)_2$-X-E) and the active site serine (or histidine) is usually the fourth ligand of the tetrahedral structure.[1-5] Previously, we added saccharides (Sac<8H) to an α-chymotrypsin-catalyzed system in the presence of phenylboronic acid, expecting that they would withdraw phenylboronic acid out of the active site through the formation of more hydrophilic complexes ($PhB^-(OH)$<8>Sac).[6] If this change is really induced, the hydrolytic activity should be regenerated. Contrary to our expectation, however, *most saccharides further intensified the inhibitory effect.* The result can be rationalized as such that the boron atom complexed with diols becomes more acidic[7-10] and therefore becomes more reactive toward the enzyme nucleophilic center (Scheme 1).

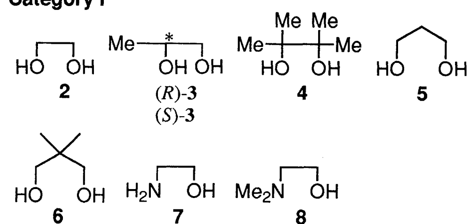


Scheme 1. E-XH and Sac<8H denote α-chymotrypsin and saccharide, respectively.

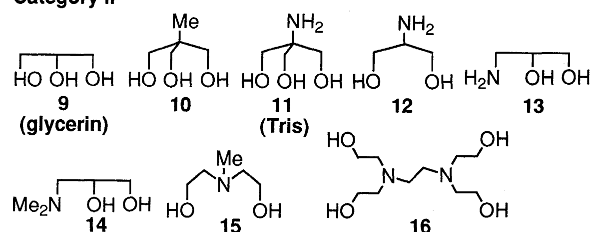
In contrast, the inhibitory effect was not intensified by added saccharides at high pH region, indicating that the nucleophilic center cannot attack boronate anions. Here, a new idea occurred to us: when tripodal compounds such as **9** - **16** are added, the boron atom is converted to the tetrahedral borate complex and the reaction with the enzyme nucleophilic center can no longer take place. As a result, the complex is withdrawn out of the active site and the enzymatic activity is regenerated. If this is the case, one can freely control the hydrolytic activity of these enzymes using phenylboronic acids and polyols: dipodal additives in category I would intensify the inhibitory effect whereas tripodal additives in category II would weaken the inhibitory effect. We test this interesting hypothesis in this paper, using phenylboronic acid derivatives (**17**) as inhibitors.



Category I



Category II



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

As shown in Figure 1A, the catalytic activity decreased with increasing **17** concentrations. The order of the inhibitory effect was **17c** > **17b** > **17a**: *i.e.*, the stronger the electron-withdrawing ability, the higher the inhibitory effect[12]. In **17c** the reaction was totally suppressed at [**17c**] = 1.23×10^{-2} mol dm^{-3}.

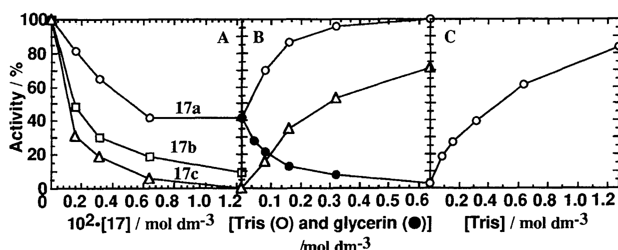


Figure 1. The control of the hydrolytic activity by the combination of **17** with Tris and glycerin; [α-chymotrypsin] = 6.08×10^{-7} mol dm^{-3}.

In the presence of 6.31×10^{-3} mol dm^{-3} **17a** the enzyme activity was reduced to 40%. The additive effect was investigated at these reaction conditions. Figure 2 summarizes the effect of additives in category I. D-Fructose (**1**) used as a representative of saccharides showed a strong inhibitory effect. Compound **2** showed a weak inhibitory effect but **5** is almost ineffective. (*R*)-**3** showed the inhibitory effect greater than (*S*)-**3**. Compounds **4** and **6** with additional methyl groups provided turbid solutions[13] and the rate measurement was not reproducible. Compounds **7** and **8** weakly recovered the enzyme activity. Conceivably, the electron-donating ability of amines weakens the acidity of the boron atom and it no longer undergoes the attack of the nucleophilic center.

Figure 3 summarizes the effect of additives in category II. As expected, **11**, **12**, **15** and **16** could regenerate the 100% enzyme activity and **10**, **13** and **14** showed the moderate regeneration effect. The sole exception in category II is glycerin **9**. The molecular model predicts that three oxygens in **9** cannot covalently interact with the same boron atom: that is, although **9** is a triol but actually acts as a diol in the complexation with **17a**.

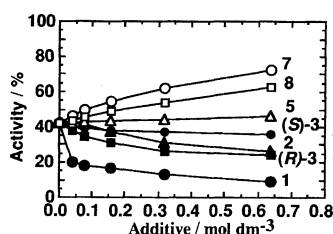


Figure 2. The effect of additives in category I

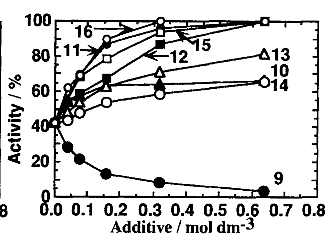
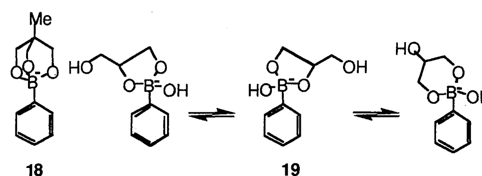


Figure 3. The effect of additives in category II

To obtain further insights into the **17**•additive complex structure we measured ^{1}H NMR spectra of equimolar mixtures (0.037 mol dm^{-3}) of **17a** plus **9** and **17a** plus **10** in D_2O (300 MHz, pD 10.9 with 0.01 mol dm^{-3} carbonate, 25 °C). Compound **10** (δ_{CH_2} 3.50 ppm) gave a new singlet resonance with the 6H integral intensity for **17a•10** at 3.78 ppm. This supports the view that three OH groups are covalently-linked to a boron atom (as in **18**). In contrast, compound **9** (δ_{CH} 3.78 ppm, δ_{CH_2} 3.63 ppm) gave broad signals at 25 °C but five separated CH and CH_2 signals at -10 °C (3.47, 3.71, 3.88, 4.05, and 4.16 ppm). This implies that two of three OH groups are covalently-linked to a boron atom and they are exchanging with each other in the NMR time-scale (as in **19**: in fact, one can count five different proton sources in the equilibria). The results establish that (i) the boron atom in the complexes with tripodal additives is converted to a tetrahedral borate complex and they no longer react with the nucleophilic center and (ii) the boron atom in the complexes with dipodal additives (including **9**) becomes more acidic and easily reacts with the nucleophilic center. Since **13** and **14** regenerate the enzyme activity, they form complexes using OH and NH_2 (or NMe_2) groups like **7** and **8** but not using two OH groups like **9**.

Now, all additives have successfully been classified. We here demonstrate the control of the hydrolytic activity by the combination of **17** as an inhibitor with several additives (Figure 1B, 1C). At [**17a**] = 6.31×10^{-3} mol dm^{-3} glycerin is added: as



seen in Figure 1B the activity can be suppressed to 3% of the regular activity in the absence of **17**. When Tris (**11**) is added to this solution, the activity can be recovered to the 83% activity (Figure 1C). At [**17c**] = 1.23×10^{-2} mol dm^{-3} where the enzymatic reaction is totally inhibited, Tris (**11**) is added: as seen in Figure 1B the activity can be recovered up to 72%. These results clearly indicate that the activity of α-chymotrypsin can be controlled in a range of 0% -100% by the present method.

In conclusion, the present study shows that phenylboronic acids are unique inhibitors, the inhibitory effect of which can be either intensified or weakened by the additives. The finding is useful not only to prove the hybridization of the boron atom in the enzyme active site but also to control the enzyme activity. We believe that this method is applicable more generally to the control of the nucleophilic enzyme activity.

References and Notes

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12 In Ref. 2 the similar substituent effect on the inhibition of the α-chymotrypsin and subtilisin activities was reported.

13 Generally saying, trigonal boron-diol complexes (less water-soluble) are not so stable as tetrahedral boron-diol complexes (more water-soluble) in aqueous solution. Compounds **4** and **6** which are frequently used as a stable protecting group for boronic acids may result in less water-soluble trigonal boron-diol complexes in a significant concentration and precipitate.