JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Phenylboronic Acid—a Potent Inhibitor of Lipase from *Oryza sativa*

M. P. RAGHAVENDRA AND V. PRAKASH*

Department of Protein Chemistry and Technology, Central Food Technological Research Institute, Mysore 570 013, India

The kinetics of inhibition of rice bran lipase (RBL) by phenylboronic acid (PBA) was studied to elucidate the nature of inhibition and the effect of the inhibitor on the structure−function of RBL. The effectiveness of an inhibitor is normally expressed by the constant K_i, which is calculated from the Lineweaver−Burk plot and found to be 1.7 mM at pH 7.4. The kinetics of inhibition by PBA was competitive, indicating the presence of serine in the active site of the enzyme. The loss of activity of RBL was concentration dependent on the inhibitor (PBA), and the inactivation followed a pseudo-first-order kinetics. Fluorescence emission measurements indicated a decrease in the fluorescence emission intensity and a red shift in the emission maximum as the inhibitor concentration was increased. The inhibition of the enzyme by PBA was also confirmed by thermal denaturation measurements, which indicated a shift in the thermal denaturation temperature of the enzyme toward lower temperatures. The far-UV-CD data suggest that there were no significant changes in the conformation of the enzyme as a result of binding of PBA. These results indicate that PBA is a potential inhibitor of RBL and binds to the enzyme in bringing about inhibition without any structural alterations.

KEYWORDS: Rice bran lipase; inhibition; activity; phenylboronic acid; thermal denaturation temperature; conformational stability

INTRODUCTION

Rice (*Oryza sativa*) is one of the most important food cereals for the majority of the world's population. Bran, which comprises the testa and pericarp, is known to be rich in a number of important components such as oil (*1*), proteins and vitamins, and neutraceuticals. The prime drawback of rice bran as a source of oil is the presence of highly active lipolytic enzymes in the bran (*1*−*5*). The lipolytic activity is observed as soon as the bran is removed from the rice, and lipolysis continues with prolonged storage (*2*), yielding high levels of free fatty acids in the oil, which becomes unsuitable for processing to an edible oil. Several methods have been applied to arrest the lipolysis in the bran including heating, hydrochloric acid treatment (*6*), or exposure to microwave and irradiation (*7*), but they have not been totally successful. Inactivation of rice bran lipase (RBL) by chemical inhibitors is expected to yield useful information of the structure−function relationship of RBL.

Phenylboronic acid (PBA) has been reported to be a reversible inhibitor of hydrolases, that is, lipases (*8*−*10*) and proteases (*11*−*14*), and inhibits the lipases by binding near or at the active site serine. The kinetics of boronic acid inhibition have been studied, in particular for pancreatic lipase (*10*) and lipoprotein lipase (*8*). It is reported that most of the boronic acids were noncompetitive inhibitors, but when the substrate was an emulsion of tributyrin and also when the substrate was a very fine emulsion of olive oil or when the pH of the reaction was raised from 7.0 to 9.5, there was a tendency toward mixed or competitive kinetics. However, the reversible inhibition of lipases with boronic acids is highly dependent on the inhibition condition used. A change in the substrate and/or pH gave a change in the inhibitory kinetics from noncompetitive to mixed or competitive kinetics (*15*).

The present study was taken up to investigate whether the inhibitor action is analogous to other lipase systems or different from those reported under the given conditions and to study the changes in the concentrations of the enzyme−inhibitor−substrate complex in order to determine the individual rate constants. Furthermore, the results were evaluated by biophysical characterization using circular dichroic (CD) spectroscopy, fluorescence spectroscopy, and thermal denaturation temperature analysis.

MATERIALS AND METHODS

Materials. Triacetin, Triton X-100, and phenylboronic acid were obtained from Sigma Chemical Co., St. Louis, MO. Buffer salts were obtained from E-Merck India Ltd., Mumbai, India. Calcium chloride (dihydrate) was from BDH (P) Ltd., New Delhi, India. All other chemicals used were of analytical grade. Quartz-double-distilled water was used throughout in all of the experiments.

Methods. *Isolation and Purification of Rice Bran Lipase.* RBL was isolated and purified according to a modified procedure (*16*). The clarified rice bran extract in 50 mM Tris-HCl buffer of pH 7.5 was

* Author to whom correspondence should be addressed (telephone +91-821-517 760; fax +91-821-516 308; e-mail prakash@cftri.com).

10.1021/jf0112926 CCC: $22.00
Published on Web 09/07/2002

first subjected to 25% ammonium sulfate precipitation, and the supernatant was used for isolation of lipase after centrifugation. The concentration of ammonium sulfate in the supernatant was then raised to 55%, and the precipitate thus obtained was collected, dissolved in the same buffer, and dialyzed at 4 °C to remove salts. The clear supernatant solution obtained after centrifugation was then subjected to ion-exchange chromatography using an DEAE-Sepharose CL-6B anion exchange column, and the enzyme was selectively eluted with 0.2 M KCl solution. The enzyme fraction eluted at 0.2 M KCl gradient was pooled and further subjected to 55% ammonium sulfate precipitation and dialyzed free of salt. The clear solution was then loaded onto a Sephadex G-75 column pre-equilibrated with 50 mM sodium phosphate buffer, pH 7.4. The enzyme fraction was pooled, concentrated, dialyzed free of salts, and lyophilized. The temperature was maintained at 4 °C throughout. The enzyme thus obtained was stored at −20 °C in a desiccator and used for further experimentation.

Protein Concentration. Protein concentration of RBL solution was determined using an extinction coefficient ($E^{1\%}_{1cm}$) of 15.25 ± 0.5 at 276 nm, which was obtained by correlation of absorption to nitrogen by micro-Kjeldahl estimation (*16*).

Lipase Assay. Lipase activity of both native and modified enzyme with PBA was carried out in a Mettler Toledo DL12 titrator by titrimetric method using triacetin as substrate at 30 °C (*17*). A 5% (w/v) solution of triacetin containing 0.1% Triton X-100 was prepared in 20 mM sodium phosphate buffer, pH 7.0. The reaction mixture containing 4 mL of substrate, 2 mL of enzyme (0.5−2.0 mg/mL), and 10 μL of 0.1 M $CaCl_2$ was incubated in Queue orbital shaker at 30 °C at 150 rpm for 4 h. The reaction was terminated by the addition of 4 mL of distilled alcohol prior to the addition of the substrate, which serves as the blank. The liberated acid was titrated against 0.05 N alkali to an end point of pH 9.5. One unit of enzyme activity was expressed as microequivalents of alkali consumed per milligram of protein per hour of incubation, according to the equation

$$\text{specific activity} = \frac{(V_2 - V_1) \times \text{N of alkali} \times 1000}{a \times \text{incubation period}} \qquad (1)$$

where V_1 and V_2 are the volumes of standard alkali (NaOH) consumed by blank and sample, respectively, N is the normality of the standard alkali, a is the amount of protein in the reaction mixture (mg/mL), and 1000 is the factor to express the activity in microequivalents of alkali consumed.

Determination of Kinetic Constants of the Rice Bran Lipase. An enzyme−inhibitor preincubation method was used to measure the residual specific activity using an emulsified substrate. For kinetic studies, 3×10^{-5} M enzyme was incubated with different concentrations of PBA at 30 °C for different time intervals. At specified time intervals, 200 μL aliquots of the enzyme were drawn and checked for the remaining activity as described above. The Michaelis−Menten constant (K_m) and the inhibition constant (K_i) of the enzyme were determined by employing the Lineweaver−Burk double-reciprocal plot obtained from the initial velocity studies using the designated substrate and inhibitor concentrations for measuring the reaction rates (*18*). The results are the average of two experiments.

Size Exclusion Chromatography Using Fast Protein Liquid Chromatography (FPLC). Size exclusion chromatography of both native and PBA-treated RBL was carried out on a Superdex-75 column using FPLC. The RBL was incubated in the presence of 3 mM PBA under nitrogen at 30 °C for 30 min in an Innova 4000 incubator shaker with gentle stirring. Both native and PBA-treated samples were dialyzed with three or four changes against respective buffers and centrifuged at 10000*g* for 10 min. Clear supernatant was loaded onto the Superdex-75 column. The protein was eluted at a flow rate of 0.5 mL/min in a 60 min run. Blanks containing 3 mM PBA were also run to give necessary corrections.

Thermal Denaturation Studies. The thermal denaturation behavior of the RBL in the absence and presence of PBA was followed using a Gilford Response-II UV−visible spectrophotometer from M/s Ciba-Corning Diagnostics. The setup consists of an electronically controlled thermal cuvette holder, which can accommodate six thermal quartz cuvettes and accurately manage increments in temperature up to 0.1 °C/min. The change in the absorbance of ∼0.3 mL of protein having an absorbance of 0.3−0.4 in each case was monitored at 287 nm as a function of temperature in the range of 30−95 °C with 1 °C increments with appropriate blanks. Spectral data were stored and analyzed using specific software with the instrument. The apparent T_m was calculated either by first-derivative plot of absorbance or van't Hoff plot (*19*). The fraction of protein in the unfolded state (F_u) is given by

$$F_u = \frac{Y_f - Y}{Y_f - Y_u} \qquad (2)$$

where Y_f is the absorbance of protein solution in the native state, Y_u is the absorbance of protein solution in the unfolded state, and Y is the absorbance of the protein solution at different temperatures. The apparent thermal denaturation temperature ($T_{m(app)}$) is defined as the temperature at which the value of F_u is 0.5. The results are the average of two experiments.

Fluorescence Spectroscopy. The fluorescence spectra of RBL in the presence and absence of PBA were recorded with a protein concentration of 0.1 mg/mL using a Shimadzu RF-5000 recording spectrofluorometer at 30 °C. The emission spectra of enzyme in buffer alone and of enzyme in the presence of PBA were recorded in the range of 300−400 nm, 10 s after excitation at 285 nm. The results are the average spectra of two experiments.

Circular Dichroic Spectroscopy. CD spectra of lipases were recorded in a Jasco J20-C automatic recording spectropolarimeter at 30 ± 0.5 °C with the slits programmed to give a 1 nm bandwidth. The far-UV-CD spectra of the native and PBA-treated RBL were measured with a protein concentration of 0.2 mg/mL in a 1 mm cell in the wavelength range of 200−260 nm. The far-UV-CD spectral data were expressed as mean residue ellipticity (degree·cm^2·$dmol^{-1}$) (*20*). The results shown are the average spectra of two experiments.

RESULTS

Effect of Phenylboronic Acid on Activity. The kinetics of inactivation of RBL has been studied by various concentrations of PBA and is shown in **Figure 1A**. The rate of inactivation is dependent on the concentration of the inhibitor used. Therefore, a greater loss of enzyme activity (80%) was obtained at 10 mM PBA concentration, and the inactivation followed pseudo-first-order kinetics as indicated by typical semilog plots of activity versus time (**Figure 1A**). The second-order rate constant (k_2) was calculated from the slope of the linear plot of the first-order rate constant (k_1) versus $[PBA]_0$ (**Figure 1B**) and found to be 1.25 mM^{-1}·min^{-1}. The reaction order (n) with respect to PBA was determined from the slope of a double-log plot of k_1 as a function of PBA concentration (**Figure 1C**), yielding a slope of 0.94, which is close to 1, indicating a reaction order of 1 with respect to PBA. This indicates the binding of PBA with the enzyme with the reaction order of 1 near the active site to bring about the inhibition.

Inhibition Constant. A Lineweaver−Burk double-reciprocal plot (*21*) of initial reaction velocity versus the specified variable substrate concentrations at a series of fixed $[I]_0$ values is shown in **Figure 2**. The velocity of the reaction increases with the substrate concentrations, depicting that the enzyme shows more affinity toward the substrate and is related to the Michaelis constant K_m. The K_m values increase from 3.5 ± 0.4 in the absence of inhibitor to 9.3 ± 0.8, 13.9 ± 0.9, 20.0 ± 1.1, and 26.0 ± 1.3 in the presence of 3, 6, 8, and 10 mM PBA, respectively. The increasing value of K_m indicates that the affinity of substrate for the enzyme decreased with increasing concentrations of PBA. Thus, kinetic analysis suggests that the inhibition is competitive. Such competitive inhibition of PBA has been observed in many other lipases (*8*, *9*, *11*, *22*). The K_i (mM) value was calculated from the direct linear plot of the slope versus inhibitor concentration $[I]_0$ (mM) and found to be

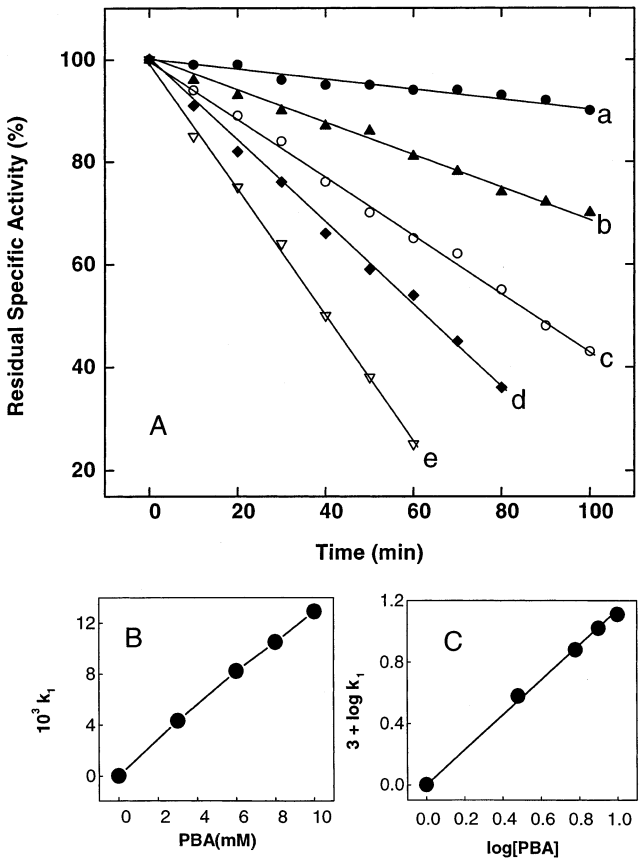

Figure 1. (A) Kinetics of inactivation of RBL by PBA in 0.05 M sodium phosphate buffer, pH 7.4: (a) control; (b) 3, (c) 6, (d) 8, and (e) 10 mM PBA concentrations. (B) Primary plot of pseudo-first-order rate constants versus PBA concentrations. (C) Double-log plot of rate constants versus PBA concentrations to calculate order of the reaction.

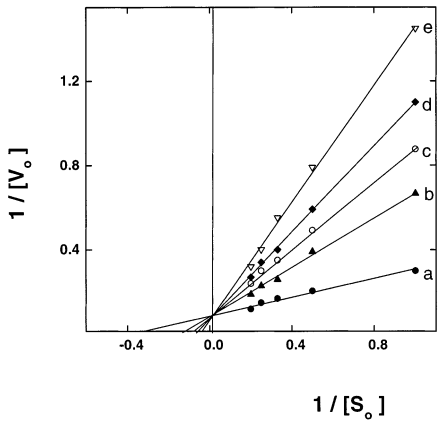

Figure 2. Lineweaver–Burk double-reciprocal plots for the inhibition of RBL at different fixed concentrations of the inhibitor PBA: (a) control; (b) 3, (c) 6, (d) 8, and (e) 10 mM PBA. The inhibitor was preincubated at 30 °C for 10 min prior to the substrate addition.

1.7 mM at pH 7.4. The results demonstrated that the inhibitor could attenuate but not overcome the activation of RBL by the substrate at these concentrations of inhibitor.

Stability of RBL. The effect of PBA on the RBL has been checked by size exclusion chromatography on a Superdex-75 column using FPLC and is shown in **Figure 3**. Both enzyme in buffer alone and PBA-treated enzyme (6 mM) eluted with an elution volume of 11.5 mL, but the intensity of the peak marginally decreases as compared to control. These data clearly indicate that there are no aggregates and enzyme is having the same exclusion size in the presence of PBA as indicated by the chromatographic pattern.

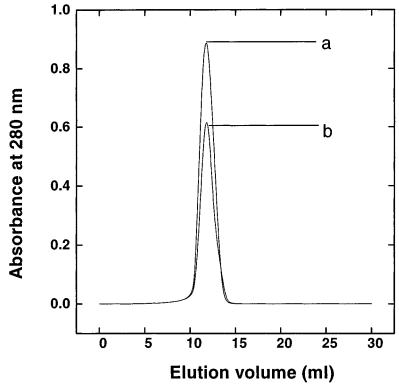

Figure 3. Elution profile of RBL (a) in the absence and (b) in the presence of 6 mM PBA concentration in 0.05 M sodium phosphate buffer, pH 7.4. In both (a) and (b), elution was monitored at 280 nm with a flow rate of 0.5 mL/min in a 60 min run.

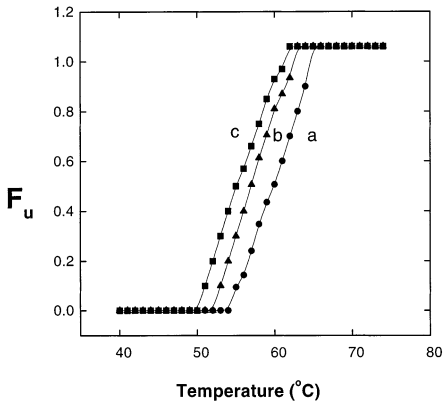

Figure 4. Thermal denaturation profile of RBL (a) in the absence and (b) in the presence of 3 mM and (c) in the presence of 6 mM PBA concentration in 0.05 M sodium phosphate buffer, pH 7.4. The absorption spectra were recorded as a function of temperature at 287 nm. F_u is the fraction of the maximum change that has taken place for any reaction, where the maximum is taken as 1.0.

Effect of Phenylboronic Acid on Thermal Denaturation. The extent of change in the conformation resulting in denaturation of the enzyme with the addition of PBA is evaluated by thermal denaturation temperature analysis. The $T_{m(app)}$ of the enzyme determined in the presence of different concentrations of PBA is shown in **Figure 4**. It is clear from the figure that $T_{m(app)}$ decreases by 3 and 6 °C from the control value of 60 ± 1 °C in the presence of 3 and 6 mM respectively, indicating the destabilization of RBL in the presence of PBA.

Effect of Phenylboronic Acid on Fluorescence Spectra. Fluorescence spectroscopy was carried out to probe the perturbation of tryptophan residues as a result of PBA interaction with the enzyme and is shown in **Figure 5**. From **Figure 5**, it can be seen that the fluorescence emission intensity decreases by 17, 28, 35, and 40% at 3, 6, 8, and 10 mM, respectively. The decrease in the fluorescence emission intensity and red shift in the emission maximum may be attributed to the perturbation of the microenvironment of the tryptophan residues at higher concentrations of PBA.

In several instances competitive inhibitors are known to bring about conformational changes in enzymes. Therefore, it is important to understand whether any significant conformational change has occurred in the enzyme as a result of the PBA–enzyme complex.

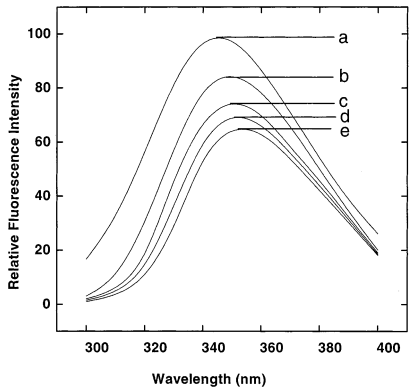

Figure 5. Fluorescence emission spectra of RBL (a) in the absence and (b) in the presence of 3, (c) 6, (d) 8, and (e) 10 mM PBA concentrations in 0.05 M sodium phosphate buffer, pH 7.4, at 285 nm in the range of 300–400 nm.

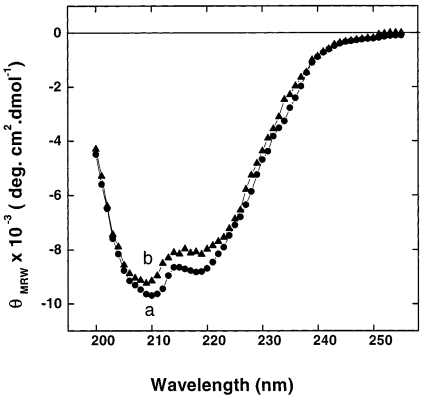

Figure 6. Far-UV-CD spectrum of RBL (a) in the absence and (b) in the presence of 3 mM PBA concentration in 0.05 M sodium phosphate buffer, pH 7.4.

Effect of Phenylboronic Acid on the Structure of Rice Bran Lipase. The conformational status of RBL is monitored as a function of PBA concentration by far-UV-CD spectroscopy and is shown in **Figure 6**. The enzyme exhibits two minima around 220 and 208 nm. In the presence of PBA, there was a small decrease in the ellipticity values in the region of 205−238 nm. Analysis of the data for secondary structure suggests 8% α-helix, 44% β-structure, and 48% aperiodic structure in buffer alone. There was no significant change in the structural features of lipase in the presence of PBA. The inhibitor has no effect on the secondary structural characters of the enzyme, but activity changes were brought about by microenvironmental changes in the active site of the enzyme or in its neighborhood.

DISCUSSION

Aliphatic and aromatic boronic acids are known to be inhibitors of serine hydrolases (*12*, *13*, *23*). The pancreatic lipase was also inhibited by boronic acids, and the derivatives of boronic acid did not interfere with the binding of the lipase to the substrate. The mechanism mainly involves blocking of the active site of the enzyme and inhibition via interaction with the lipase-active site, which is the serine residue (*10*).

The activity of RBL is inhibited by the various concentrations of PBA, and the slopes are more pronounced at higher concentrations. This indicates the alteration of the surface parameters of the enzyme by the inhibitor. The inhibition followed first-order kinetics, and these kinetic data were analyzed by the use of a double-reciprocal plot; the type of reaction may be represented as

$$E + S + I \rightleftharpoons ES\ (\text{active}) + EI\ (\text{inactive}) \rightarrow E + P \quad (3)$$

where E, S, I, and P represent enzyme, substrate, inhibitor, and products and EI and ES are the enzyme−inhibitor and enzyme−substrate complexes, respectively. Here, the inhibitor interacts with the enzyme form, which is a precursor of the S-binding form. Furthermore, it is assumed that either S or I may be present on the enzyme surface, but not simultaneously. This may arise when S and I compete for the active site or binding of I so as to cover the active site.

PBA has been shown to represent an analogue of the tetrahedral intermediate arising in the active of serine-histidine hydrolases during the catalytic process (*11*, *24*, *25*). This intermediate precedes the formation of an acyl-enzyme (*11*). Therefore, at fixed concentration of inhibitor it is possible to drive all of the inhibition from the enzyme by the addition of sufficient substrate. Hence, V_{max} was unchanged in the presence of the inhibitor; that is, all lines shared a common *y*-intercept, and this clearly proves that the affinity of the inhibitor is more effective toward the lipase rather than substrate and further supported by the value of the inhibition constant K_i.

The reaction between PBA and enzyme was reversible, because the enzyme regains activity after the removal of PBA by extensive dialysis. This clearly reveals that PBA is not tightly bound to the enzyme by any covalent bonds. It was reported that in the case of lipoprotein lipase the presence of apolipoprotein C-II partly reverses the inhibition of lipoprotein lipase by PBA (*8*). The inhibition is supposed to occur through a tetrahedral complex with the active site consisting of the serine and histidine residues (*26*).

The size exclusion chromatographic experiments show that there is no change in the size of the molecule, and no aggregation of the enzyme could be seen. The fluorescence emission spectral measurements showed a decrease in the fluorescence emission intensity accompanied by progressive red shift, indicating that the tryptophan groups are experiencing a higher polar environment in the presence of PBA concentrations due to their exposure from the interior of the protein to the bulk solvent (*27*, *28*). These changes in the tryptophan fluorescence of RBL may also have an effect on the thermal stability of RBL. The thermal denaturation result shows that the PBA destabilizes the RBL, indicated by a shift in the $T_{m(app)}$ from a control value of 60 ± 1 °C by 6 °C at 6 mM PBA. However, the effect of PBA on the activity of RBL is accompanied by only a small change in the secondary structure of the enzyme only at higher concentrations of PBA.

The above results indicate that PBA inactivates RBL and decreases the structural stability of the enzyme. The inactivation by the addition of PBA depends on the concentration of the inhibitor, and the extent of effectiveness of the inhibitor is also evident by the K_m and K_i values—it is competitive in nature. Furthermore, the inhibitory effect of PBA is reversible, but at high concentrations, the loss of activity is not completely reversible, and there are no structural changes in enzyme at either low or high concentration of the inhibitor. On the other hand, changes in both the fluorescence and thermal denaturation temperature confirm that minor structural changes do occur without any aggregation of the molecule.

Therefore, the above data unequivocally show that above 6 mM PBA there is a significant change in the structural profile of lipase, and this probably involves aromatic residues such as tryptophan as evidenced by fluoresence measurements. Above a 6 mM concentration of PBA, even though there is no

secondary structural change in the enzyme, the microenvironmental changes in and around residues such as tryptophan are very evident. This may also reflect the status with reference to the environment of serine and histidine residues bringing about the inactivation of the enzyme in these high concentrations of PBA.

ABBREVIATIONS USED

RBL, rice bran lipase; PBA, phenylboronic acid; $[I]_0$, inhibitor concentration; UV-CD, ultraviolet circular dichroic; FPLC, fast protein liquid chromatography; F_u, fraction unfolded; $T_{m(app)}$, apparent thermal denaturation temperature.

LITERATURE CITED

(1) Raghavendra Rao, S. N.; Ananthchar, T. K.; Desikachar, H. S. R. Oil content of bran from rice milled to different degrees of polishing. *J. Food Sci. Technol*. **1965**, *2*, 115−116.

(2) Hirayama, O.; Matsuda, H. Purification and characterization of lipolytic acylhydrolases from rice bran. *Nippon Nogei Kagaku Kaishi* **1975**, *49*, 569−576.

(3) Aizono, Y.; Funatsu, M.; Fujiki, Y.; Watanabe, M. Purification and characterization of rice bran lipase II. *Agric. Biol. Chem.* **1976**, *40*, 317−324.

(4) Shastry, B. S.; Raghavendra Rao, M. R. Chemical studies on rice bran lipase. *Cereal Chem.* **1976**, *53*, 190−200.

(5) Fujiki, Y.; Aizono, Y.; Funatsu, M. Chemical properties of major subunit of rice bran lipase. *Agri. Biol. Chem.* **1978**, *42*, 599−606.

(6) Nasirullah; Krishnamurthy, M. N.; Nagaraja, K. V. Effect of stabilization on the quality characteristics of rice bran oil. *J. Am. Oil Chem. Soc.* **1989**, *66*, 661−663.

(7) Thomas, P. P.; Gopalkrishnan, N.; Damodaran, A. D. Rice bran lipase inactivation by microwaves for low free fatty acid oil. *Oleagineux* **1991**, *46*, 245−246.

(8) Vainio, P.; Virtanen, J. A.; Kinnunen, P. K. J. Inhibition of lipoprotein lipase by benzene boronic acid. Effect of apolipoprotein C−II. *Biochim. Biophys. Acta* **1982**, *711*, 386−390.

(9) Abouakil, N.; Lombardo, D. Inhibition of human milk bile salt dependent lipase by boronic acids. Implication to the bile salts activator effect. *Biochim. Biophys. Acta* **1989**, *1004*, 215−220.

(10) Garner, C. W. Boronic acid inhibitors of porcine pancreatic lipase. *J. Biol. Chem.* **1980**, *255*, 5064−5068.

(11) Koehler, K. A.; Lienhard, G. E. 2-Phenylethaneboronic acid, a possible transition state analog for chymotrypsin. *Biochemistry* **1971**, *10*, 2477−2483.

(12) Philipp, M.; Bender, M. L. Inhibition of serine proteases by arylboronic acids. *Proc. Natl. Acad. Sci. U.S.A.* **1971**, *68*, 478−480.

(13) Antonov, V. K.; Ivanina, T. V.; Ivanova, A. G.; Berezin, I. V.; Levashov, A. V.; Martinek, K. Binding-catalysis relationship in α-chymotrypsin action as revealed from reversible inhibition study of phenylalkylboronic acids. *FEBS Lett.* **1972**, *20*, 37−40.

(14) Antonov, V. K.; Ivanina, T. V.; Berezin, I. V.; Martinek, K. *n*-Alkylboronic acids as bifunctional reversible inhibitors of α-chymotrypsin. *FEBS Lett.* **1970**, *7*, 23−25.

(15) Woolley, P.; Petersen, S. B. In *Lipases*; University Press: Cambridge, U.K., 1994.

(16) Rajeshwara, A. N.; Prakash, V. Purification and characterization of lipase from rice (*Oryza sativa* L). *Nahrung* **1995**, *39*, 406−418.

(17) Sudhindra Rao, K.; Rajendran, S.; Rajeshwara, A. N.; Prakash, V. Structural stability of lipase from wheat germ in alkaline pH. *J. Protein Chem*. **1991**, *10*, 291−299.

(18) Allison, R.; Purich, D. L. Practical considerations in the design of initial velocity enzyme rate assays. *Methods Enzymol*. **1979**, *63*, 3−22.

(19) Pace, C. N.; Shirley, B. A.; Thomson, J. A. Measuring the conformational stability of a protein. In *The Protein Structure: A Practical Approach*; Creighton, T. E., Ed.; IRL Press: Oxford, U.K., 1989; pp 311−330.

(20) Yang, J. T.; Kubota, S. *Microdomains in Polymer Solution*; Dublin, P. L., Ed.; Plenum Press: New York, 1985.

(21) Lineweaver, H.; Burk, D. The determination of enzyme dissociation constant. *J. Am. Chem. Soc*. **1934**, *56*, 658−666.

(22) Crompton, I. E.; Cuthbert, B. K.; Lowe, G.; Waley, S. G. β-lactamases inhibitors: The inhibition of serine β-lactamases by specific boronic acid. *Biochem. J*. **1988**, *251*, 453−459.

(23) Koehler, K. A.; Hess, G. P. A new specific and reversible bifunctional alkylborinic acid inhibitor of acetylcholinesterase. *Biochemistry* **1974**, *13*, 5345−5350.

(24) Kraunt, J. Serine proteases: Structure and mechanism of catalysis. *Annu. Rev. Biochem.* **1977**, *46*, 331−358.

(25) Matthews, D. A.; Alden, R. A.; Birktoft, J. J.; Freer, S. T.; Kraunt, J. X-ray crystallographic study of boronic acid adducts with subtilisn BPN (Novo) A. Model for the catalytic transition state. *J. Biol. Chem.* **1975**, *250*, 7120−7126.

(26) Brockerhoff, H. A model of pancreatic lipase and the orientation of enzymes at interfaces. *Chem. Phys. Lipids* **1973**, *10*, 215−222.

(27) Teale, F. W. J. The ultraviolet fluorescence of proteins in neutral solution. *Biochem. J.* **1960**, *76*, 381−390.

(28) Shifrin. S.; Luborsky, S. W.; Grochowski, B. J. L-Asparaginase from *Escherichia coli* B. Physicochemical studies of the dissociation process. *J. Biol. Chem.* **1971**, *246*, 7708−7713.

Received for review October 1, 2001. Revised manuscript received July 24, 2002. Accepted July 26, 2002. M.P.R. gratefully acknowledges CSIR, New Delhi, India, for providing the Research Associateship during the course of study.

JF0112926