A New Strategy for Determining Optimum pH of Isozymes

Kil-Joong Yoon

Division of Applied Sciences, Cheongju University, Cheongju 360-764, Korea
Received February 16, 2004

A hydrogenperoxide sensor containing peroxidase extracted from horseradish was constructed and pH effect on its sensing ability was investigated. Current profiles of the biosensor with pH and the electrophoretic analysis showed that horseradish peroxidase consists of two isozymes. Assuming that it is a hypothetical two-isozyme mixture, the current profiles were deconvoluted into two Gaussians. Application of the new Michaelis-Menten equation connoting pH concept to this system enabled to find all the related dissociation constants of the isozyme-substrates and the isozyme-proton complexes and to determine pHs for the maximal isozyme activities.

Key Words : Biosensor, Enzyme electrode, Hydrogen peroxide, Peroxidase, Isozyme

Introduction

The most striking characteristics of enzymes are their immense catalytic power and high specificity in the biological system. The structure and the function of an enzyme are determined by the sequences of amino acids of which the protein is made. The active site of an enzyme, which takes up a relatively small part of the total volume of the enzyme, is a three-dimensional entity and a crevice formed by residues that come from different parts of the linear amino acid sequences. So a change in pH can have a number of distinct effects on enzymes, e.g. inactivation of the enzyme, a change in the ionization state of the enzyme or a change in the equilibrium position outside a certain pH range. This means that enzyme-catalyzed reactions are dependent on proton concentration in the biological system. In 1913, Michaelis and Menten proposed a simple model to account for the kinetic properties of many enzymes. But they had no cognizance of pH effect that exerts a far-reaching influence on the reaction rates. Even though reaction rate is not dependent on pH only, their having no consideration of pH effect is a crucial weak point in their establishment. But application of the new Michaelis-Menten equation, which considered pH effect, to our system enables to find all the dissociation constants of the related chemical species in our biological system.[1-4]

Quantitative determination of hydrogen peroxide is very important in many fields of industry, biochemistry and environmental chemistry.[5,6] Here if the reaction is especially involved in redox process, electrochemical methods are still more efficient tools in the quantitative analysis of biological material. Lots of electrochemical approaches to sense hydrogen peroxide have ever been carried out.[7a-e] Consequently, a lot of biosensors which are modified with the biocatalytic materials such as animal or plant tissue[8a-f] have been constructed and their electrochemical behaviors, for example, response time, potential and pH dependence, long term stability, optimization of mediator concentration, effect of the tissue composition and so forth, have been investigated. We had ever defined a pH-dependent current function, $G(H^+)$, to determine the optimum pH of enzyme electrochemically when a biosensor has only one catalytic component and already reported the results. But pH-dependent current profile of the peroxide sensor containing cabbage tissue[9] showed that its intensity does not depend on only one enzyme, but on two active isozymes in our previous study.[10-15] Because we have not ever had any appropriate method to the analysis of such a system as has more than one catalytic enzyme, two new assumptions, which are very central to the present work, were supposed to treat with our system, These are as follows: 1) The reduction function of cabbage tissue based transducer catalyzed by peroxidase is Gaussian distribution with pH. 2) pH-dependent current profile of cabbage root-tissue based amperometric electrode is the sum of the two Gaussians, which show two current peaks at two different pHs. Then, we developed a carbon paste electrode with the commercially available peroxidase extracted from horseradish because horseradish and cabbage belong to the same taxonomical family, cruciferae.[16] It showed the same phenomena as cabbage tissue based sensor did. Based on the assumptions mentioned above, pH-dependent current profiles of the enzyme electrode with horseradish peroxidase at various monitoring concentrations of substrate were resolved into two Gaussians. And application of the deconvoluted data to the new Michaelis-Menten equation enabled to determine optimum pH of each isozyme activity. This article is related to the approach for determining optimum pHs of isozymes in the system which has more than one enzyme.

Experimental Section

Apparatus. The electrodes were connected to a BAS Model EPSILON cyclic voltammograph (Bioanalytical System, Inc., U. S. A.) for CV diagram. An Ag/AgCl reference (BAS MF2052) and a Pt counter electrode (BAS MW1032) were used. And EG&G Model 362 (Princeton Applied Research, U. S. A.) scanning potentiostat was used for other amperometric measurements. The output was recorded on a KIPP &

ZONEN X-t recorder (BD111, Holland). The pHs of the electrolytic solutions were checked with a Digital pH/mV/ Temp meter (Suntex SP-701, Taiwan). The electrophoresis of enzyme was run on a Dual Gel Caster (Mighty Small II kit, SE 250, Hoefer Scientific Inc. U. S. A.).

Chemicals. Graphite powder (: ≤ 0.1 mm) and mineral oil were purchased from Fluka and Sigma, respectively. Hydrogen peroxide (Junsei, EP, 35%), peroxidase (Sigma, from horseradish, abbr. HRP, E.C. 1.11.1.7) and guaiacol (Sigma) were used as received. Electrolyte was NaCl (Shinyo Pure Chem. ≥ 99.5%). pH constancy of electrolytic solution (0.1 M NaCl) was achieved by adequate adding a dilute HCl (Oriental Chem. Co.) and a dilute NaOH (Shinyo Pure Chem.) solution.

Electrode and procedures. The electrode material used in this work was prepared as follows.[10] 0.91 g of graphite powder and 10 mL of chloroform (containing 0.09 g ferrocene) were mixed and then dried throughly. 100 mg of the ferrocene immobilized graphite powder was added to 100 μL of 1.0% HRP solution prepared in advance and air dried subsequently. Carbon paste was made by mixing graphite powder with mineral oil at a 55 : 45 ratio (wt/wt). The final carbon paste had the following composition: 49.6% graphite powder, 4.9% ferrocene, 45% mineral oil, 0.5% HRP by weight. The working electrode was prepared by packing this carbon paste into a 6 mm i.d. polyethylene tube which is provided with the ohmic contact.[10] The signal was measuerd as follows. The enzyme electrode was placed in 10 mL of 0.1 M NaCl solution under constant stirring and at an applied potential of -275 mV *vs*. Ag/AgCl. And the difference between signals without and with adding substrate solution was considered to be current due to the reduction of hydrogen peroxide catalyzed by HRP on a strip chart recorder. In addition, electrophoretic expansion of peroxidase was performed in 10% native polyacrylamide gel and visualized in the 50 mM phosphate buffer (pH 7.4) containing 15 mM guaiacol and 5 mM hydrogen peroxide. All data were computed with KaleidaGraph 3.0 and Origin 7.0.

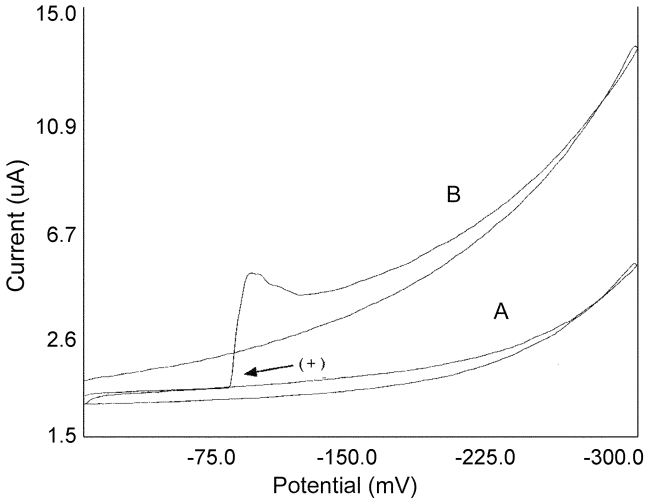

Figure 1. Cyclic voltammograms for HRP modified enzyme electrode without (A) and with (B) adding 200 μL of 1.0 M H_2O_2, in 0.1 M NaCl with a potential scan rate of 10 mV/s. The (+) indicates the addition of H_2O_2.

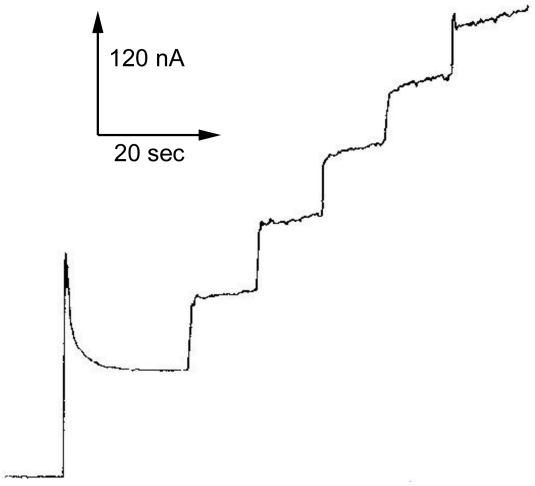

Figure 2. Typical amperogram at pH 6.14 and at -275 mV for successive increments of 50 μL of 1.0 M H_2O_2.

Results and Discussion

Figure 1 compares two cyclic voltammograms of the enzyme electrode modified with HRP in 0.1 M NaCl solution (pH 6.14) without (A) and with (B) 0.02 M H_2O_2. Even though the shape of the voltammogram obtained after adding substrate is very similar to that of the CV obtained before adding substrate, the former reveals higher reduction current than the latter as soon as substrate is added.

This indicates that peroxidase exerts immense catalytic power. The difference in the current between A and B, which is considered to be signal in Figure 1, showed an ohmic behaviour {y = -3.06-0.0377x, R = 0.993, y: current (nA), x: potential (mV)} with the applied potential in the working potential range. Based on the above results, the operating potential of -275 mV was chosen in order to make a compromise between a big signal and a low noise. Figure 2 is a typical strip chart recording obtained with the HRP enzyme electrode upon successive additions of standard hydrogen peroxide to working solution. It shows that the response is linear up to the maximum working concentration of hydrogen peroxide. The linearity of signal current with substrate concentration is y = -0.05+0.0296x {R = .999, y: current (nA), x : added vol. (μL) of 0.1 M H_2O_2} in the range of our working concentration. Table 1 gives the pH-dependence of the reduction currents catalyzed by HRP at a variety of substrate concentrations. Plots of currents against pH will therefore be of the form shown in Figure 3. And they show two clear peaks at two different pH values. The obserbation of these two peaks is a good evidence that two principal species are present. Figure 4 shows the dark bands of the stained protein separated at three different concentrations of peroxidase. The solutions used in electrophoresis contain peroxidase of 0.5 (lane 1), 1.0 (lane 2) and 2.0 μg/

Table 1. Effect of pH on the biocatalytic current

[S], mM	pH											
	2.33	3.47	4.66	6.14	6.70	7.93	8.94	9.78	10.86	11.81	12.42	
8.4	60	177	231	222	174	99	126	129	90	60	8	nA
9.9	70	201	264	252	192	120	144	144	102	75	9	
12.0	82	225	303	291	216	144	168	162	117	93	11	
15.0	94	264	348	333	252	180	198	192	135	117	15	

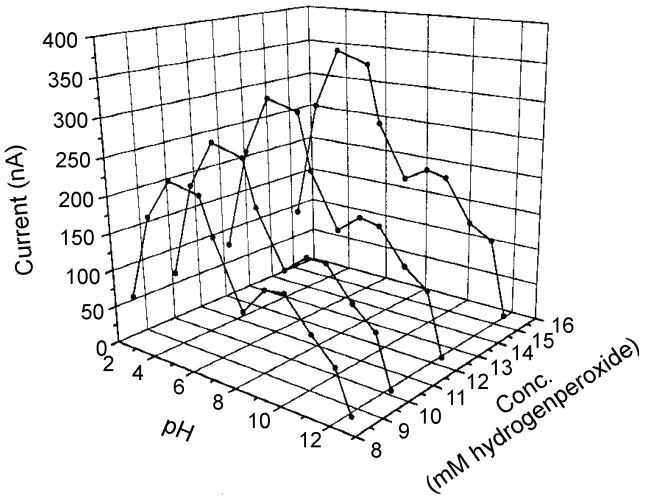

Figure 3. Dependence of the biocatalytic current on pH at a variety of substrate concentrations. Other conditions are the same as in Figure 2.

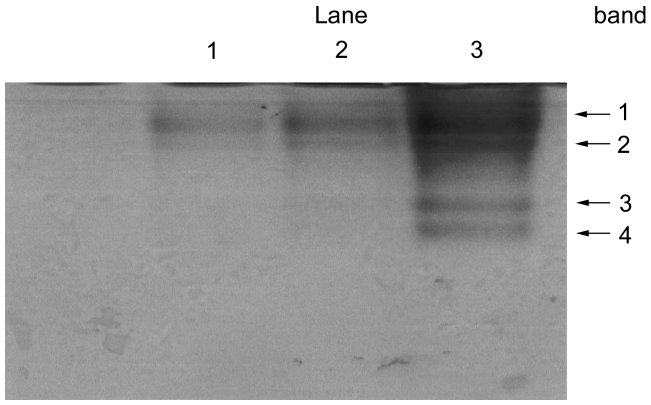

Figure 4. Native polyacrylamide gel electrophoresis of the peroxidase extracted from horseradish.

mL (lane 3). Two stained proteins (band 1 and band 2) are observable in lane 1and lane 2, respectively. And four bands are stained at the high concentration of peroxidase (lane 3). But lane 3 shows that the intensities of the two new bands (band 3 and band 4) which have the higher electrophoretic mobility are much weaker than those of band 1 and band 2. This makes it possible to regard band 1 and band 2 as major bands. Two experimental results above suggest probably that two isozymes are present in the modified biosensor. Those made us assume that the current profiles in Figure 3 are the sums of two Gausians. Our assumption being quite within the bounds of possibility, a good deal of effort has been directed toward resolving them. So every pH dependent profile of current in Table 1 has been deconvoluted into two Gausian products and the results obtained are presented in Table 2. Enzymes which are active in the region of the low and high pH are referred to as isozyme A and B in this paper, respectively. The relationships[2] of $[H^+]$, K_m and V_{max} derived from the new Michaelis-Menten theory connoting pH concept can be written as follows,

$$K_m = K_s \frac{1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}}{1 + \frac{[H^+]}{K_{es1}} + \frac{K_{es2}}{[H^+]}} \quad (1)$$

$$V = \frac{V_{max}}{1 + \frac{[H^+]}{K_{es1}} + \frac{K_{es2}}{[H^+]}} \quad (2)$$

From equation (1) and (2), we can therefore write

$$\frac{V}{K_m} = \frac{V_{max}}{K_s} \frac{1}{1 + \frac{[H^+]}{K_{e1}} + \frac{K_{e2}}{[H^+]}} \quad (3)$$

Table 2. Computer deconvolution of the pH-dependent current profiles

[S], mM	pH												
	Isozyme A							Isozyme B					
	2.33	3.47	4.66	6.14	6.70	7.93	8.94	8.94	9.78	10.86	11.81	12.42	
8.4	70	161	240	209	167	68	22	98	121	95	45	6	nA
9.9	88	186	279	236	183	76	25	117	139	109	55	8	
12.0	100	213	319	270	208	83	28	125	157	125	59	9	
15.0	104	248	362	305	237	94	32	153	184	147	73	10	

Table 3. LogV_{max} and log(V_{max}/K_m) determined by the Lineweaver-Burk plot

	pH											
	Isozyme A							Isozyme B				
	2.33	3.47	4.66	6.14	6.70	7.93	8.94	8.94	9.78	10.86	11.81	12.42
logV_{max}	2.504	2.902	3.023	2.878	2.707	2.253	1.877	2.656	2.725	2.680	2.447	1.924
$\log\frac{V_{max}}{K_M}$	3.050	3.384	3.574	3.539	3.467	3.118	2.572	3.180	3.270	3.151	2.810	1.910

Table 3 lists the logarithms of V_{max} and V_{max}/K_m that Lineweaver-Burk plots give at various pHs for two isozyme A and B.

Using the above equations and Table 3, we calculate all the dissociation constants (K_{e1}, K_{e2}, K_{es1}, K_{es2}, K_s, $K_{s'}$, $K_{s''}$) of chemical species which are present in our system,

$$K_{e1} = \frac{[EH][H^+]}{[EH_2]};\ K_{e2} = \frac{[E][H^+]}{[EH]};\ K_{es1} = \frac{[EHS][H^+]}{[EH_2S]}$$

$$K_{es2} = \frac{[ES][H^+]}{[EHS]};\ K_{s''} = \frac{[EH_2][S]}{[EH_2S]};\ K_s = \frac{[EH][S]}{[EHS]}$$

$$K_{s'} = \frac{[E][S]}{[ES]}$$

This can be done as follows.

1) When K_{es1} >> [H^+] >> K_{es2}, a commom logarithm of the approximated equation (2) is

$$\log V = \log V_{max} \quad (4)$$

A plot of log$V$ against a fluent, pH yields a straight line with a slope of 0 and this straight line cuts a vertical shaft at logV_{max}.

2) When [H^+] >> K_{es1}, its common logarithm is given by the expression,

$$\log V = \log V_{max} + \log K_{es1} + pH \quad (5)$$

Thus, a curve of log$V$ against pH is here a straight line with a one-unit slope and intersects the axis of ordinates at logV_{max} + logK_{es1}. In other words the log$V$ increases by 1 for every pH increase of 1 in the region.

3) When K_{es2} >> [H^+] , similar considerations apply also to the region, then equation (2) follows

$$\log V = \log V_{max} - \log K_{es2} - pH \quad (6)$$

A plot of log$V$ against pH will be a straight line with slope of -1 and intercept of logV_{max} – logK_{es2} on the vertical axis. In short, equation (4), (5) and (6) are the calculated straight lines tangent to the fitted quadratic curves of the plot, logV_{max} vs. pH, which intersect at a pH value equal to the pK. For isozyme A and B, it is easily shown that equation (4) and (5), and (4) and (6) intersect at a pH value equal to pK_{es1} and pK_{es2}, respectively, at the top of Figure 5.

Also it is possible to use equation (3) in order to determine K_{e1} and K_{e2} in a fashion that is similar to the procedure mentioned previously.

4) When K_{e1} >> [H^+] >> K_{e2}, a common logarithm of the equation (3), which was approximated, is

$$\log\frac{V}{K_m} = \log\frac{V_{max}}{K_s} \quad (7)$$

The curve of log(V/K_m) against pH is a straight line with a slope of 0 and this straight line cuts a vertical shaft at log(V_{max}/K_s). Here, the value of K_s may readily be obtained by putting log(V_{max}/K_s) equal to the intercept.

5) When [H^+] >> K_{e1}, a common logarithm of the equation (3) is given by the expression,

$$\log(V/K_m) = \log(V_{max}/K_s) + \log K_{e1} + pH \quad (8)$$

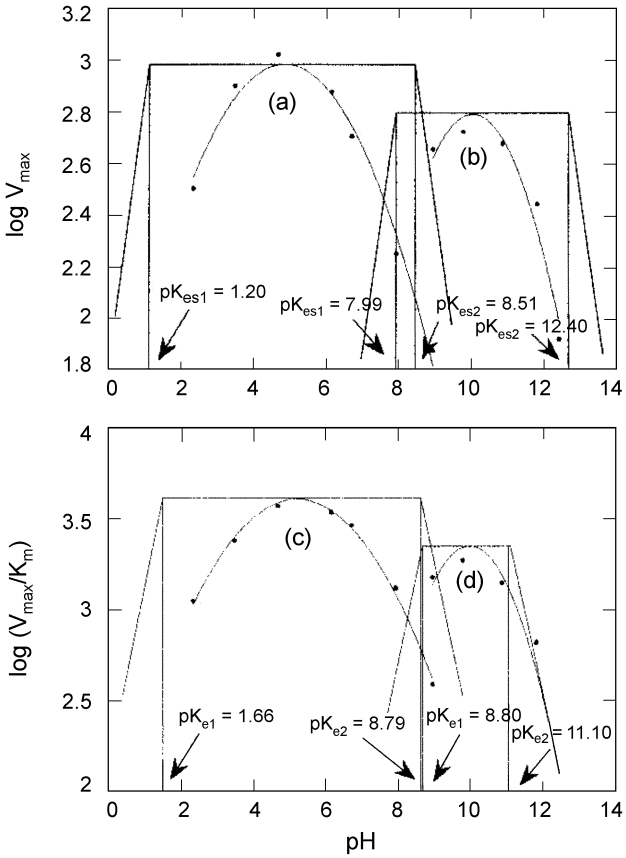

Figure 5. Effect of pH on logV_{max} and log(V_{max}/K_m) of enzyme-catalyzed reactions. Quadratic equations, (a) y = -0.0687x^2 + 0.665x + 1.38; (b) y = -0.127x^2 + 2.53x – 1.38; (c) y = -0.706x^2 + 0.734x + 1.71; (d) y = -0.211x^2 + 4.20x – 17.6, here, x is the axis value (pH) of abscissa and y, that of ordinates.

Table 4. Gathering of thermodynamic parameters of isozymes

	Isozyme	
	A	B
K_{es1}	6.3×10^{-2}	1.0×10^{-8}
K_{es2}	3.1×10^{-9}	4.0×10^{-13}
K_{e1}	2.2×10^{-2}	1.6×10^{-9}
K_{e2}	1.6×10^{-9}	7.9×10^{-12}
K_s	0.23	0.25
$K_{s'}$	0.12	4.9
$K_{s''}$	0.66	1.6
V_{max} (nA)	9.8×10^2	5.0×10^2

This is a straight line with a one-unit slope and an intercept at $\log(V_{max}/K_s) + \log K_{e1}$.

6) When $K_{e2} >> [H^+]$, logarithmic expression of the equation (3),

$$\log(V/K_m) = \log(V_{max}/K_s) - \log K_{e2} - \text{pH} \qquad (9)$$

will be a straight line with slope of -1 and intercept of $\log(V_{max}/K_s) - \log K_{e2}$ on the vertical axis. Equation (7), (8) and (9) were illustrated in figure 5-(c) and (d). It shows that they intersect at a pH value equal to pK_{e1} and pK_{e2} for enzyme A and B, respectively.

Using the relationships, $\frac{K_{es1}}{K_{e1}} = \frac{K_{s''}}{K_s}$ and $\frac{K_{es2}}{K_{e2}} = \frac{K_s}{K_{s'}}$, enables us to calculate $K_{s''}$ and $K_{s'}$. Table 4 lists all the thermodynamic parameters calculated.

When we designate pH dependent fractional composition as F, the general form of F for the polyprotic acid H_nA is

$$F_{H_{n-j}A} = \frac{K_1 K_2 \ldots K_j [H^+]^{n-j}}{D}$$

where $D = [H^+] + K_1 [H^+]^{n-1} + K_1K_2 [H^+]^{n-2} + \cdots + K_1K_2K_3 \cdots K_n$.

Plots of F versus pH show that the maxima values of F_{EH}

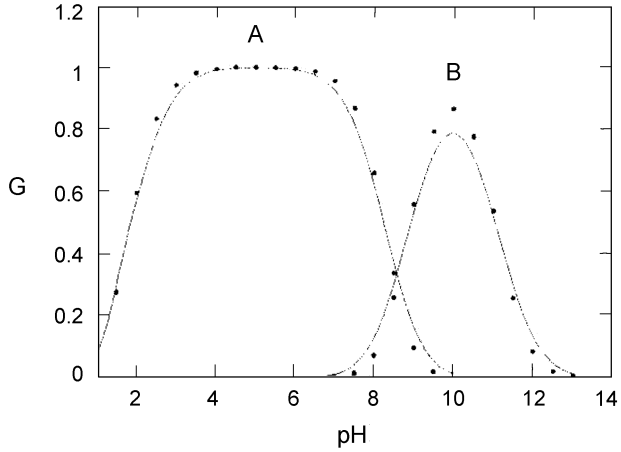

Figure 7. pH-dependence of G (H^+).

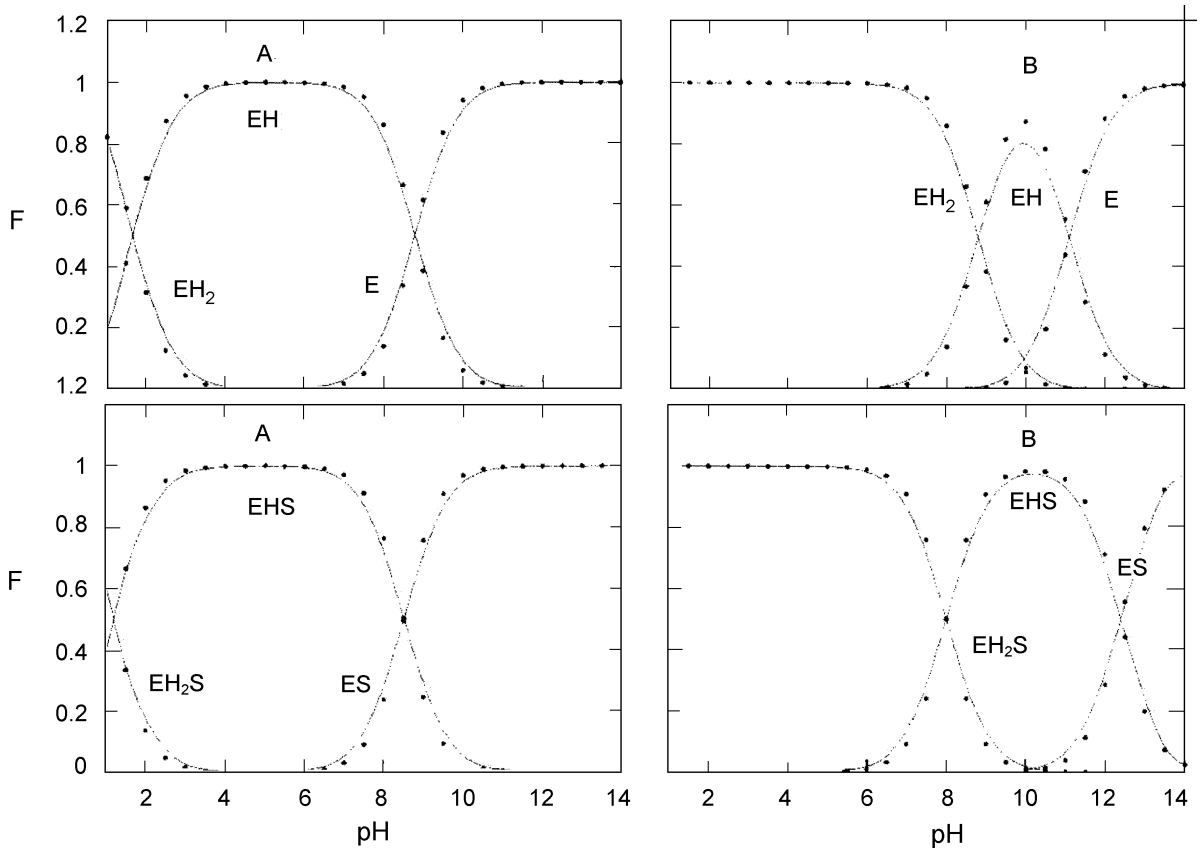

Figure 6. Fractional composition diagrams for peroxidase-proton and -substrate complexes.

and F_{EHS} are between pH 4 and pH 6 and between pH 9 and pH 11 as shown in Figure 6. This coincides with the fact that Figure 3 already showed two peak currents around between pH 4 and 8, and between pH 8 and 11. It is perhaps thought that the enzymatic catalysis is rather through the process, EH + S $\rightarrow$ EHS $\rightarrow$ EH + product. Presumably, it is informative and clear that the reaction rate is attributed to the joint contribution of EH and EHS. So a new functions connoting the characteristics of the joint contribution for isozyme A and B, $G(H^+) = F_{EH} \cdot F_{EHS}$, can be defined. Figure 7 shows that maximum values of $G(H^+)$ occur at around pH 5 and at pH 10. Conclusively it seems likely that these are optimum pHs of peroxidase isozymes extracted from horseradish.

Conclusions

The pH-dependent current profile of the biosensor immobilizing the peroxidase extracted from horseradish showed two peaks and its electrophoretic expansion showed two major bands. Coincidence of the two events above enabled us to assume that the system consists of two isozymes and to resolve the current profiles into two Gausians. The application of the new Michaelis-Menten equation to the two Gausians made determine the optimum pHs of isozymes. It is believed that this electrochemical method will be helpful to determine the optimum pH of other isozymes in many fields.

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