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Kinetic Studies of the Urease-Catalyzed Hydrolysis of Urea in a Buffer-Free System

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ABSTRACT

The kinetics of urea hydrolysis catalyzed by urease, mainly in the absence of buffers by use of the self-buffer effect of the products, was investigated. The effect of pH, temperature, and concentration of enzyme, substrate, product, salt ions, and buffers on the kinetic behavior of urease was examined. A kinetic model of a modified Michaelis-Menten form, incorporating substrate and product inhibition, pH dependence, and temperature effect, was developed to describe the reaction rate. Experimental data indicated that urease in a buffer-free solution was less susceptible to the inhibition of substrate product. The Michaelis constant keeps almost constant with the variation of pH and temperature, and increases with the addition of buffers and salts. The data also suggested that the noncompetitive pattern of the product inhibition, which is not significantly affected by temperature, increases gently with increasing pH. A Monod form rate expression was proposed to analyze the pH effect on the maximum rate. The proposed kinetic model was also examined by the long-time experiments in which pH, substrate, and product concentration varied obviously during the reaction course.

Index Entries: Enzyme kinetics; soluble urease; urea hydrolysis; buffer-free system; pH control by CO_2 addition.

Nomenclature: C_{NH_3}, initial ammonia concentration in the reaction solution, M; C_{urea}, urea concentration in the reaction solution, M;

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C_{urease}, urease concentation in the reaction solution, mg/L; *Ea*, activation energy, kJ/mol; $[H^+]$, hydrogen ion concentration, M; k_0, frequency factor, mol NH_3/s/g urease; $K_{es,1}$, $K_{es,2}$, empirical parameters in Eq. (4) and Eq. (10), *M*; K_M, Michaelis constant, *M*; K_p, product inhibition constant, *M*; K_s, substrate inhibition constant, *M*; *P*, product concentration, *M*; *S*, substrate concentration, *M*; *T*, temperature, K or °C; *t*, time, s; *V*, reaction rate, mol NH_3/s/g urease; V_m, maximum reaction rate, mol NH_3/s/g urease; V_{m_0}, maximum reaction rate without pH inhibition, mol NH_3/s/g urease; $V_{m,p}$, maximum reaction rate at product concentration P, mol NH_3/s/g urease; V_m(pH), maximum reaction rate at pH, mol NH_3/s/g urease; α, empirical exponent in Eq. (4) and Eq. (10); β, empirical exponent in Eq. (4) and Eq. (10).

INTRODUCTION

Urease is a very interesting enzyme because of the high efficiency of its action. It deserves attention not only because of its historical importance, since it was the first enzyme to be isolated in crystalline form (*1*), but also because of its wide applications in urea sensors for the routine determination of urea in blood, in urine, and in waste water (*2–8*); in the treatment of uremia (*9–12*); in the treatment of waste water containing urea from fertilizer plants (*13–16*); and in the study of enzymatic kinetics as a model enzyme, soluble or immobilized (*17–28*).

The kinetics of the urease-catalyzed hydrolysis of urea has been investigated by many researchers (ref. *29* and refs. cited therein). Now it is commonly recognized that ammonium carbamate is the true product of the urease-catalyzed hydrolysis of urea, and final products, bicarbonate and ammonium ions, are formed by nonenzymatic and buffer-dependent decomposition of ammonium carbamate. Early papers on the kinetics of urease-catalyzed urea hydrolysis frequently reported the buffer effects as well as the effects of some metal ions and some organic substances (*17,25–28*). Recently, Huang and Chen (*29,30*) found that the inhibition of urease by phosphate is of a partially mixed type and close to the partially competitive, whereas that by citrate is uncompetitive. Goldstein et al. (*21*) found that the pattern of product inhibition also depends on the nature of the buffer used. Their experimental data indicated that product inhibition on urease-catalyzed hydrolysis of urea by ammonium ions in citrate buffer is competitive, whereas in phosphate buffer, the inhibition by ammonium ions is noncompetitive. Meanwhile, the products HCO_3^- and NH_4^+ were found in phosphate and maleate buffers, and ammonium carbamate in virtually quantitative yields was obtained in citrate and Tris buffers (*20*). Thus, it seems that although the inhibition of ammonium is noncompetitive, that of carbamate is competitive, but in contrast to the result found in earlier literature (*18,20,21,24*) that the product strongly

inhibits the reaction rate, Huang and Chen (*29,30*) concluded from the experimental data that ammonium ions exhibited no significant influence on the rate of urea hydrolysis by urease.

Although a large number of studies have been reported on the kinetics of urea hydrolysis by urease, the experiments were performed at a given temperature, pH value, ionic strength, urease concentration, or within a narrow range of them, and in a certain buffer system, usually at a low product concentration. Only recently, some researchers (*31,32*) described the reaction rate by a modified Michaelis-Menten equation that takes into account inhibitions by product and substrate, and pH-dependent kinetics.

Furthermore, because of the alkaline characteristic of the urea hydrolysis, most studies were performed with concentrated buffers to keep the pH around a preset value. Although buffer ions usually inhibit urease (*26,29,33*), only a few works on urease can be found in a buffer-free system (*22,34,35–38*); also, a high ion strength decreases the activity of urease (*27,34*); and the toxicity of heavy metal ions to urease is well known (*35,36,39,40*).

Therefore, in order to increase the specific activity of urease, a test system must be chosen that has the appropriate range of substrate concentration, pH value, and temperature, a low or zero concentration of product, and must be free from any other inhibiting anions and cations. Waste water containing urea, ammonia, and carbon dioxide drained from fertilizer plants is just such a system. There are no metal ions, and economically, it is unreasonable to add buffer salts to the waste water only to recover ammonium salt.

In the literature, the urease activity or the initial rate of urease-catalyzed hydrolysis of urea has been measured by the following methods:

1. Determination of ammonia either by
   a. Phenol-nitroprusside colorimetric method (*21,23,29,30,41,42*);
   b. Nesslerization methods (*17,27*);
   c. Enzymatic analytical methods (*31*);
   d. Ammonia or ammonium electrode (*24,43,44*); or
   e. The titration of ammonia (*45–48*);
2. Determination of carbon dioxide (*49*);
3. The pH-stat assay (*22,35,37,38,50*);
4. The colorimetric determination of urea concentration (*51,52*);
5. Thermochemical method (*20*); and
6. Conductivity measurement (*53,54*).

However, up to now, no comparison among them has been mentioned.

We have studied the effects of pH, temperature, concentration of urease, substrate, product, buffer, and salt ions on the kinetic behavior of urease-catalyzed urea hydrolysis. The kinetics of urease in the aqueous solution free from buffers and any other ions is the main interest of our study. To make full use of the self-buffer effect of the urea hydrolysis

products, NH_3 and CO_2, the controlled addition of CO_2 gas, instead of any other buffer species or pH stat by HCl, is used to control the pH value to obtain kinetic data. This study provides the basic kinetics for the treatment of waste water containing urea.

MATERIALS AND METHODS

Reagents and Apparatus

Urease, Type IX, powder from jack beans, was supplied by Sigma Chemical Co. (St. Louis, MO). Urea, analytically pure, and ammonium carbamate, analytically pure, were from E. Merck (Darmstadt, Germany). All other chemicals were of the purest grade commerically available. A multifunctional autotitrator/pH controller SM Titrino 702 from Metrohm Ltd. (Switzerland) was used in this study.

Preparations of Solutions

Ammonium carbonate solutions were prepared by dissolving a given amount of ammonium carbamate in distilled water (because ammonium carbamate can slowly revert to ammonium carbonate at room temperature). For the preparation of urease solution, instead of using buffer salts, some of ammonium carbonate solution was diluted to the concentration of $10^{-4}M$. Dithiothreitol (DTT) ($5 \times 10^{-4}M$) and EDTA ($10^{-3}M$) were also added to protecting urease from toxic substances, and the solution was brought to pH 7 with pH controller by addition of CO_2 gas, another product of urease-catalyzed urea hydrolylsis that forms a buffer with NH_3. This was done to keep the optimal pH range for urease and either to avoid the buffer effect or to decrease the inhibition of ammonium ions on urease. Because only 1 or 2 mL of urease solution would be added to a reactor of 250 mL, the effects of both DTT and EDTA can be neglected. Then, urease was dissolved in the above solution to a concentration of 0.1–2.5 g/L, and correspondingly, 0.4–10 mg/L in the reactors. The urease preparation stored at 0–4°C remains active for several days.

Choice of Reaction Rate Measuring Methods

By our preliminary experiments, we recognized that the average error of the colorimetric methods (*see* Introduction), 1a, 1b, and 4, for a stationary sample of a given constant concentration is around 3%. Clearly, for the dynamic samples taken from the reactor, the error will be bigger. So these methods are unsuitable for samples with either high concentration of ammonia to study product inhibition (measuring ammonia concentration difference), or high concentrations of urea to study the

maximum rate or substrate inhibition (measuring urea concentration difference). Method 1c at low-level ammonia has the same problem for the samples with high concentration of ammonia. The big error on measurements of carbon dioxide concentration mainly comes from its evaporation. The manufacturers of ammonium electrode (W. Ingold AG, Industrie Nord, Switzerland) declare an error around 2%. As for pH-stat method, it introduces other ions into the reactor, changing ion strength during the rection with time, which is undesirable.

Therefore, the titration of ammonia was selected as the method to determine the concentration of ammonia and the reaction rate, because by use of the autotitrator, the total ammonia concentrations in the aqueous solution of 0.001–0.1*M* can be measured with the relative error of $<0.2\%$. This is more accurate than other methods we tested. In addition, this method is less affected by the presence of CO_2 and salts of strong acids. Also, measuring ammonia concentration change is insensitive to the low concentration of buffers.

Urease Kinetics

The urease kinetics was studied by the initial rate method. An aqueous solution, 250 mL, with the given concentration of ammonium carbonate and urea was placed in the reactor (a glass vessel with a stirring bar in a temperature-controlled bath within an error of ± 0.1°C). After stirring for several minutes to attain thermal equilibrium, CO_2 gas was bubbled into the reactor to adjust the pH value from original ca 9.0 to the value desired. The controlled addition of CO_2 gas using the autotitrator/pH controller was also used to control pH within an error of ± 0.01 during the reaction. Then, the reaction was initiated by injection of a required amount of urease solution (1 or 2 mL), after having removed the same volume of solution from the reactor. After mixing for 15 s, the first sample of 1–5 mL was removed and injected into 1 mL $10^{-3}M$ $CuCl_2$ stirred solution (the amount of Cu^{2+} ions in this mixture would be enough to inactivate all urease [*40*]). Since the addition of the Cu^{2+} has no influence on the titration of total ammonia by HCl, the error resulting from using base or acid to stop reaction is avoided (*46–48*). Then, at appropriate time tervals of 1, 2, or 3 min, the same volume of reaction mixture was taken and injected into 1 mL $CuCl_2$ solution to stop the reaction. The samples were titrated with 0.01 or 0.02*M* HCl using the autotitrator. The initial rate for each run was determined by least-squares methods using four sampling points taken within the early 3, 6, or 9 min. Each experiment was repeated at least three times under the same condition. The data reported here are average values. The relative error of initial rate obtained this way in most of cases was $<1\%$. The rate was thought to respond to the average concentration of urea and ammonium from the first sample to the fourth.

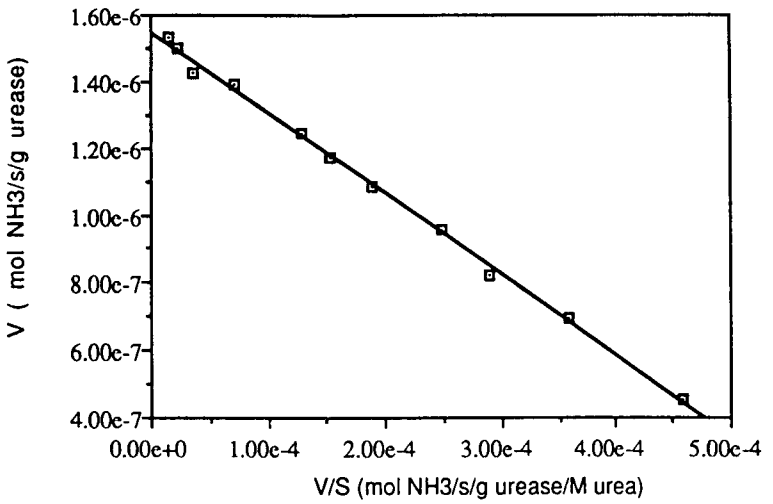

Fig. 1. Eadie-Hofstee plot for urease activity (pH = 7.00, T = 25°C, C_{urease} = 1 mg/L, C_{NH_3} = 0.001M).

RESULTS AND DISCUSSION

Effect of Urease Concentration

The experiments to establish the effect of the urease concentration on the rate of urea hydrolysis by urease showed that the reaction rate is proportional to urease concentration from 0.4 to 10 mg/L in reaction solution, indicating that the specific activity of urease remains constant within the examined urease concentration range.

Kinetic Studies

Urease activity was firstly determined at pH 7 and 25°C for several urea concentrations (0.005–0.1M). When the experiments were carried out in the absence of any other buffer salts, the solution pH increased rapidly up to 8.7–9.3. It was difficult at the initial stages of the reaction to control the pH at low preset values within a small error by the controlled addition of CO_2 gas only. Therefore, 0.0005M of ammonium carbonate was used as buffer after having adjusted the pH to the preset value by adding CO_2. It will be seen that the inhibition of ammonium at so low a concentration is quite small. The Michaelis-Menten kinetic parameters V_m and K_M derived from Eadie-Hofstee linearization (Fig. 1) are 1.545×10^{-6} mol NH_3/s/mg urease and 0.00247M, respectively. For comparison, K_M values reported in literature are listed in Table 1.

Figure 1 indicates that, at a low concentration of urea, the kinetic behavior of urease in the system of low ion strength and no buffer also obeys the Michaelis-Menten equation.

Table 1
K_M Values for Soluble Urease

Test condition	K_M, M	References
22°C, pH 7.05, no buffer, pH stat	0.00125	*38*
30°C, pH 7.00, no buffer, pH stat	0.00225	*35*
38°C, pH 7.00, no buffer, pH stat	0.0018	*22*
38°C, pH 7.00, no buffer, pH stat	0.00328	*37*
28°C, pH 7.00, 0.1*M* citrate	0.0012	*21*
28°C, pH 7.00, 0.1*M* phosphate	0.0095	*21*
25°C, pH 7.00, 0.1*M* phosphate	0.020	*29*
25°C, pH 7.00, Cphosphate →	0.0042	*29*
25°C, pH 7.00, *0.1M* citrate	0.0038	*29*
37°C, pH 7.40, 0.1*M* phosphate	0.031	*23*
27°C, pH 7.00, 0.1*M* phosphate	0.6073	*59*

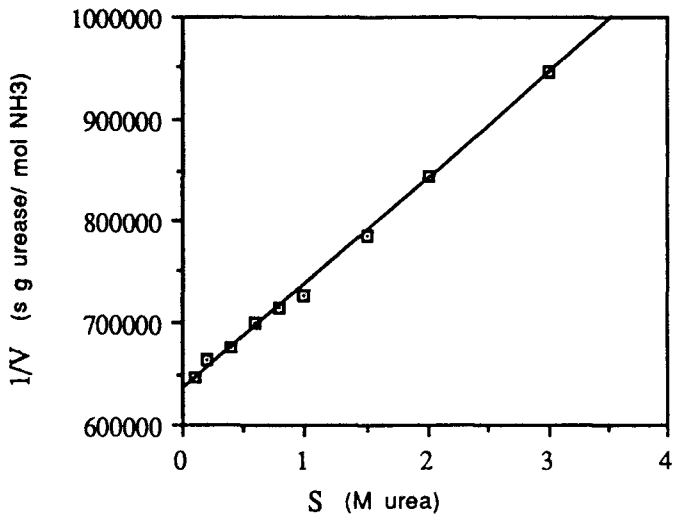

Fig. 2. Lineweaver-Burk plot for the determination of substrate inhibition (pH = 7.00, T = 25°C, C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*).

Substrate Inhibition

The inhibition of urease by urea at a high urea concentration can be described by the following equation (*21,29*):

$$V = (V_m / 1 + S/K_s) \quad (1)$$

where K_s is the substrate inhibition constant. Using the Lineweaver-Burk plot (Fig. 2), the experimental data were fitted to the above equation and K_s was determined to be 6.18*M*, which is near to values recently reported (*29*) (6.42*M* in 0.1*M* phosphate and 6.25*M* in 0.1*M* citrate), but higher than the value in the earlier publications 3.0 (*55*) and 2.81*M* (*24*).

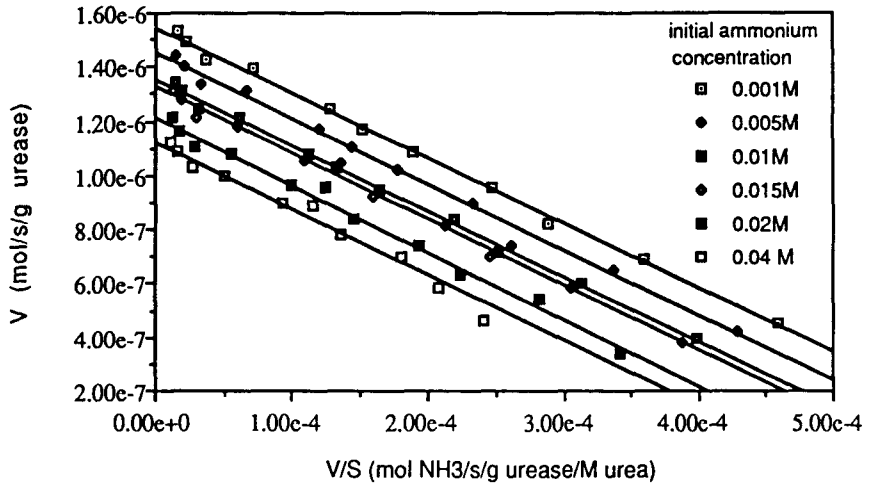

Fig. 3. Eadie-Hofstee plot for urease activity at various ammonium concentrations (pH = 7.00, T = 25°C, C_{urease} = 1 mg/L).

Product Inhibition

The Michaelis-Menten kinetic parameters were determined at various ammonia concentrations at pH 7. From Fig. 3 it can be seen that the enzyme inhibition by ammonia in the absence of buffers is strictly noncompetitive up to the maximum examined ammonium concentration of 0.04*M*. This is in accordance with the results in phosphate buffers in which NH_4^+ is the real existing form of ammonia (*20,21*). In the range of temperature and pH values studied, ammonia exists mainly in the form of ammonium ion, since in the CO_2- NH_3-H_2O coexisting system, the following reactions are established (*22,56*):

$$NH_2CONH_2 + H_2O \rightarrow 2NH_3 + CO_2 \quad (a)$$

$$NH_3 + H^+ \leftrightarrow NH_4^+ \quad (b)$$

$$CO_2 + H_2O \leftrightarrow H^+ + HCO_3^- \quad (c)$$

$$HCO_3^- \leftrightarrow H^+ + CO_3^{2-} \quad (d)$$

$$NH_3 + CO_2 \leftrightarrow NH_2COO^- + H^+ \quad (e)$$

$$H_2O \leftrightarrow H^+ + OH^- \quad (f)$$

For reference, the equilibrium concentrations of these components were calculated and are listed in Table 2. It can be concluded that within the temperature and the ratio of NH_3 to CO_2 in the reaction we studied, most of ammonia exists in the form of NH_4^+ or NH_3, and at pH = 7, mainly in the form of NH_4^+. Hence, the inhibition of ammonium is suggested as:

$$V = [V_m / (1 + K_M/S)(1 + P/K_p)] \quad (2)$$

Table 2
The Equilibrium Concentration
of the Components in the System of NH_3-CO_2-H_2O

T°C	25		40	
$C_{NH_3,\ total}$	0.01*M*	0.01*M*	0.01*M*	0.01*M*
$C_{CO_2,\ total}$	0.01217*M*	0.005*M*	0.01178*M*	0.005*M*
C_{NH_3}	5.665×10^{-5}	4.652×10^{-3}	1.534×10^{-4}	4.814×10^{-3}
C_{NH_4+}	9.939×10^{-3}	5.342×10^{-3}	9.837×10^{-3}	5.174×10^{-3}
C_{NH_2COO-}	4.077×10^{-6}	6.918×10^{-6}	9.619×10^{-6}	1.195×10^{-5}
C_{CO_2}	2.234×10^{-3}	6.821×10^{-6}	1.946×10^{-3}	1.594×10^{-5}
C_{H+}	1.000×10^{-7}	6.544×10^{-10}	1.000×10^{-7}	1.676×10^{-9}
C_{HCO_3-}	9.927×10^{-3}	4.653×10^{-3}	9.819×10^{-3}	4.799×10^{-3}
C_{CO_32-}	4.648×10^{-6}	3.328×10^{-4}	5.914×10^{-6}	1.725×10^{-4}
pH	7.000	9.184	7.000	8.776

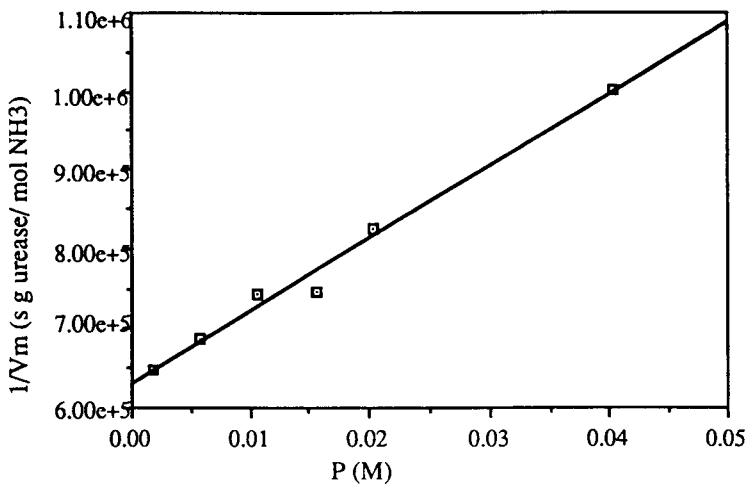

Fig. 4. Plot for urease activity at various ammonium concentrations (pH = 7.00, T = 25°C, C_{urease} = 1 mg/L).

where K_p is the inhibition constant of product. When substrate concentrations, S, is large enough, Eq. (2) can be rewritten as:

$$V_{m,p} = [V_m / (1 + P/K_p)] \quad (3)$$

where $V_{m,p}$ represents the V_m at product concentration P. From $V_{m,p}$ obtained from Fig. 3, V_m and K_p can be determined, as in Kinetic Studies for the determination of substrate inhibition constant, to be 1.585×10^{-6} mol NH_3/s/g urease and 0.0693*M*, respectively (*see* Fig. 4). The K_p value is higher than earlier reported values (*21,24*), indicating that the inhibition of urease by ammonium used is not serious, which agrees with recent publications (*29,30,43*).

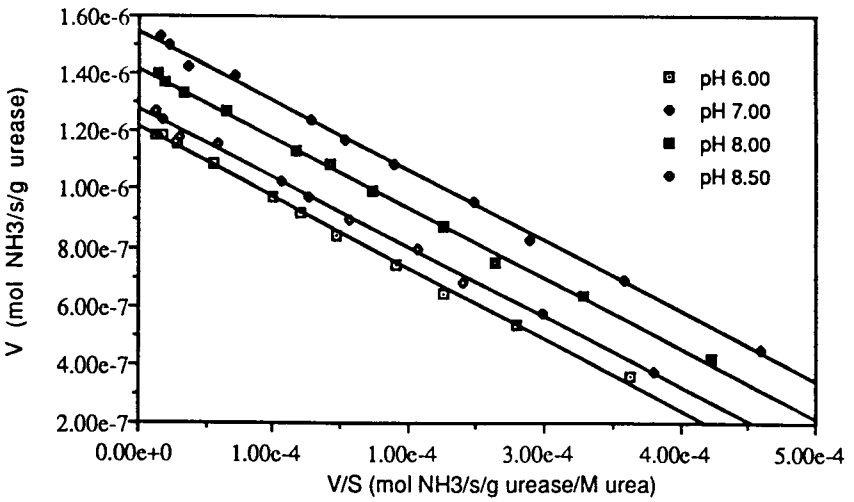

Fig. 5. Eadie-Hofstee plot for urease activity at various pH values (T = 25°C, C_{NH_3} = 0.001M, C_{urease} = 1 mg/L).

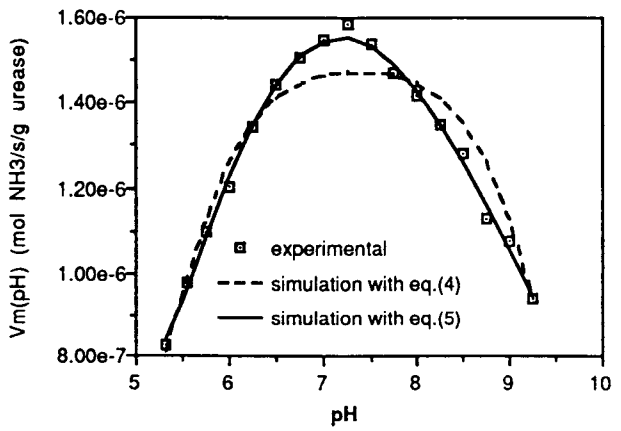

Fig. 6. The maximum reaction rate at various pH values (T = 25°C, C_{urease} = 1 mg/L, C_{NH_3} = 0.001M).

pH Effect

pH Effect on the Kinetic Parameters

The Michaelis-Menten kinetic parameters were estimated at various pH values. The selected data are shown in Fig. 5, from which it can be seen that the inhibition by H^+ or OH^- is only of the noncompetitive type, in contrast to the mixed type reported elsewhere (*31,32*). The pH-dependent maximum reaction rate V_m(pH) is shown in Fig. 6.

$$V_m(\text{pH}) = [V_{m0} / (1 + K_{es,1}/(H^+) + (H^+)/K_{es,2})] \quad (4)$$

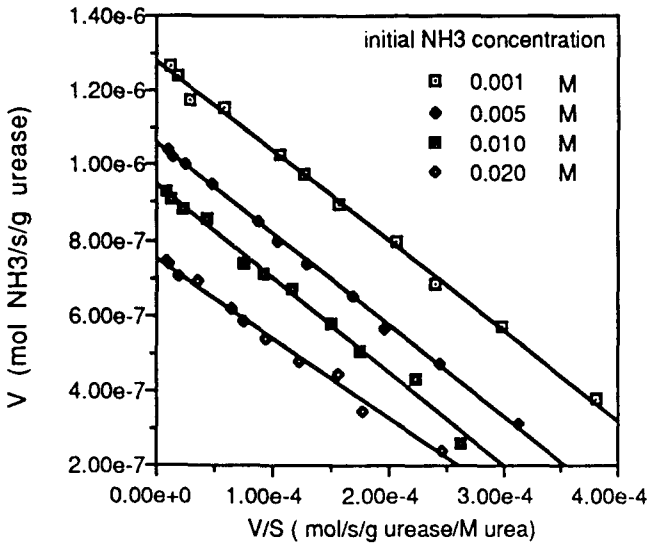

Fig. 7. Eadie-Hofstee plot for urease activity at various NH_3 concentrations (T = 25°C, pH = 8.5, C_{urease} = 1 mg/L).

Although Eq. (4) quantitatively describes the other pH-dependent kinetic data (*31,32*), it was not suitable to the present data as shown in Fig. 6. So an equation similar to Monod equation (*57,58*) was suggested to describe the pH-dependent maximum rate:

$$V_m(\text{pH}) = \{V_{m0} / 1 + [K_{es,1}/(H^+)]^{\alpha} + [(H^+)/K_{es,2}]^{\beta}\} \quad (5)$$

where V_{m0} is termed the maximum reaction rate without pH inhibition, $K_{es,1}$, $K_{es,2}$ are so-called ionization equilibrium constants of enzyme–substrate complex, and α, β are empirical exponents. This model can fit the data perfectly (as shown in Fig. 6), and the parameters V_{m0}, $pK_{es,1}$, $pK_{es,2}$, α, and β, determined by the optimization technique were 2.118×10^{-6} mol NH_3/s/g urease, 9.07, 5.62, 0.373, and 0.564, respectively. Estimated $pK_{es,1}$ and $pK_{es,2}$ can be compared with reported values (*31*), $pK_{es,1}$ = 9.18, and $pK_{es,2}$ = 6.00. The optimum pH was determined to be 7.20.

pH Effect on Product Inhibition

Eadie-Hofstee plot for urease at various product concentrations and at pH 8.5 and 6.25 is shown in Figs. 7 and 8, respectively. From these figures, it can be seen that product inhibition is still of the noncompetitive type at a wide range of pHs. The values of K_p at different pH values were determined as in Product Inhibition, listed in Table 3, and plotted in Fig. 9. As shown, K_p is extremely affected by pH value, i.e., the product inhibition increases with pH value. It is possible that the higher the pH value, the easier the urease and urease–urea complexes attacked by ammonia or ammonium ions. At pH values higher than 8.0, K_p keeps almost constant. Even so, K_p is higher than previously reported values (*21,24*).

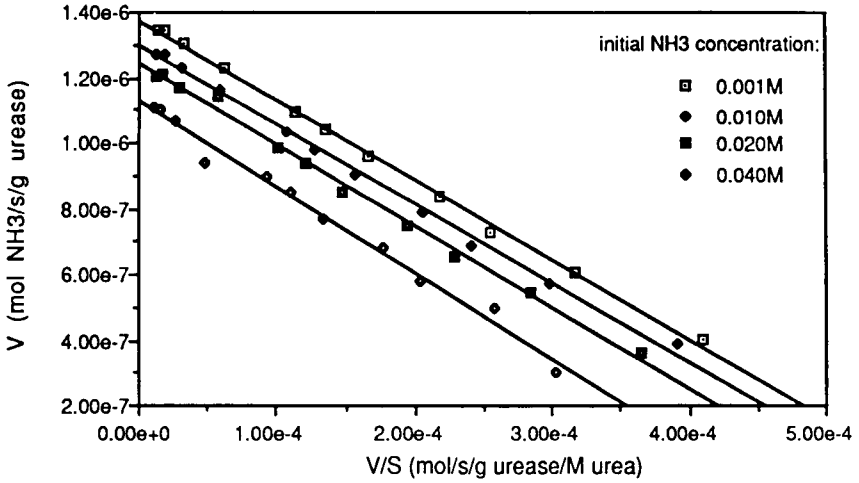

Fig. 8. Eadie-Hofstee plot for urease activity at various NH_3 concentrations (T = 25°C, pH = 6.25, C_{urease} = 1 mg/L).

Table 3
The Product Inhibition Constant K_p at Various pH Values

pH	K_p, M
6.25	0.1785
6.5	0.1194
7.0	0.0693
7.5	0.0386
8.0	0.0311
8.5	0.0327
8.75	0.0298
9.0	0.0310

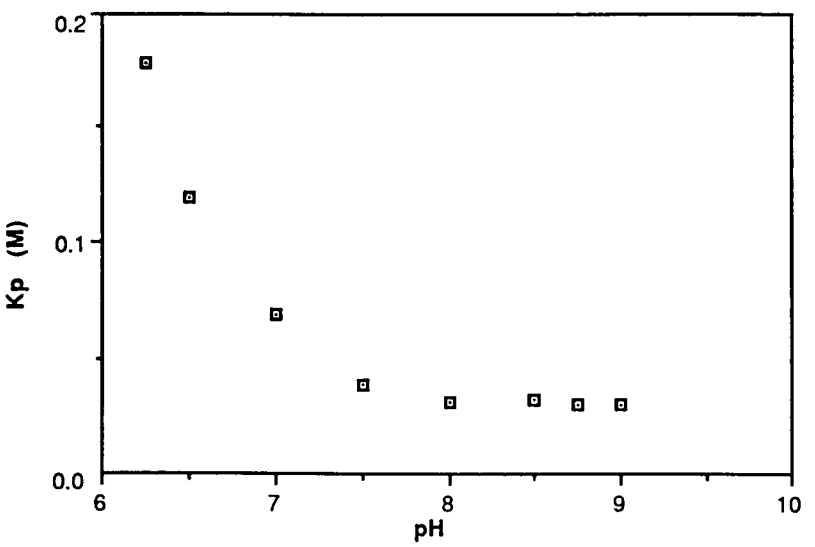

Fig. 9. Plot of K_p vs pH (T = 25°C, C_{urease} = 1 mg/L).

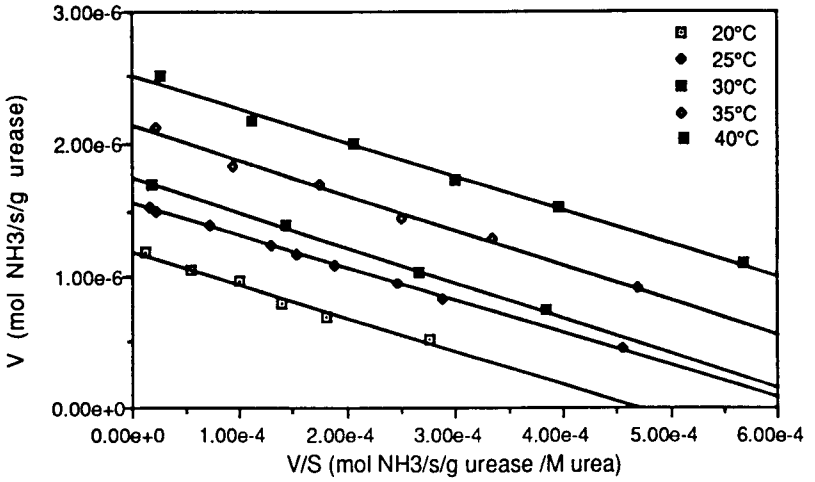

Fig. 10. Eadie-Hofstee plot for urease activity at various temperatures (pH = 7.00, C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*).

Table 4
Influence of Temperature on V_m and K_M

T, °C	20	25	30	35	40
V_m mol NH_3/s/g urease	1.223×10^{-6}	1.545×10^{-6}	1.800×10^{-6}	2.192×10^{-6}	2.578×10^{-6}
K_M (m*M*)	2.53	2.47	2.65	2.62	2.51

Temperature Effect

Temperature Effect on V_m and K_M

The Eadie-Hofstee plots for urease activity at various temperatures (20–60°C) are shown in Fig. 10, and the values of V_m and K_M are listed in Table 4, from which it can be seen that K_M is almost constant within the experimental error with temperature. An Arrhenius equation was used to describe the variation of V_m with temperature:

$$V_m = k_0 e^{-} (Ea / RT) \quad (6)$$

As shown in Fig. 11, a linear relationship of $\ln V_m$ to T^{-1} was obtained up to about 40°C. At higher temperatures, deviations from this linear relation were observed that were owing to the thermodenaturation of urase. k_0 and Ea obtained using the data within 20–40°C were 0.200 mol NH_3/s/g urease and 29.1 kJ/mol, respectively. The activation energy is slightly lower than the reported values 32.6 kJ/mol (*29*) and 35.8 kJ/mol (*23*), respectively, and may be the result of the lower sensitivity of urease to temperature in the absence of buffers.

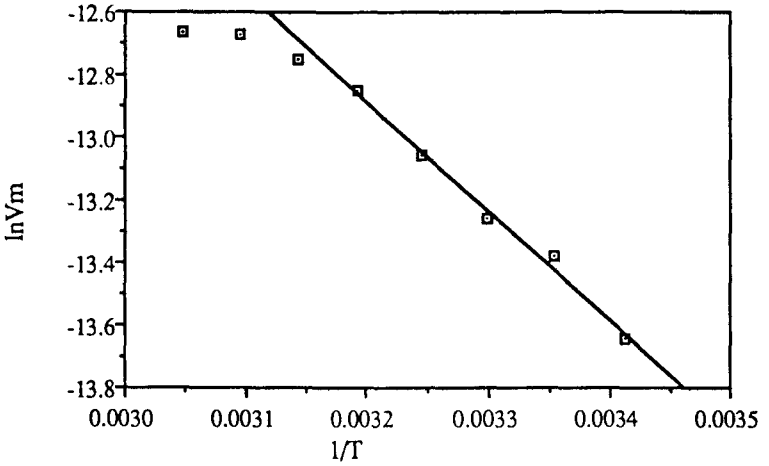

Fig. 11. Arrhenius plot for urease maximum rate (pH = 7.00, C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*).

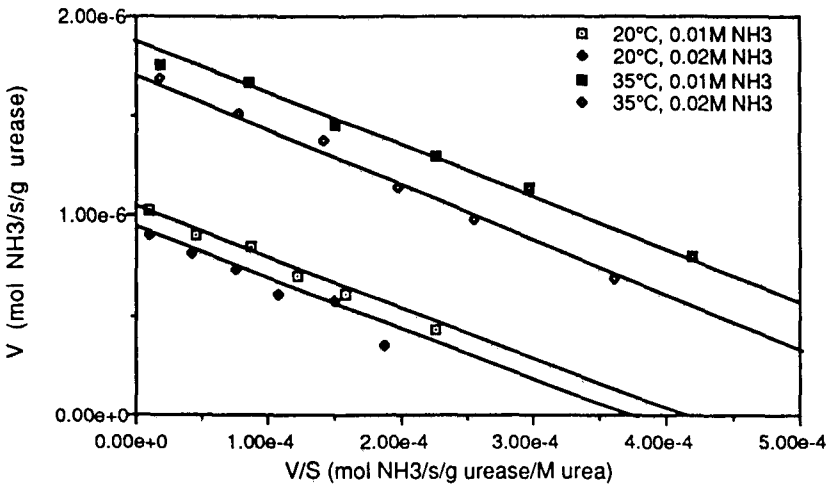

Fig. 12. Plot for urease activity at various temperatures and initial ammonium concentrations (pH = 7.00, C_{urease} = 1 mg/L). The points were experimental data, and the lines represented the simulated values from V_m and K_M at the temperatures indicated and K_p at 25°C.

Temperature Effect on the Product Inhibition

The product inhibition was also examined at various temperatures (Fig. 12). The experimental data and the simulation values seem consistent using K_p obtained at 25°C, which means that K_p keeps constant with the variation of T.

Temperature Effect on the pH Effect

The urease activity plotting to pH at higher temperatures is shown in Fig. 13. The optimum values of parameters in Eq. (5) for these data are listed in Table 5.

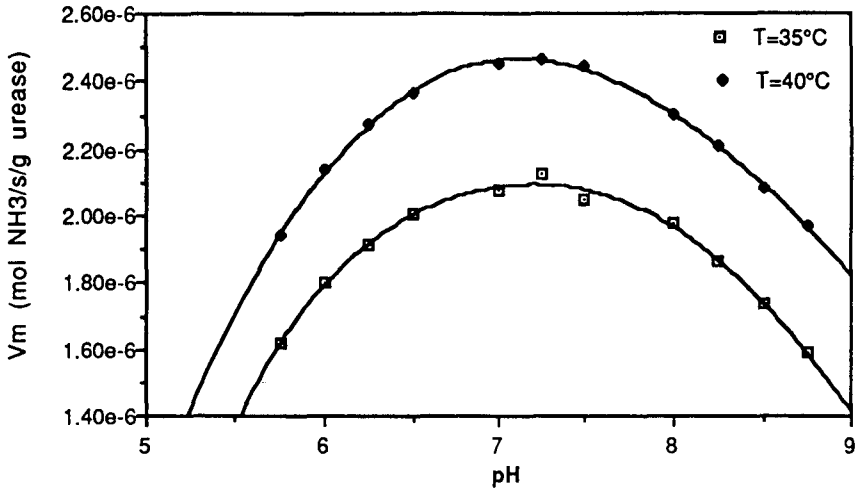

Fig. 13. Plot of V_m vs pH values. The points are experimental data, and the lines are the best fit by Eq. (5) (C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*). V_m was derived from the rate of C_{urea} = 0.1*M* by using Eq. (2).

Table 5
The Parameters of Eq. (5) at 35 and 40°C

T°C	V_{m0}	$pK_{es,1}$	$pK_{es,2}$	α	β
35	2.372×10^{-6}	9.43	5.28	0.489	0.717
40	2.793×10^{-6}	9.58	5.15	0.542	0.769

The Influence of Both Temperature and pH on Product Inhibition

In Fig. 14, the points were the experimental data at pH = 8.5 and T = 35°C. At initial ammonium concentrations of 0.01*M*, the line was the value simulated by Eq. (2) and (4) using the pH effect parameters obtained above at T = 35°C and the K_p value at pH 8.5 and T = 25°C. It also proved that K_m is kept almost constant with varying of both pH and temperature, whereas K_p is almost constant with varying temperature.

Other Influences on Urease Activity

The Deactivation of Urease

The activity of urease was measured after several aging periods. The results are shown in Fig. 15. A half-life of 110 h can be obtained from this thermostability test.

The Influence of Ion Strength on Urease Activity

Urease activity was determined in the presence of some ions as shown in Fig. 16. At low ion strength, the activation of urease can occur, whereas at high ion strength, urease is inhibited (*26*). The type of inhibi-

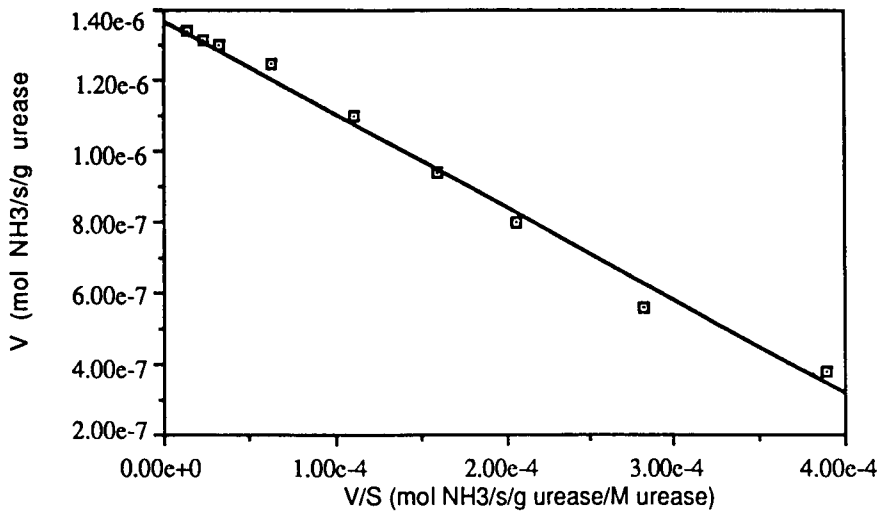

Fig. 14. Eadie-Hofstee plot of urease activity (C_{urease} = 1 mg/L, C_{NH_3} = 0.01*M*, pH = 8.5, *T* = 35°C; the points: experimental data, the line: the simulated values).

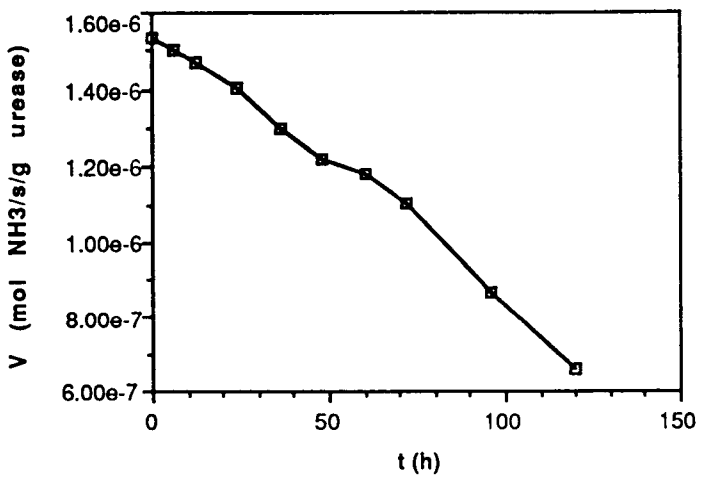

Fig. 15. Urease activity varying with time (testing condition: *T* = 25°C, pH = 7.0, C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*, C_{urea} = 0.1*M*; aging condition: *T* = 25°C, pH = 7.0, C_{NH_3} = 0.001*M*, C_{urease} = 1 mg/L).

tion depends on the ion strength instead of ion type, and at higher pH values, the inhibition becomes more pronounced. At a given ion strength, Michealis-Menten equation is still effective, as can be seen from Fig. 17. At ion strength 0.1*M*, V_m and K_M were 1.503 × 10^{-6} mol NH_3/s/g urease and 0.0039*M*, respectively. Compared to their value without ions, V_m decreases and K_M increases. So as far as kinetics is concerned, the ion-free system is beneficial to urease.

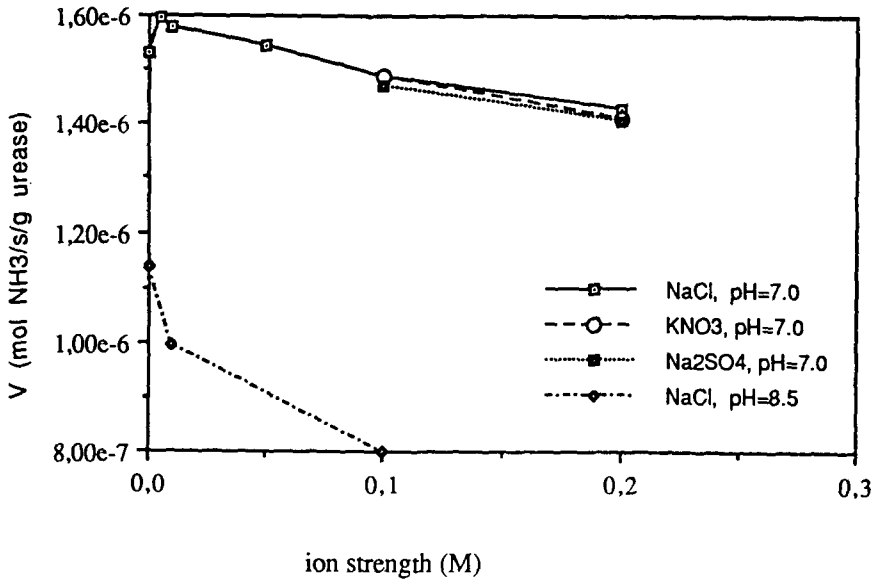

Fig. 16. Urease activity varying with ion strength (T = 25°C, pH = 7.0, C_{urease} = 1 mg/L, C_{NH_3} = 0.001M, C_{urea} = 0.1*M*).

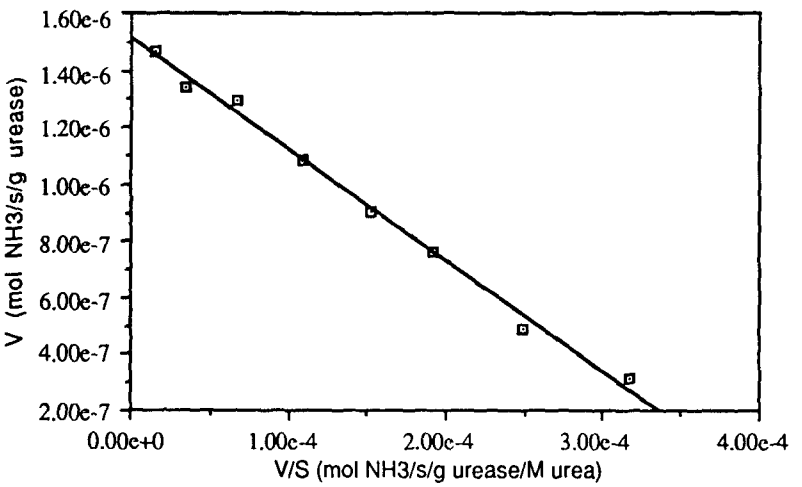

Fig. 17. Eadie-Hofstee plot for urease activity (pH = 7.00, T = 25°C, C_{urease} = 1 mg/L, C_{NH_3} = 0.001*M*; C_{NaCl} = 0.1*M*).

Buffer Effect

The urease activity was detected in the presence of buffer. From Fig. 18, it can be concluded that phosphate salt inhibits urease competitively, which is inconsistent with the conclusion of Huang and Chen (*29*). It can also be concluded that product inhibition is noncompetitive even in the presence of buffer ions. From the data, K_p was determined to be 0.0684*M*,

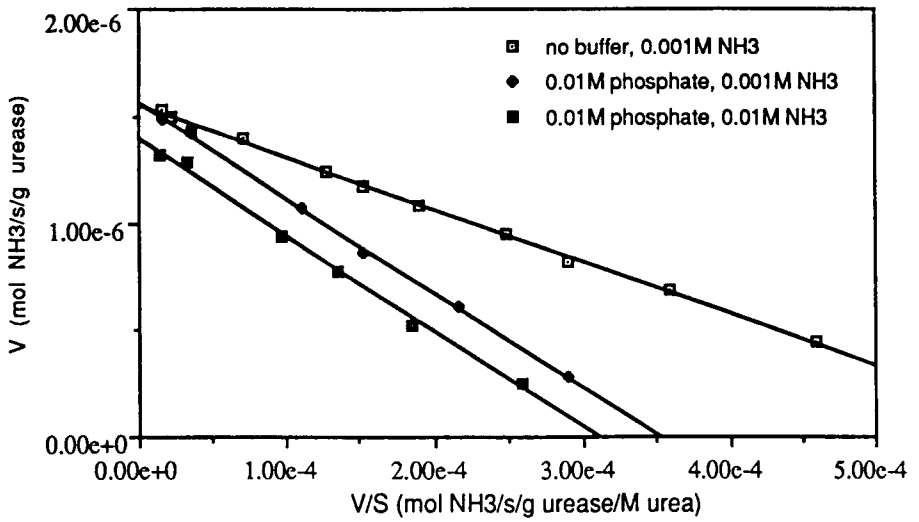

Fig. 18. Eadie-Hofstee plot for urease (pH = 7.00, T = 25°C, C_{urease} = 1.0 mg/L, C_{NH_3} = 0.001M).

which is quite close to the value of 0.0693M in the same conditions without buffers, so the existence of buffer ions does not affect the product inhibition effect.

Experimental Verification of the Kinetics Model by the Long-Term Behavior of Urease-Catalyzed Urea Hydrolysis

As mentioned above, the kinetics data were obtained by the initial rate methods. However, in practice, the kinetic behavior of urea hydrolysis over the whole course of the reaction is more important. pH, as well as substrate concentration, varies a great degree, especially in the system without pH control. Furthermore, endogenous ammonia instead of exogenous, first existing in the form of ammonium carbamate, probably results in a different pattern of product inhibition. From these considerations, the long-term behavior of urea hydrolysis by urease in buffer-free solutions has been studied experimentally and theoretically, based on the kinetic parameters obtained above.

Theoretically, the following assumption can be proposed to describe the hydrolysis of urea by urease in buffer-free system: (1) the urea hydrolysis obeys the kinetic model we obtained by the initial rate method; and (2) the ionic reactions are fast compared to the rate of urea hydrolysis and be assumed to be at equilibrium in the reaction solution. Therefore, the following equations are used to describe the reaction:

$$(dC_{urea} / dt) = (1 / 2) V \quad (7)$$

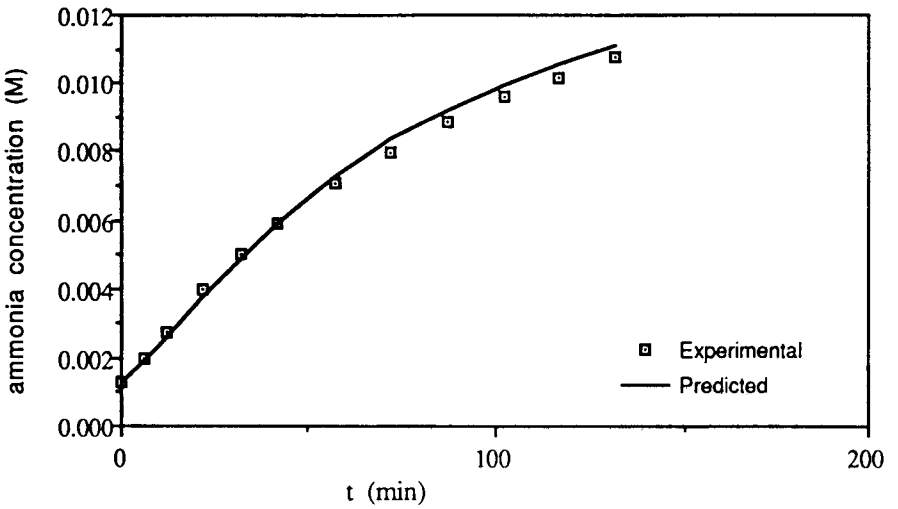

Fig. 19. Time-course of urea of hydrolysis by urease (T = 25°C, C_{urease} = 2.0 mg/L, S_0 = 0.006M, C_{NH_3} = 0.001M, initial pH: 6.00).

$$(dC_{NH_3} / dt) = V \tag{8}$$

$$(dC_{CO_2} / dt) = (1 / 2)\, V \tag{9}$$

here:

$$V = k_0 e^{-} (Ea / RT) / \{1 + [K_{es,1}/(H^+)]^{\alpha} + [(H^+)/K_{es,2}]^{\beta}\} \\ [(1 + K_M/S + S/K_s)(1 + P/K_p)] \tag{10}$$

and P is the total concentration of ammonia. K_p was treated as a function of pH from the data in Table 3.

By imposing the initial conditions of t = 0, S = S_0, P = P_0 (or C_{NH_3} = $C_{NH_3,0}$), and C_{CO_2} = $C_{CO_2,0}$, and combining with ion equilibrium Eqs. (a)–(f), total material balance equations, and electroneutralic equation, the above differential equations can be solved by the Runge-Kutta method.

The experimental data and the theoretical curve of ammonia concentration and pH against reaction time are shown in Figs. 19–22. It can be seen that the ammonia concentration profile fits well to the theoretical predictions. However, in the neutral region, the experimental pH values obviously deviate from the theoretical predictions. The reasons are probably that, within the ratio of NH_3 to CO_2 in the time-course experiment, the saturate pressure of CO_2 is far higher than that of NH_3. For example, at T = 25°C and pH = 6.00, the pressure ratio of CO_2 to NH_3 is up to 2.7 × 10^6. Even at the product self-buffer point, ca. 8.7–9.2, the pressure ratio is higher than 2.3, so the evaporation of CO_2 out from the reaction solution is not avoided, especially when pH is low. Furthermore, in the NH_3–CO_2–H_2O coexisting system, it is known by calculation that at the

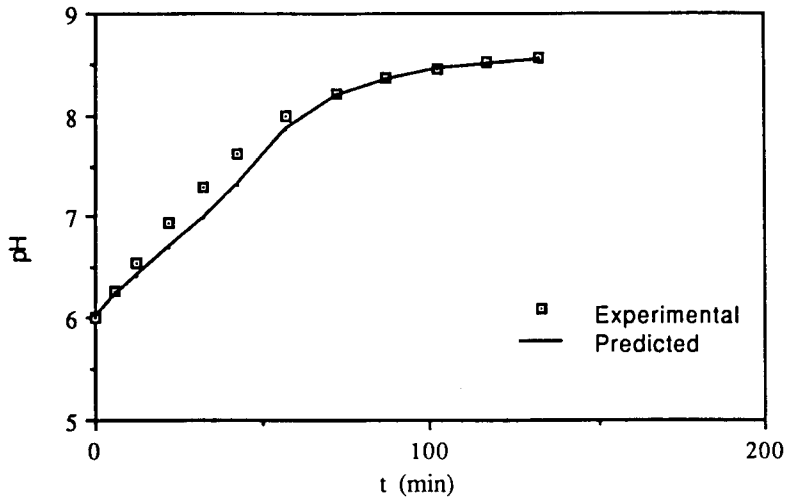

Fig. 20. Variation of pH with time for urea hydrolysis by urease (T = 25°C, C_{urease} = 2 mg/L, S_0 = 0.006M, C_{NH_3} = 0.001M, initial pH: 6.00).

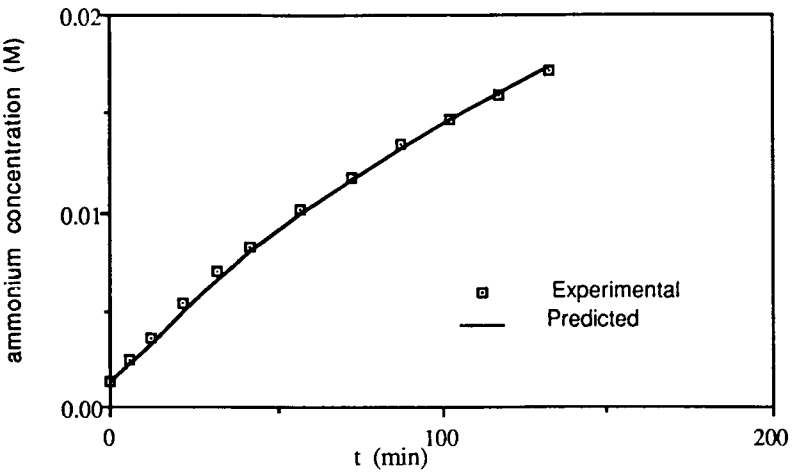

Fig. 21. Time-course of urea of hydrolysis by urease (T = 25°C, C_{urease} = 2.0 mg/L, S_0 = 0.1M, C_{NH_3} = 0.001M, initial pH: 6.00).

pH of 6.5–8, a slight change of the ratio of NH_3 to CO_2 will result in a large change of pH. Therefore, it is probable that at this pH range, the experimental pHs are higher than the predictions. In spite of the deviations, the variations of both pH and ammonia concentration during long-term hydrolysis of urea by urease in buffer-free system were in general agreement with the theoretical results compared with that in buffers (*30*).

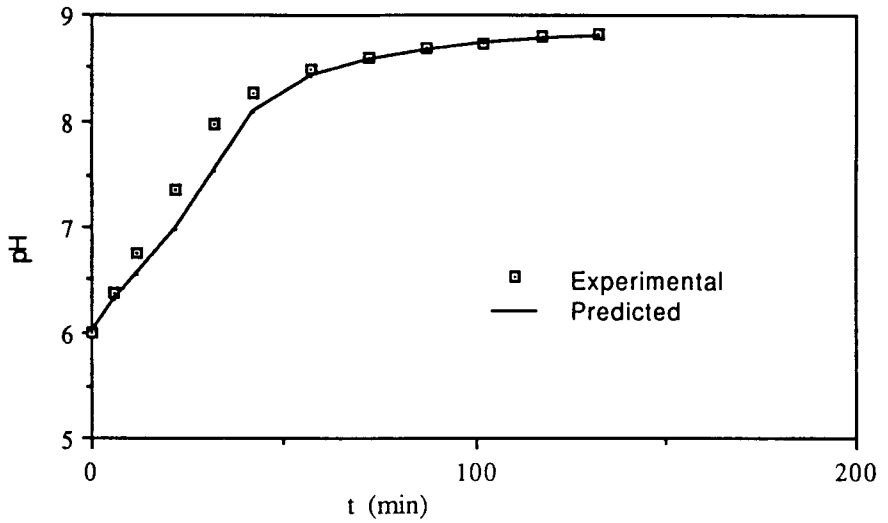

Fig. 22. Variation of pH with time for urea hydrolysis by urease (T = 25°C, C_{urease} = 2 mg/L, S_0 = 0.1M, C_{NH_3} = 0.001M, initial pH: 6.00).

CONCLUSIONS

The kinetics of the hydrolysis of urea by urease, mainly in the absence of buffers and other ions, was investigated. It is indicated that the specific activities of urease in buffer-free solutions remain constant within the urease concentration range of 0.4–10 mg/L, and urease exhibited maximum activity at pH 7.2. The kinetics obeys a Michaelis-Menten mechanism, and Michaelis constant remains almost constant with the change of pH and temperature. The substrate inhibition was experimentally studied. The product inhibition was shown to be of the noncompetitive type. The product inhibition constant is bigger than previously reported and independent of temperature and buffer, but quite sensitive to the variation of pH. An equation of the Monod type was developed to analyze the pH effect on the maximum reaction rate. The maximum reaction rate is found to follow an Arrhenius equation up to about 40°C. The inhibitive effect of buffer salts and other ions on urease was also detected.

As a summary, an extended Michaelis-Menten reaction rate expression, which incorporates pH-dependent kinetics, substrate inhibition, and noncompetitive product inhibition, is proposed to describe the kinetic behavior of urease-catalyzed urea hydrolysis in a buffer-free system:

$$V = k_0 e^{-} (Ea / RT) / \{1 + [K_{es,1}/(H^+)]^{\alpha} + [(H^+)/K_{es,2}]^{\beta}\} \\ [(1 + K_M/S + S/K_s)(1 + P/K_p)] \qquad (10)$$

Table 6
The Parameter Values of Eq. (10)

Parameters	Units	Values
k_0	mol NH_3/s/g urease	0.267
E_a	kJ/mol	29.1
K_M	M	0.00256
K_s	M	6.18
K_p	M	$27.8 - 13.1 \times pH + 2.32 \times (pH)^2 - 0.183 \times (pH)^3 + 0.0054 \times (pH)^4$
$pK_{es,1}$		9.07 (25°C), 9.43 (35°C), 9.58 (40°C)
$pK_{es,2}$		5.62 (25°C), 5.28 (35°C), 5.15 (40°C)
α		0.373 (25°C), 0.489 (35°C), 0.542 (40°C)
β		0.564 (25°C), 0.717 (35°C), 0.769 (40°C)

The model parameters are listed in Table 6.

The examination of the above kinetic model and model parameters by the long-time experiments in which pH value, substrate concentration, and product concentration varied obviously during the reaction course showed the suitability of the model suggested. By increasing the specific activity of urease, the low ion strength and buffer-free system is beneficial, since it decreases the sensitivity of soluble urease to the inhibition effects of substrate, product, and pH.

The above study provides a basic kinetics for the treatment of waste water containing urea, ammonia, and carbon dioxide. However, since the self-buffer point of product is around pH 9 and, in the original waste, the ratio of ammonia to carbon dioxide is bigger than 2, the urease with optimum pH in alkaline region should be selected as biocatalyst, preferentially as an immobilized preparation.

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