Acid-Base Equilibria and Related Properties of Chitosan

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(Received August 26, 1982)

The pK_a of $-NH_3^+$ group of chitosan in water was 6.2, while that of D-glucosamine-HCl, monomer of chitosan, was found to be 7. 8. The difference of pK_a values between chitosan and D-glucosamine was attributed to the strong electrostatic interaction between $-NH_3^+$ groups in chitosan. The apparent binding constant of Cu^{2+} to D-glucosamine was estimated to be 1×10^4. For chitosan, no significant binding of Cu^{2+} to the polymer was observed when pH<5, but strong cooperative binding was observed near pH 5.1. The mechanism of such cooperativity was proposed. Chitosan in solution exhibited typical polyelectrolytic behaviors: viscosity increases with increased amount of charged group, and decreases with addition of salt. The concentration dependence of viscosity was measured, and the Huggins parameters and intrinsic viscosity were calculated at various ionic strength. The results were interpreted in terms of molecular properties of the chitosan molecule.

Introduction

Chitosan, poly-β(1-4)-2-amino-2-deoxy-D-glucose, is obtained by deacetylation of chitin, poly-N-acetyl-D-glucosamine, which occurs widely in lower animals, fungi and etc. Most common commercial sources of chitin are exoskeletons of crabs and shrimps. Neither chitin nor chitosan have fixed stoichiometries: chitin contains a few free amine groups, while N-deacetylation of acetamido moiety is not complete in chitosan.[1] Chitosan is not soluble in pure H_2O and organic solvent, but soluble in organic acids, and mineral acids at specific conditions. Recently, great interests are given to the chemistry of chitosan, mainly due to the wide applicability of the modified biopolymer. For example, this is used as food additives, anticoagulent, supporting materials for chromatographies, chelating polymers for harmful metal removal, membranes for various biomedical applications and etc.[1-4] Most of these applications of chitosan are based on the polyelectrolytic nature and chelating ability of amine group of the macromolecule. In an acidic solution, amine groups of chitosan are protonated to $-NH_3^+$, and thus polyelectrolytic and chelating properties of chitosan are mainly governed by acidity of the $-NH_3^+$ group. Several investigators reported pK_a and some of the related properties of chitosan[5], but the results are sketchy and, sometimes, contradictory to each other. Thus, we decided to study acid-base equilibria and related properties of chitosan systematically to provide better understanding on the chemical properties of the polymer, and to obtain informations which can extend applicability of chitosan.

Experimental Methods

Chitosan was obtained from Tokyo Kasei and purified as follows. 1 % chitosan solution in 0.05 *N* HCl was prepared and filtered through glass wool to remove insoluble particles. Chitosan was precipitated from the filtrate by addition of 0.1 *N* NaOH to pH 8, was suspended in distilled water with several changes of water for two days, was filtered and dried under reduced pressure. Chitosan solutions were prepared by dissolving a proper amount of the purified chitosan in 0.01 *N* HCl containing desired amounts of NaCl (for ionic strength) and/or Cu^{2+} (for Cu^{2+} binding studies).

D-Glucosamine-HCl was purchased from Wako Pure Chemical Ind., and used as received. Acid-base equilibria and the effect of Cu^{2+} on the equilibria were studied by potentiometric titration with NaOH using a pH meter. Viscosity was measured at 25.0°C using a Ostwald type viscometer. For intrinsic viscosity measurements, iso-ionic dilutions were performed. Other chemicals were Reagent Grade and used without purification.

Results and Discussions

Acid-Base Equilibrium Titration of chitosan solution with excess amount of HCl is expected to be similar with that of the mixture of a strong acid (excess HCl) and a weak acid ($-NH^{3+}$ of chitosan). In this case, it is generally approximated that the amount of the weak acid titrated is equal to the amount of the base consumed between first and second inflection points of the titration curve.[6] Figure 1 shows a titration curve of chitosan in 0.01 *N* HCl, together with those of D-glucosamine-HCl and 0.01 *N* HCl as a reference. The degree of the N-deacetylation in chitosan used in this experiment was calculated from first and second inflection points of the titration curve, and found to be 64 %. The pK_a value of $-NH_3^+$ group can be estimated from the midpoint of the titration curve, but better method of evaluation is to use Katchalsky-Spitinik equation (1)[7], in which electrical free energy change during neutralization of a polyelectrolyte is considered;

$$pH = pK_a + n \log [\alpha/(1-\alpha)] \quad (1)$$

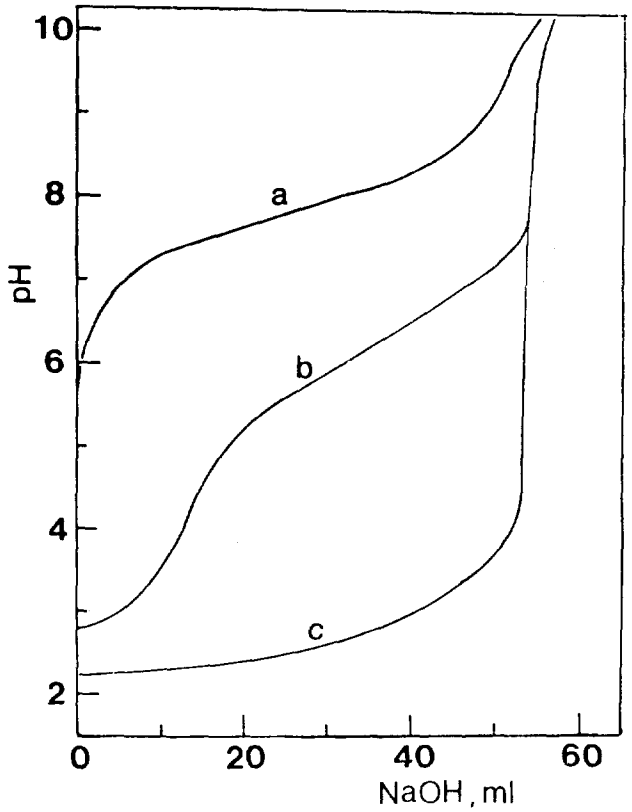

Figure 1. Potentiometric titration curves of (a) 100 mg of D-glucosamine-HCl in 50 m*l* of water; (b) 100 mg of chitosan in 50 m*l* of 0.01*N* HCl; (c) 50 m*l* of 0.01 *N* HCl. The concentration of NaOH was 0.095*N*.

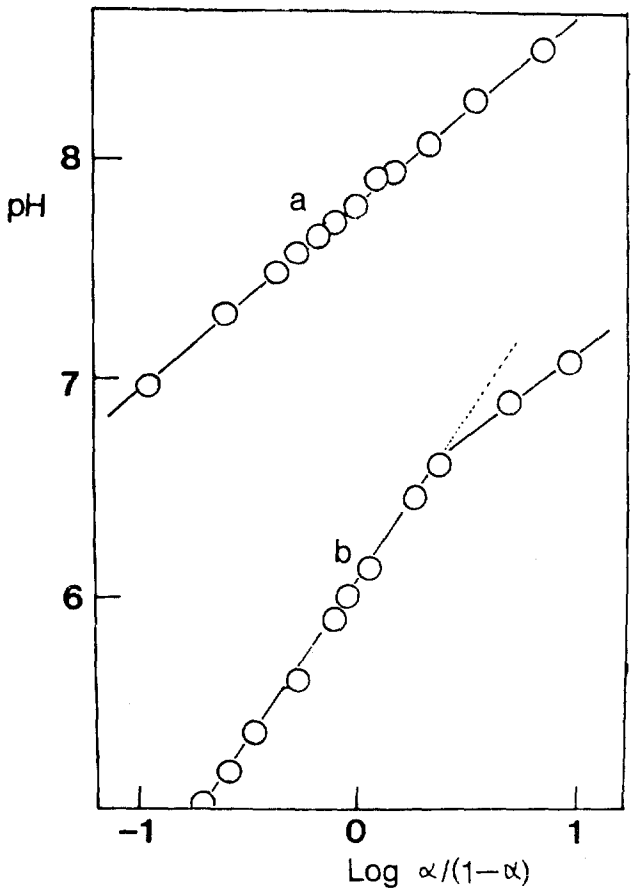

Figure 2. Titration data plotted according to equation 1. (a) D-glucosamine-HCl, (b) chitosan in HCl solution.

where α is the degree of neutralization and can be calculated from the titration curve at each pH value. n is a empirical parameter related to the free energy change during titration. The pK_a and n values are determined from the intercept and the slope of the plot of pH versus log $[\alpha/(1-\alpha)]$ as shown in Figure 2. The pK_a of $-NH_3^+$ group in chitosan was found to be 6.1 when $\alpha<0.72$, and 6.7 when $\alpha>0.72$. The pK_a of its monomer, D-glucosamine, was determined to be 7.8. These values are in good agreement with earlier reported values of 6.3 for chitosan,[8] and 7.5 for D-glucosamine.[9] The pK_a of chitosan was slightly dependent on salt concentration of the solution: for example, the value was 6.4 in 0.1 *M* NaCl. Strong electrostatic interaction among the neighboring charged-NH_3^+ groups in chotosan can account for the much lower pK_a of chitosan, compared to that of D-glucosamine HCl. This interaction is also reflected in the high value of n, 1.47, for chitosan. The break of linearity in data for chitosan in Figure 2 indicates that molecular conformation of the molecule is changed near $\alpha=0.72$. In fact, this α value coincides with the pH value of 6.6, at which chitosan starts to form precipitate during titration. One of the possible explanation of the molecular conformation change which leads to the formation of precipitate and to the change of pK_a value is the formation of intermolecular hydrogen bonding involving $-NH_2$ group when $\alpha>0.72$.This precipitate of hydrogen-bonded chitosan may have similar structure to those found in chitin.

Effects of Cu^{2+} on the Acid-base Equilibrium. Free-NH_2 groups of chitosan are good ligands for various metal ions, and this chelating property can account for wide range of applications of chitosan.[1a] Since hydronium ions compete with metal ions in binding to $-NH_2$, the chelation can be studied indirectly from effects of metal ions on the acid-base equilibrium of the ligand moiety. Cu^{2+} is an ideal candidate for this purpose, because $-NH_2$ is a good ligand for the ion and Cu^{2+} complexes are shown to serve as a standard for other metal complexes.[10]

Distinctly different effects of Cu^{2+} on the titration curves were revealed as shown in Figure 3. Generally, the competition of H^+ and Cu^{2+} for common binding site, $-NH_2$ group, can be expressed as follows;

$$RNH_3^+ \rightleftharpoons RNH_2 + H^+ \; ; \; K_a = \frac{[RNH_2][H^+]}{[RNH_3^+]}$$

$$RNH_2 + Cu^{2+} \rightleftharpoons RNH_2Cu^{2+} \; ; \; K_c = \frac{[RNH_2Cu^{2+}]}{[RNH^2][Cu^{2+}]}$$

Other complexes having higher amine-to-Cu^{2+} ratios also can be considered. However, we can assume that only 1:1 complex is formed when $[RNH_2] \ll [Cu^{2+}]$: this condition is met at the initial stage of titration, *e. g.* $pH \ll pK_a$. For a solution containing 0.002 mole/*l* Cu^{2+}(Figure 3), the titration curve for D-glucosamine-HCl is apparently shifted to the lower pH values in the amount of 1.6 pH unit, compared to the one taken in the absence of Cu^{2+}. This result reflects that, when the same amount of D-glucosamine is neutralized, the $[RNH_3]$ to $[RNH_2]$ ratio in the presence of 0.002 mole/*l* Cu^{2+} is $10^{1.6}$ times larger than the one expected from the acid-

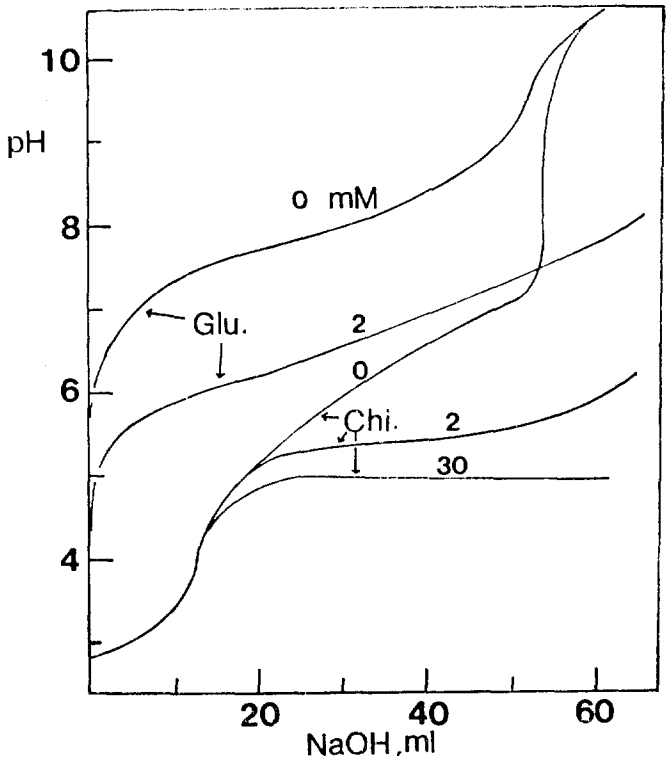

Figure 3. The effect of Cu^{2+} on the titration curves of D-glucosamine-HCl and chitosan. $[Cu^{2+}]$ are shown in the Figure. in m*M* and other conditions are same as Figure. 1.

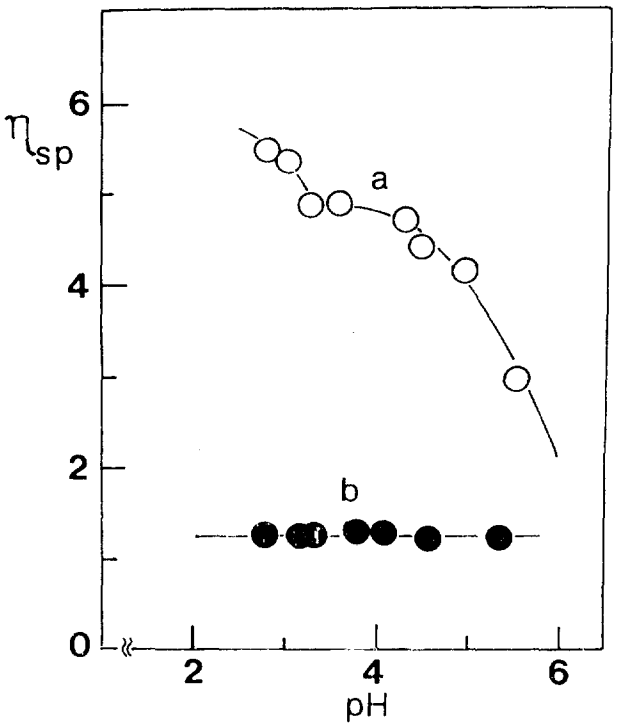

Figure 4. The variation of viscosity of chitosan solution (2.0g/dl) on pH at 25 °C. (a) NaCl is absent, (b) [NaCl]= 0.1 mole/*l*.

base equilibrium only. This implies that $[RNH_2Cu^{2+}]/[RNH^2]$ is about $10^{1.6}$, and thus K_c for D-glucosamine-Cu^{2+} complex is approximately 1×10^4 $(M/l)^{-1}$. Unlike D-glucosamine, the effect of Cu^{2+} on the titration curve for chitosan is negligible when pH$<$5,but pH is nearly invariable with added NaOH near pH=5.1. This behavior can be interpreted in terms of cooperative binding of $Cu^{2+}$to chitosan as shown below:

$$Cu^{2+} + \begin{matrix}-NH_3^+\\-NH_3^+\end{matrix} \quad \underset{\leftarrow}{\overset{OH^-}{\rightarrow}} \quad \begin{matrix}-NH_2Cu^{2+}\\-NH_3^+\end{matrix}$$

I II

$$\underset{\leftarrow}{\overset{OH^-}{\rightarrow}} \quad \begin{matrix}-NH_2\\-NH_2\end{matrix}\!\!\diagdown\!\!\diagup Cu^{2+}$$

III

When most of the amine groups of chitosan (I) are present as $-NH_3^+$ (pH$<$5), the formation of chitosan-Cu^{2+} complex(II) would increase the electrostatic repulsion between charged groups, and thus the complex is very difficult to be formed. Therefore, the presence of the metal ions gives little effect on the acidity of $-NH_3^+$ groups in chitosan at the early stage of titration. However, when significant amount of $-NH_3^+$ groups are neutralized and the complex II is formed, another neighboring amine group is chelated to the Cu^{2+} resulting complex III. This cooperative binding reduces the electrostatic repulsion and increases the stability of the complex formed.

Viscosity of Chitosan: Viscosity of chitosan in solution exhibits typical polyelectrolytic behaviors. In the absence of NaCl, the specific viscosity, $\eta_{sp}=[\eta_{solution}/\eta_{soluent}]-1$ increases with protonation of amine group in the pH range[4-6] as shown in Figure 4, due to the increased effective volume of the molecule by the charge repulsion. The η_{sp} also increases as pH of the solution is lowered from 3.5. This implies that, in addition to $-NH_2$ group, other group is protonated in this pH range. However, the exact nature of this additional protonation is not clear from this study. The parallelism between the degree of protonation and η_{sp} of chitosan solution in the pH 4-6 is different from the report of Filar and Wirick,[11] who showed a maximum near pH 3. 6. This discrepancy between two results seems to arise from the difference in the experimental method: Filar and Wirick followed the variation of viscosity of chitosan with pH, varied by addition of acetic acid to chitosan-acetate solution, and thus the overall solvent composition as well as pH is changed.

In contrast to the absence of NaCl, the viscosity of chitosan solution was shown to be nearly independent of pH, when NaCl is added to the solution in the concentration of 0.1 mole/*l*. This result indicates that, at such a high concentration of NaCl, the charge of $-NH_3^+$ of chitosan is shielded very well by association of counter ion (Cl^-), and therefore the molecular dimension of the macromolecule is virtually independent of degree of dissociation of amine group.

The concentration dependence of viscosity of chitosan in solution was studied to obtain molecular parameters, and plotted according to the Huggins' equation (2) in Figure 5.

$$\eta_{sp}/c=[\eta]+k'[\eta]^2c \qquad (2)$$

where c denotes the concentration of a solute, and k' is Huggins' constant. When salt is absent or in the low concentration, the reduced viscosity (η_{sp}/c) was increased from values

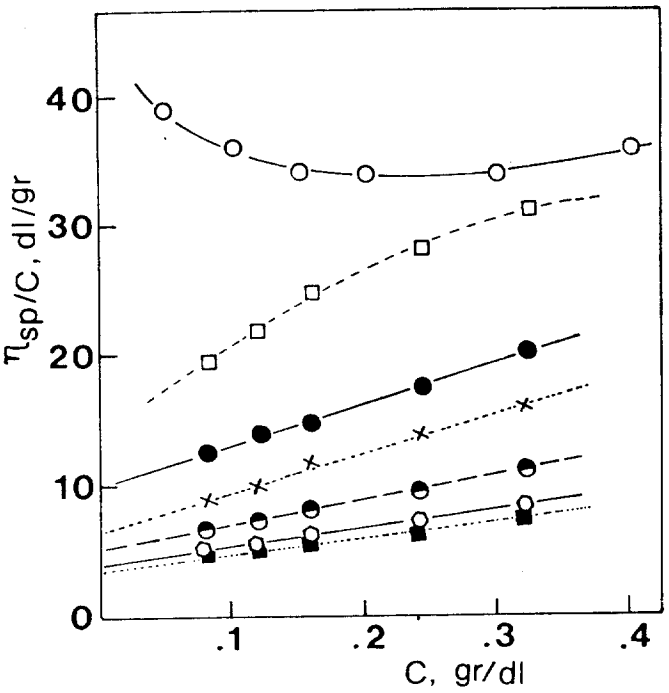

Figure 5. Reduced viscosity *vs.* concentration for chitosan solutions at 25 °C and pH 3.2. The NaCl concentrations are 0.000, 0.003, 0.010, 0.020, 0.053, 0.203 and 0.403mole/l (from top to bottom).

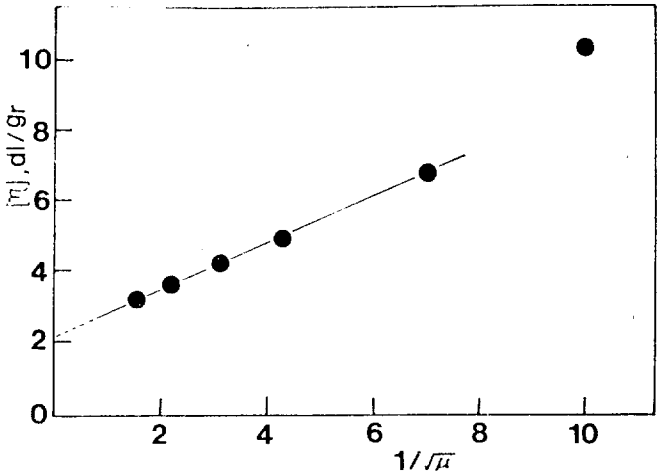

Figure 6. Intrinsic viscosity of chitosan at pH 3.2 against $1/\sqrt{\mu}$

expected from the Eq. 2 as the solution is diluted: most of polyelectrolytes exhibit similar behavior due to the increased charge repulsion in a dilute solution. However, when sufficient amount of salt is added to neutralize this charge effect, the viscosity varies according to Eq. 2, and the intrinsic viscosity, $[\eta]$, and k' can be calculated from the plot. The results are summarized in Table 1. The k' values in Table 1 indicate that a chitosan molecule in a dilute salt solution exists as a flexible chain, but it becomes harder as the ionic strength of the solution increased, probably, because the association of counter ions produces the ionic atmosphere which resists the deformation of the molecule electrostatically. It was shown that the k' value for a solid polymer particle is approximately 2.0,[12] and the same for a flexible polymer in a good solvent is often 0.35.[13] Pals and Hermans[14] suggested that the variation of $[\eta]$ is approximatelyl inear

TABLE 1: Viscosity Parameters of Chitosan in Solution at Various Ionic Strength

$\mu\times10^2$	$[\eta]$	$k'[\eta]^2$	k'
1.0	10.3dl/gr	30.7 (dl/gr)^2	0.29
2.0	6.8	28.5	0.62
5.3	4.9	19.5	0.81
10.3	4.3	16.1	0.87
20.3	3.6	14.8	1.14
40.3	3.3	12.9	1.18

wiht $1/\sqrt{\mu}$, where μ is the ionic strength. Indeed, the plot of data in Table 1 (Fig. 6) yielded good straight line, when $\mu > 2.0\times10^{-2}$ and the extrapolated value of $[\eta]$ was 210 cm^3/g. The intrinsic viscosity is related to the mean-square root of the end-to-end distance, $\langle R^2\rangle^{1/2}$, and the average molecular weight, $\bar{M}_n$, of the solute molecule by Flory-Fox' theory;[15]

$$[\eta]=\langle R^2\rangle^{3/2}\phi/\bar{M}_n \tag{3}$$

where ϕ is the Flory parameter and is 3.7×10^{21} for a non-ionic polymer in a good solvent.[16] The end-to-end distance of chitosan in its unexpanded state would be close to the value at infinite ionic strength, and that value can be estimated by substituting 1.2×10^5 for $\bar{M}_n$ of chitosan[17], and 3.7×10^{21} for Flory parameter into Eq. 3. The end-to-end distance of unexpanded chitosan estimated by this approximation was 190 Å. This value corresponds only 0.3 Å per saccharide for the extension to a given direction: axial length of carbohydrates is usually 4~5 Å per saccharide.[18] In an expanded state, this distance would be slightly larger, because higher intrinsic viscosity and smaller ϕ values are used in Eq. 3. But the order of magnitude cannot be changed. Therefore, we can soundly assume that chitosan molecule exists in a solution as a highly coiled one.

Acknowledgement. The authors gratefully acknowledge the financial support provided by the Korea Science and Engineering Foundation for this study.

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Enzymatic Synthesis of Cephaloglycin

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Cephaloglycin was synthesized directly from D-α-phenylglycine methyl ester and 7-aminocephalosporanic acid using whole cell enzyme of *Xanthomonas citri* (IFO 3835). Some optimal conditions for cephaloglycin synthesis were investigated, and yield improvements for its production by several methods were attempted. Using the whole cell enzyme system, the reaction kinetic model for cephaloglycin synthesis is proposed, and the kinetic constants for D-α-phenylglycine methyl ester hydrolysis, cephaloglycin synthesis, and cephaloglycin hydrolysis were determined. The K_m values of D-α-phenylglycine methyl ester, 7-aminocephalosporanic acid, and cephaloglycin were 11 mM, 24 mM, and 167 mM, and K_i value of D-α-phenylglycine was 15 mM, respectively. The pattern of product inhibition was found to be competitive one.

Introduction

New antibiotics discovered each year continue to increase linearly but those that were put into commercial production declined rapidly after 1950's,[1] because it became increasingly difficult to isolate new antibiotics sufficiently superior to existing products to warrant its introduction into clinical practice. A significant effort was focused on the modification of side groups that are capable of increasing the antibiotic potency or spectrum. A variety of semisynthetic cephalosporins have also been created by the substitution of appropriate side chains[2-4]. A large number of microorganisms have been found to have the synthetic activity of cephalosporin derivatives from 7-amino-3-cephem nucleus and several side chain compounds[5-16]. This biological synthesis of cephalosporins is relatively simple and convenient due to its mild condition, one step reaction, and high conversion, whilst the chemical process requires fairly large number of reaction steps for protection of reactive functional groups. Considering the merits of enzymatic synthesis, studies on the properties of cephalosporin synthesizing enzyme and the methods of increasing the conversion for production of cephaloglycin were undertaken.

Materials and Methods

Materials. The D-α-phenylglycine methyl ester (PGM) was prepared from D-α-phenylglycine (PG) (Aldrich Chemical Co., WI) and methanol using thionyl chloride. The 7-aminocephalosporanic acid (7-ACA) was kindly supplied from Wyeth Laboratory Inc., PA and the authentic cephaloglycin (CEG) was obtained from Sigma Chemical Co., MO. All other chemicals used were analytical reagents grade.

Experimental. A mutant of *Xanthomonas* citri (IFO 3835) was used throughout this work. The culture medium and fermentation conditions used, and the method of whole cell enzyme preparation were the same as those described previously[9]. To 1 m*l* of whole cell suspension in 0.1 *M* sodium phosphate buffer (pH 6.4) 2 m*l* of substrate in the same buffer solution was added and the reaction mixture was incubated at 37 °C for 5 min. The products were then assayed. Since both hydrolysis and synthesis of CEG occured simultaneously in this reaction system, the optimal conditions had to be determined by measuring the total accumulated concentration of CEG. The effect of enzyme loading on the reaction profile was also evaluated. The whole cell enzyme was concentrated and the conversion resulted from using the concentrated enzyme was compared with those using the original enzyme strength. Acetone treated whole cell enzyme was also evaluated for its possible improvements in the reaction rate and permeability of substrate and product. In order to suppress hydrolysis of PGM to PG by water, some organic solvents were selected based on their properties like Lewis basicity, relative ability of proton donor and degree of dipole moment[19], and added 10 % (v/v) to the reaction systems. The cumulative yield of CEG was measured and the conversion was compared with that achieved in aqueous reaction system. For kinetic study, the products in reaction mixture were analysed using high performance