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Hydrolysis of 4-Methylumbelliferyl Tetra-*N*-acetyl-β-chitotetraoside by Lysozyme and Its Inhibition by *N*,*N*′,*N*″-Triacetylchitotriose

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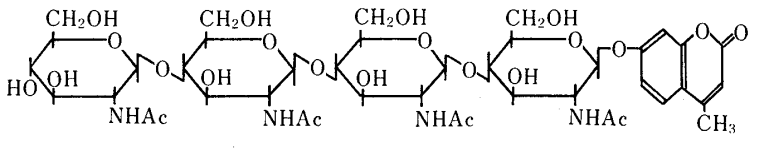
The hydrolytic activity of lysozyme towards 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside (4-MU-$(GlcNAc)_4$) was little affected by ionic strength, though the activity of lysozyme towards cell suspension of *Micrococcus lysodeikticus* varied markedly with ionic strength. About 40—60% of lysozyme activity with 4-MU-$(GlcNAc)_4$ as a substrate was inhibited by 0.1 mM *N*,*N*′,*N*″-triacetylchitotriose ($(GlcNAc)_3$), but the lytic activity of lysozyme towards *M. lysodeikticus* was little affected. The kinetics of hydrolysis of 4-MU-$(GlcNAc)_4$ by hen egg-white (HEW) lysozyme and human placental (HP) lysozyme and the inhibition of this hydrolysis by $(GlcNAc)_3$ were investigated. The K_s values for 4-MU-$(GlcNAc)_4$ of HEW- and HP-lysozymes were 19.7 and 27.9 μM, respectively, and the V_{max} values were 0.124 and 0.0833 nmol/min/mg, respectively. The k values of both enzymes were very low, implying a poor orientation of the scissile bond in the substrate molecule with respect to the active site of lysozyme. $(GlcNAc)_3$ inhibited lysozyme with hyperbolic mixed-type inhibition. The inhibition reduced the V_{max} values of both lysozymes. The K_s value of HEW-lysozyme was increased by the addition of the inhibitor, whereas the K_s value of HP-lysozyme was decreased. The K_i value was 29.6 μM for HEW-lysozyme and 106 μM for HP-lysozyme.

Keywords——lysozyme; 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside; kinetics; inhibition; synthetic substrate; lysozyme fluorometric assay

Lysozyme has usually been assayed by turbidimetry with dried cell powder of *Micrococcus lysodeikticus* as the substrate.[1] However, as the substrate is not always uniform, this assay does not give consistent results. Lysozyme assay using *M. lysodeikticus* as a substrate is known to be influenced markedly by the ionic strength of the medium.[2] In addition, lysozyme has transglycosylation activity[3] and the products formed from polysaccharide of cell wall by lysozyme can repeatedly serve as substrates or acceptors. In order to overcome these problems and thus to assay lysozyme activity accurately, a synthetic substrate having a definite and uniform structure is required. Recently, the synthesis of 4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside (4-MU-$(GlcNAc)_4$) (Chart 1) and the application of this compound in a fluorometric assay of lysozyme were reported by Inaba *et al.*[4] Lyzozyme was assayed by measuring the fluorescence intensity of 4-methylumbelliferone released from 4-MU-$(GlcNAc)_4$ after enzymatic hydrolysis. It is emphasized in their report that 4-MU-



4-methylumbelliferyl tetra-*N*-acetyl-β-chitotetraoside

Chart 1

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$(GlcNAc)_4$ is an effective and useful substrate.

The kinetics of hydrolysis of 4-MU-$(GlcNAc)_4$ by lysozyme have not previously been investigated. In this paper, kinetic studies of the lysozyme-catalyzed hydrolysis of the new synthetic substrate, 4-MU-$(GlcNAc)_4$, and inhibition of the hydrolysis by *N,N′,N″*-triacetylchitotriose are described.

Experimental

Materials——Hen egg-white lysozyme (HEW-lysozyme) and *Micrococcus lysodeikticus* cells (spray-dried) were purchased from Seikagaku Kogyo Co., Ltd. *N*-Acetyl-D-glucosamine was purchased from Wako Pure Chemical Industries Ltd. *N*-Acetylmuramic acid was obtained from Aldrich Chemical Company Inc. *N,N′*-Diacetylchitobiose ($(GlcNAc)_2$) and *N,N′,N″*-triacetylchitotriose ($(GlcNAc)_3$) were purchased from Sigma Chemicals Co. Human placental lysozyme (HP-lysozyme) (2× crystallized) and 4-MU-$(GlcNAc)_4$ were kindly donated by Dr. Hasegawa, Fundamental Research Laboratories of Pharmacology, Green Cross Corporation.

Turbidimetric Assay of Lysozyme Activity——The lysozyme activity was determined at 35 °C by observing spectrophotometrically the decrease in turbidity which occurred during the lysis of a suspension of cells of *M. lysodeikticus* with a Union High-Sens SM-401 spectrophotometer equipped with a National X-Y recorder. Thirty mg of dried cells of *M. lysodeikticus* was suspended in 100 ml of 0.035 M phosphate buffer (pH 6.2). Enzyme solution (1.4 μM) was prepared by dissolving crystalline lysozyme in 0.035 M phosphate buffer (pH 6.2). The enzyme reaction was initiated by adding 0.02 ml of enzyme solution to a mixture of the suspension of bacterial cells (2.7 ml) and water (0.3 ml), and the decrease of turbidity at 640 nm was followed for 10 min.

Fluorometric Assay of Lysozyme Activity——Reaction mixture (3.0 ml) containing 4-MU-$(GlcNAc)_4$ (0.004—0.025 mM) and lysozyme (8.8×10^{-9} mol) in 0.05 M sodium citrate buffer (pH 5.2) was incubated at 30 °C. After 1 h, the fluorescence intensity of 4-methylumbelliferone liberated was measured at 445 nm with excitation at 320 nm in a Hitachi 650-10S fluorescence spectrophotometer equipped with a temperature-controlled cuvette chamber and a Hitachi 056 recorder.

Results and Discussion

Effect of Substrate Concentration, Ionic Strength and Various *N*-Acetylsaccharides on Lysozyme Activity

Before inhibition studies and determination of the kinetic constants of HEW- and HP-lysozymes, the time course and the pH-activity relationship for the hydrolysis of 4-MU-$(GlcNAc)_4$ by the enzymes were examined. The hydrolysis by the lysozymes proceeded linearly over a period of at least 2 h under the conditions of 0.02 mM 4-MU-$(GlcNAc)_4$ and 2.1×10^{-8} mol of enzyme. Both HEW- and HP-lysozyme gave bell-shaped activity–pH curves with a maximum at around pH 5.2.

When the dependence of the observed initial rate of the lysozyme-catalyzed hydrolysis of 4-MU-$(GlcNAc)_4$ upon substrate concentration was expressed in a Lineweaver–Burk plot, a straight line was obtained up to the substrate concentration of about 2.5×10^{-5} M, indicating that the enzymatic process obeys simple Michaelis–Menten kinetics in this low concentration range. Upward curvature of the plot was observed in the substrate concentration range higher than about 2.5×10^{-5} M, indicating that high substrate concentrations give rise to substrate inhibition. The kinetic parameters for hydrolysis of 4-MU-$(GlcNAc)_4$ by HEW- and HP-lysozyme and its inhibition by $(GlcNAc)_3$ were calculated by using the data in the low substrate concentration range where the simple Michaelis–Menten equation seems applicable. The K_m values for 4-MU-$(GlcNAc)_4$ of HEW- and HP-lysozyme were 19.7 and 27.9 μM, respectively.

The inhibitory effect of NaCl on lysozyme activity was determined by both the turbidimetric method using *M. lysodeikticus* and the fluorometric method using 4-MU-$(GlcNAc)_4$ as a substrate (Fig. 1). The lytic activities against *M. lysodeikticus* were inhibited at above 0.08 M NaCl, and 0.2 M NaCl inhibited the enzyme activities by about 70% (HEW-lysozyme) or 85% (HP-lysozyme). On the other hand, hydrolytic activities towards 4-MU-

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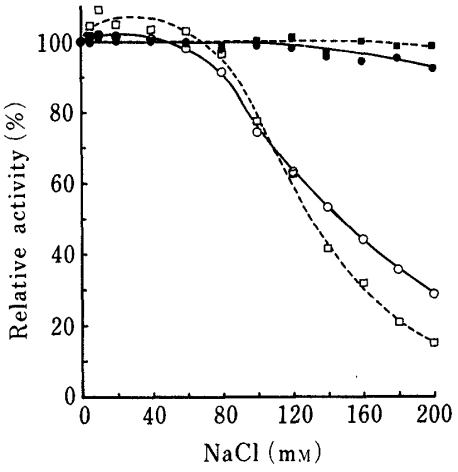

Fig. 1. Effect of Concentration of Sodium Chloride on Lysozyme Activities Measured by Turbidimetric and Fluorometric Methods

The enzyme activities were assayed as described in Experimental except that various concentrations of sodium chloride were added.
○●, HEW-lysozyme; □■, HP-lysozyme; ○□, turbidimetric method; ●■, fluorometric method.

TABLE I. Inhibition by Compounds Structurally Related to 4-MU-$(GlcNAc)_4$

Compound	Concentration (mM)	Remaining activity (%)			
		Fluorometry[a]		Turbidimetry[b]	
		HEW-lysozyme	HP-lysozyme	HEW-lysozyme	HP-lysozyme
None	—	100	100	100	100
N-Acetyl-D-glucosamine	1	98.5	96.0	97.8	95.9
N-Acetylmuramic acid	1	99.3	94.1	102.2	98.3
$(GlcNAc)_2$	0.1	96.5	92.1	96.5	97.1
	0.4	78.4	71.4	93.1	88.0
$(GlcNAc)_3$	0.03	62.7	76.0	99.4	99.2
	0.1	37.4	63.8	92.7	93.2

a) 4-MU-$(GlcNAc)_4$ was used as a substrate.
b) Dried cells of *M. lysodeikticus* were used as a substrate.

$(GlcNAc)_4$ were little affected by the addition of NaCl.

Four compounds structurally related to the synthetic substrate were tested for ability to inhibit the lysozyme-catalyzed hydrolysis of 4-MU-$(GlcNAc)_4$ and the lysis of *M. lysodeikticus* cells (Table I). In both assay methods, the activities of both enzymes were little affected by *N*-acetyl-D-glucosamine and *N*-acetylmuramic acid. $(GlcNAc)_2$ was slightly inhibitory (about 20—30% inhibition at 0.4 mM), and $(GlcNAc)_3$ (0.1 mM) diminished the activities of HEW- and HP-lysozymes by about 60 and 40%, respectively, in the fluorometric method. In the case of the turbidimetric method, the enzymes were hardly inhibited by these *N*-acetylsaccharides.

Kinetic Studies

When the lysozyme activities were assayed with 4-MU-$(GlcNAc)_4$ as a substrate and $(GlcNAc)_3$ as an inhibitor and the data were plotted by the method of Lineweaver and Burk,[5] mixed-type inhibitions were observed on both enzymes, as shown in Figs. 2 and 3. In the case of HEW-lysozyme, the apparent K_m value was increased and the apparent V_{max} value was decreased by addition of the inhibitor, while the apparent K_m and V_{max} values of HP-lysozyme were decreased by the inhibitor. Replots of the slope and intercept in Figs. 1 and 2 *versus* inhibitor concentration were hyperbolic for both enzymes (Fig. 4). Therefore, according to the theory of Segel,[6] the inhibition was not a linear mixed-type inhibition (a mixture of partial competitive inhibition and pure noncompetitive inhibition), but was a hyperbolic mixed-type

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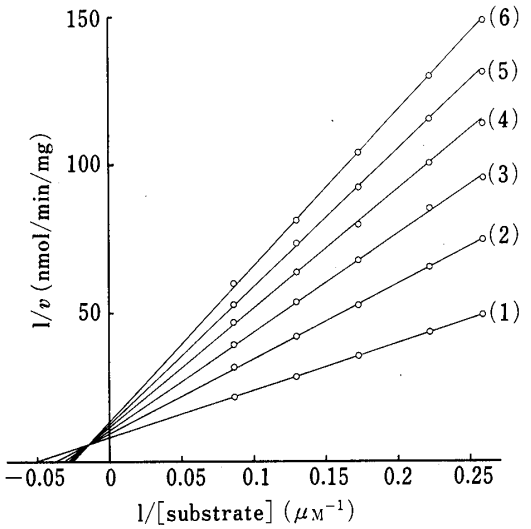

Fig. 2. Effect of Substrate Concentration on the Rate of Hydrolysis of 4-MU-$(GlcNAc)_4$ in the Presence or Absence of $(GlcNAc)_3$ by HEW-Lysozyme

The enzyme activities were assayed as described in Experimental except that various concentrations of $(GlcNAc)_3$ were added. The concentrations of $(GlcNAc)_3$ were: 1, 0 mM; 2, 0.02 mM; 3, 0.04 mM; 4, 0.06 mM; 5, 0.08 mM; 6, 0.1 mM.

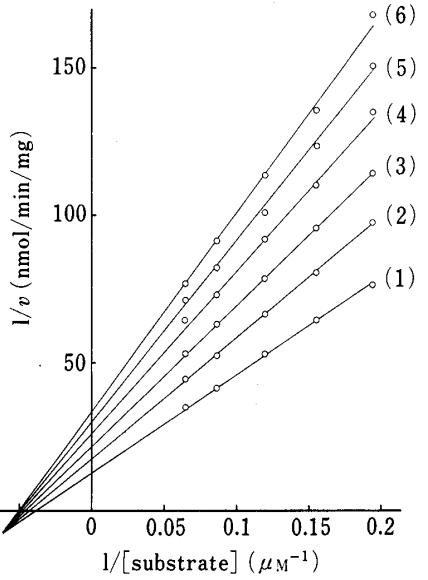

Fig. 3. Effect of Substrate Concentration on the Rate of Hydrolysis of 4-MU-$(GlcNAc)_4$ in the Presence or Absence of $(GlcNAc)_3$ by HP-Lysozyme

The enzyme activities were assayed as described in Experimental except that various concentrations of $(GlcNAc)_3$ were added. The concentrations of $(GlcNAc)_3$ were; 1, 0 mM; 2, 0.04 mM; 3, 0.08 mM; 4, 0.12 mM; 5, 0.016 mM; 6, 0.2 mM.

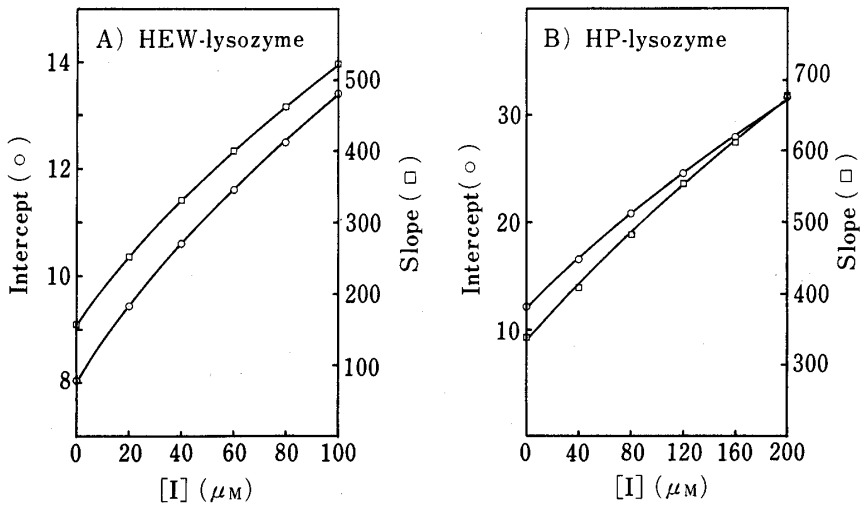

Fig. 4. Replot of Intercepts and Slopes in Figs. 2 and 3 *versus* Inhibitor Concentration

A, HEW-lysozyme; B, HP-lysozyme.

inhibition (a mixture of partial competitive inhibition and partial noncompetitive inhibition). Both enzyme-substrate (ES) and enzyme-substrate-inhibitor (ESI) complexes in this inhibition system can form products at different rates. The hyperbolic mixed-type inhibition can be expressed in a general way as shown in Chart 2. K_s is the dissociation constant for substrate (S), K_i is the dissociation constant for inhibitor (I), and α is the factor by which K_s is changed when the enzyme (E) is bound with I. For the four enzyme species (E, ES, EI and ESI) to be at equilibrium, the dissociation constant for I must be changed to αK_i when S binds to the

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enzyme. In other words, the overall equilibrium constant for the formation of ESI must be the same regardless of the path (E→ES→ESI or E→EI→ESI). Further, k is the rate constant for the breakdown of ES to E+P, and βk is that for the breakdown of ESI to EI+P.

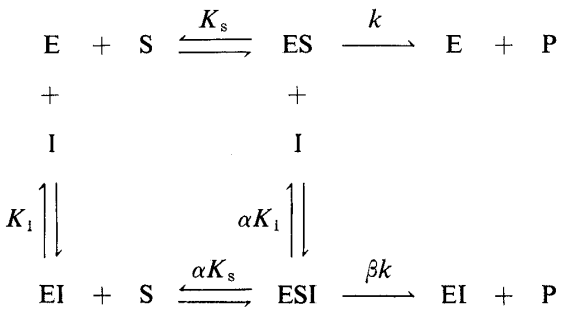

Chart 2

The general velocity equations may be derived as follows.

$$v = k[\mathrm{ES}] + \beta k[\mathrm{ESI}] \tag{1}$$

$$\frac{v}{[\mathrm{E}]_t} = \frac{k[\mathrm{ES}] + \beta k[\mathrm{ESI}]}{[\mathrm{E}] + [\mathrm{ES}] + [\mathrm{EI}] + [\mathrm{ESI}]} \tag{2}$$

$$\frac{v}{[\mathrm{E}]_t} = \frac{k\frac{[\mathrm{S}]}{K_s}[\mathrm{E}] + \beta k\frac{[\mathrm{S}][\mathrm{I}]}{\alpha K_s K_i}[\mathrm{E}]}{[\mathrm{E}] + \frac{[\mathrm{S}]}{K_s}[\mathrm{E}] + \frac{[\mathrm{I}]}{K_i}[\mathrm{E}] + \frac{[\mathrm{S}][\mathrm{I}]}{\alpha K_s K_i}[\mathrm{E}]} \tag{3}$$

The V_{max} is taken as $k[\mathrm{E}]_t$, and accordingly, we have

$$\frac{v}{V_{max}} = \frac{\frac{[\mathrm{S}]}{K_s} + \frac{\beta[\mathrm{S}][\mathrm{I}]}{\alpha K_s K_i}}{1 + \frac{[\mathrm{S}]}{K_s} + \frac{[\mathrm{I}]}{K_i} + \frac{[\mathrm{S}][\mathrm{I}]}{\alpha K_s K_i}} \tag{4}$$

Equation (4) may be rearranged into equation (5).

$$\frac{v}{V_{max}} = \frac{[\mathrm{S}]}{K_s\frac{\left(1+\frac{[\mathrm{I}]}{K_i}\right)}{\left(1+\frac{\beta[\mathrm{I}]}{\alpha K_i}\right)} + [\mathrm{S}]\frac{\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}{\left(1+\frac{\beta[\mathrm{I}]}{\alpha K_i}\right)}} \tag{5}$$

The reciprocal form of equation (5) is:

$$\frac{1}{v} = \frac{K_s}{V_{max}}\frac{\left(1+\frac{[\mathrm{I}]}{K_i}\right)}{\left(1+\frac{\beta[\mathrm{I}]}{\alpha K_i}\right)}\frac{1}{[\mathrm{S}]} + \frac{1}{V_{max}}\frac{\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}{\left(1+\frac{\beta[\mathrm{I}]}{\alpha K_i}\right)} \tag{6}$$

From equation (6), at any inhibitor concentration,

$$\text{Intersection with } 1/[\mathrm{S}] \text{ axis} = -\frac{\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}{K_s\left(1+\frac{[\mathrm{I}]}{K_i}\right)} \tag{7}$$

$$\text{Intersection with } 1/v \text{ axis} = \frac{1}{V_{max}}\frac{\left(1+\frac{[\mathrm{I}]}{\alpha K_i}\right)}{\left(1+\frac{\beta[\mathrm{I}]}{\alpha K_i}\right)} \tag{8}$$

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In the case of HEW-lysozyme, a plot of the apparent K_s ($K_{s_{app}}$) *versus* inhibitor concentration ([I]) is hyperbolic (Fig. 5, inset). If ΔK_s is defined as the difference between $K_{s_{app}}$ and K_s, a general reciprocal equation relating ΔK_s and [I] is obtained as follows:

$$\frac{1}{\Delta K_s}=\frac{\alpha K_i}{K_s(\alpha-1)}\frac{1}{[I]}+\frac{1}{K_s(\alpha-1)} \tag{9}$$

A plot of $1/\Delta K_s$ *versus* 1/[I] is a straight line with a slope of $\alpha K_i/K_s(\alpha-1)$ and a vertical-axis intercept of $1/K_s(\alpha-1)$. When $1/\Delta K_s=0$, the intercept on the 1/[I] axis gives $-1/\alpha K_i$. A plot of the apparent V_{max} ($V_{max_{app}}$) *versus* [I] is also hyperbolic (Fig. 5, inset). If ΔV_{max} is defined as the difference between V_{max} and $V_{max_{app}}$, the reciprocal equation is:

$$\frac{1}{\Delta V_{max}}=\frac{\alpha K_i}{V_{max}(1-\beta)}\frac{1}{[I]}+\frac{1}{V_{max}(1-\beta)} \tag{10}$$

The plot is linear with a slope of $\alpha K_i/V_{max}(1-\beta)$ and an intercept on the $1/\Delta V_{max}$ axis of $1/V_{max}(1-\beta)$. When $1/\Delta V_{max}=0$, the intercept on the 1/[I] axis gives $-1/\alpha K_i$. The quantity α

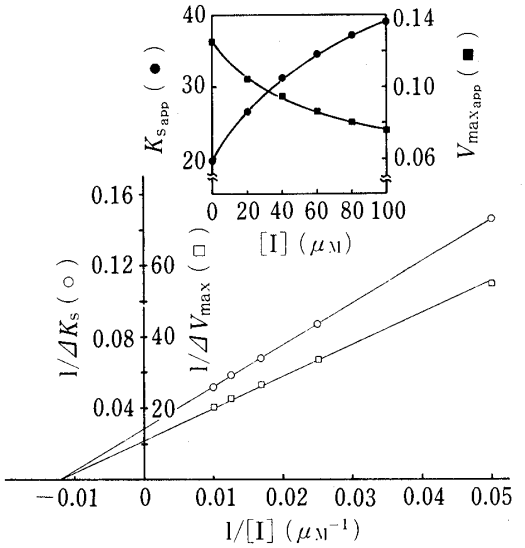

Fig. 5. Replot of the Reciprocal of ΔK_s and ΔV_{max} of HEW-Lysozyme from Fig. 2 as a Function of the Reciprocal of the Inhibitor Concentration

The inset shows a replot of the apparent K_s and V_{max} values from Fig. 2 as a function of inhibitor concentration.

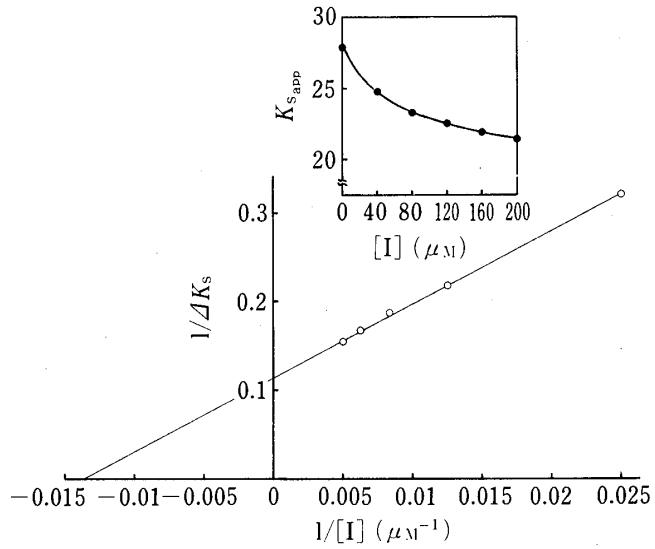

Fig. 6. Replot of the Reciprocal of ΔK_s of HP-Lysozyme from Fig. 3 as a Function of the Reciprocal of the Inhibitor Concentration

The inset shows a replot of the apparent K_s value from Fig. 3 as a function of inhibitor concentration.

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TABLE II. Kinetic Parameters for the Hydrolysis of 4-MU-$(GlcNAc)_4$ by Lysozyme and Its Inhibition by $(GlcNAc)_3$

Parameter	HEW-lysozyme	HP-lysozyme
K_s (μM)	19.7	27.9
V_{max} (nmol/min/mg)	0.124	0.0823
k (s^{-1})	2.96×10^{-5}	2.01×10^{-5}
K_i (μM)	29.6	106
K_s' (μM)	53.0	19.1
k' (s^{-1})	0.825×10^{-5}	0.408×10^{-5}
K_i' (μM)	79.5	72.9
α	2.69	0.687
β	0.279	0.203

K_s', k' and K_i' represent αK_s, βk and αK_i, respectively.

can be determined from the intercept on the $1/\Delta K_s$ axis of the $1/\Delta K_s$ *versus* 1/[I] plot. The value of β can be determined from the $1/\Delta V_{max}$ axis intercept of the $1/\Delta V_{max}$ *versus* 1/[I] plot. With α and β known, K_i can be calculated from the slopes or intercepts on the 1/[I] axis. On the other hand, in the case of HP-lysozyme, the catalytic rate decreased and the affinity increased in the presence of the inhibitor. The $K_{s_{app}}$ *versus* [I] plot was hyperbolic, and decreased to a limit as [I] increased (Fig. 6, inset). If ΔK_s is defined as $K_s - K_{s_{app}}$, the reciprocal equation becomes:

$$\frac{1}{\Delta K_s} = \frac{\alpha K_i}{K_s(1-\alpha)} \frac{1}{[I]} + \frac{1}{K_s(1-\alpha)} \tag{11}$$

Replots of $1/\Delta K_s$ *versus* 1/[I] of HEW- and HP-lysozyme are shown in Figs. 5 and 6, respectively.

The kinetic parameters calculated according to the equation described above are listed in Table II.

The K_s value for 4-MU-$(GlcNAc)_4$ of HEW-lysozyme is about 1.4 times smaller than that of HP-lysozyme, while the k value of the former is about 1.5 times larger than that of the latter. Because lysozyme does not possess a binding site for the 4-methylumbelliferyl moiety of the substrate, it seems likely that the remarkably low k value for 4-MU-$(GlcNAc)_4$ of lysozyme implies a poor orientation of the scissile bond in the substrate molecule with respect to the active site of lysozyme. The K_i value of $(GlcNAc)_3$, which inhibited lysozyme with a hyperbolic mixed type inhibition, for HEW-lysozyme was about 3.5 times smaller than that for HP-lysozyme. The factor α, by which K_s or K_i is changed when I or S occupies the enzyme, was more than 1 in HEW-lysozyme and less than 1 in HP-lysozyme. Therefore, the dissociation constant of the ESI complex ($K_s' = \alpha K_s$) of HEW-lysozyme is larger than that of the ES complex (K_s), namely $K_s < K_s'$, while K_s' of HP-lysozyme was smaller than K_s, namely $K_s > K_s'$. A similar tendency was observed in the inhibitor constant, namely, $K_i < K_i'$ ($= \alpha K_i$) in the case of HEW-lysozyme and $K_i > K_i'$ for HP-lysozyme. The K_s value of HEW-lysozyme was smaller than that of HP-lysozyme, but the K_s' value of the former was larger than that of the latter. The K_i value of HP-lysozyme was larger than that of HEW-lysozyme, though both enzymes have similar K_i' values. The V_{max} value of HEW-lysozyme was about 1.5 times larger than that of HP-lysozyme. Thus, different values of kinetic parameters were observed in the two kinds of lysozyme and these differences may result from differences in the structures of the active sites of HEW- and HP-lysozyme.

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