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Kinetic Measurement of the Interaction between a Lysozyme and Its Immobilized Substrate Analogue by Means of Surface Plasmon Resonance

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A method for evaluating the association and dissociation rate constants of interaction between a lysozyme and its substrate analogue, an immobilized *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, by means of surface plasmon resonance has been developed. Site-specific immobilization of *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, which is a product of *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside, on carboxymethyldextran linked to the surface of the cuvette of the instrument, IAsys, was carried out by catalysis with EDC/NHS. The kinetic parameters of the interaction between hen or human lysozyme and the immobilized substrate analogue indicated that a larger dissociation constant of the human lysozyme-immobilized substrate analogue complex depended on a smaller association rate constant. The kinetic parameters of the interaction between the immobilized substrate analogue and a mutant hen lysozyme, in which Arg14 and His15 are deleted, with higher activity than the wild type hen lysozyme were measured. It was suggested that the higher activity of the mutant lysozyme was due to faster removal of the substrate from the active site cleft and/or the formation of a stabler and better complex as to hydrolysis.

Key words: association rate constant, dissociation rate constant, lysozyme, site-specific immobilization, surface plasmon resonance.

Analysis of the interaction between a protein and its ligand is a fundamental approach for elucidating a biological phenomenon. The affinity of the interaction between them is often evaluated by spectrophotometry or fluorescence spectroscopy. On the other hand, few kinetic parameters of such an interaction have been obtained, even though they provide valuable information, such as that obtained on kinetic analysis of enzymatic function. Recently, a biosensor based on surface plasmon resonance (SPR) was developed and used to determine the kinetic parameters of such an interaction (*1-3*).

A chicken type lysozyme is an enzyme that hydrolyzes a copolymer of *N*-acetyl muramic acid and *N*-acetyl glucosamine, and a polymer of *N*-acetyl glucosamine (*4*). In particular, the function and structure of hen or human lysozyme have been extensively investigated for a long time because their X-ray crystallographic structures had been determined (*5, 6*). However, even in the case of these enzymes there have been a few reports on kinetic parameters of the interactions of proteins and their ligands. These lysozymes were shown to form a non-productive complex with a substrate analogue, $(NAG)_3$ (*7, 8*). Therefore, in this paper, we describe an analytical method involving a biosensor, IAsys, for evaluating the kinetic parameters of the interaction between lysozymes and *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, which is immobilized on the detecting surface of the cuvette in the biosensor.

MATERIALS AND METHODS

Materials—Six times recrystallized hen lysozyme was kindly donated by QP Company (Tokyo). Three times recrystallized human lysozyme was purchased from Green Cross (Osaka). Del 14.15 hen lysozyme was prepared according to the literature (*9*). *p*-Nitrophenyl-tri-*N*-acetyl-β-chitotrioside, EDC, and NHS were purchased from Seikagaku Kogyo (Tokyo), the Protein Research Foundation (Osaka), and Nacalai Tesque (Kyoto), respectively. Other reagents used were of the highest grade available.

Preparation of a Substrate Analogue Attachable to the Detecting Surface of the Cuvette in the Biosensor—One milligram of *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside was dissolved in 1 ml of 0.1 M phosphate buffer, pH 8. To the solution 6 mg of $Na_2S_2O_4$ was added, followed by stirring at room temperature. The reaction was followed by monitoring the disappearance of the starting material by RP-HPLC. The column (Wakosil 5C18, 4.6×250 nm) was isocratically eluted with 1% acetonitrile containing 0.1% HCl. The desired product, *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, was collected, lyophilized, and rechromatographed. The column (Wakosil 5C18, 4.6×250 nm) was

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Abbreviations: Del 14.15 hen lysozyme, a mutant lysozyme from which Arg14 and His15 are deleted; EDC, *N*-ethyl-*N'*-(3-dimethylaminopropyl)carbodiimide hydrochloride; EDTA, *N,N,N',N'*-ethylenediaminetetraacetic acid; $(NAG)_3$, tri-*N*-acetyl-β-chitotrioside; NHS, *N*-hydroxysuccinimide; PBS/T buffer, 0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% Tween; RP, reversed phase; SPR, surface plasmon resonance.

eluted with a gradient generated from 30 ml of 1% acetonitrile and 30 ml of 40% acetonitrile containing 0.1% HCl in order to remove a trace of $Na_2S_2O_4$ completely before immobilization of the desired product on the detecting surface of the cuvette in the biosensor.

Immobilization of p-Aminophenyl-tri-N-Acetyl-β-Chitotrioside in the Cuvette—Biospecific-interaction analysis was carried out using a biosensor, IAsys (Fisons), based on the principle of surface plasmon resonance (*10, 11*). First, PBS/T buffer [0.05 M phosphate buffer, pH 7.2, containing 0.15 M NaCl and 0.05% (v/v) Tween] was added to the cuvette placed in the instrument and then the solution was kept at 20°C for 10 min. After removing the solution, carboxymethyldextran, which is linked to the detecting surface of the cuvette in the instrument, was activated by the addition of a mixture of 0.4 M EDC and 0.1 M NHS solutions. After removing the solution, lyophilized *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside (200 μl, 2.0-2.5 μmol) dissolved in 0.1 M formate buffer, pH 3, was added to the cuvette in order for its amino group to react with the activated carboxymethyldextran. After 12 h, the cuvette was washed with PBS/T buffer, and then the cuvette was refilled with a 1 M ethanolamine solution, pH 8.5, for 2 min. Finally, the ethanolamine solution was removed and the cuvette was filled with PBS/T buffer before the kinetic experiments.

Kinetic Measurement of the Interaction between Lysozymes and the Immobilized Substrate Analogue—The interaction between lysozymes and the immobilized substrate analogue was monitored at 20°C as the change in the SPR response. The association-rate for the binding between lysozymes and the immobilized substrate analogue can be expressed as the following equation,

$$dR/dt = k_{ass}[C](R_{max} - R) - k_{diss}R, \quad (1)$$

where k_{ass} is the association-rate constant, k_{diss} the dissociation-rate constant, R_{max} the maximum binding capacity (in resonance units) of the immobilized substrate analogue surface, as determined by saturation with lysozymes, R the amount of bound lysozyme measured as the SPR response (resonance units) at time t, and [C] the concentration of lysozyme added to the cuvette (the immobilized substrate analogue). A linear plot of dR/dt *versus* R yields

$$\text{slope} = -(k_{ass}[C] + k_{diss}), \quad (2)$$

$$y \text{ intercept} = k_{ass}[C]R_{max}, \quad (3)$$

dR/dt being obtained from measurements of the slope at multiple time points on the real-time association curve. By plotting the slopes of the dR/dt *versus* R lines as a function of the lysozyme concentrations [C], a new line is obtained, and the association-rate constant, k_{ass}, can be obtained as the slope.

When the lysozymes added to the cuvette are washed with PBS/T buffer, the dissociation is then observed according to

$$dR/dt = -k_{diss}\ R, \quad (4)$$

which on integration leads to

$$\ln(R_0/R_n) = k_{diss}(t_n - t_0), \quad (5)$$

where R_n is the response at time n, and R_0 the response at an arbitrary starting time, time zero. The dissociation-rate constant, k_{diss}, is thus obtained as the slope of the $\ln(R_0/R_n)$ *versus* $(t_n - t_0)$ plot. The dissociation rate constant, k_{diss}, is directly obtained using the FAST FIT program (Fisons).

The affinity constant, K_d, is then calculated from k_{diss}/k_{ass}.

Protocol for Evaluation of k_{ass} and k_{diss} on the Interaction between Lysozymes and an Immobilized Substrate Analogue—The cuvette was filled with 200 μl of 10 mM acetate buffer, pH 5, and thermostated at 20°C over 15 min. An appropriate volume of the acetate buffer was removed from the cuvette and the same volume of a lysozyme solution (0.5-2.0 μM), where lysozyme was dissolved in 10 mM acetate buffer, pH 5, and pre-incubated at 20°C, was added to the cuvette (association reaction). The association reaction was monitored for 15 min. Then, the lysozyme solution was removed with a sucker, and 200 μl of 10 mM acetate buffer, pH 5, which had been pre-incubated at 20°C, was immediately added to the cuvette (dissociation reaction). The dissociation reaction was monitored for 5 min. After the association and dissociation reactions, in order to remove lysozyme which had bound to the matrix on the cuvette surface (carboxymethyldextran phase) electrostatically or non-specifically, the immobilized substrate analogue was regenerated by two step regeneration; the detecting surface of the cuvette in the biosensor was washed with a 8 M urea solution (0.58 M Tris-HCl buffer containing 8.1 M urea, pH 8.6, containing 5 mM EDTA) containing 16 mM 2-mercaptoethanol, followed by replacement with a 10 mM HCl solution. Each washing operation was repeated three times. After regeneration, the cuvette was refilled with 10 mM acetate buffer, pH 5, as fast as possible. The surface concentration of the immobilized substrate analogue was estimated to be about 5 ng/mm².

RESULTS

Immobilization of a Substrate Analogue of Lysozymes in the Detecting Surface of the Cuvette in a Biosensor—*p*-Nitrophenyl-tri-*N*-acetyl-β-chitotrioside is often used as a substrate analogue of lysozymes (*12, 13*). Since the hen and human lysozymes are known to form a non-productive complex with tri-*N*-acetyl-β-chitotrioside (*7, 8*), we decided to combine *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, the reduced product of *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside, with carboxymethyldextran linked to the detecting surface of the cuvette in the biosensor. For reduction of the nitro group, the *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside was reacted with an excess amount of $Na_2S_2O_4$ at pH 8 and room temperature. The reaction was followed by monitoring the disappearance of the starting material by RP-HPLC. The starting material disappeared immediately (within 1 min), and then the reaction mixture was applied to the RP-HPLC column. Three peaks were eluted faster than the starting material on the RP-HPLC column (Fig. 1). By measuring ultraviolet spectra of the derivatives in these peaks, the spectrum of the derivative in peak c in Fig. 1 was found to be similar to that of aniline but different from that of *p*-nitrophenol (data not shown). The derivative in peak c in Fig. 1 was suggested to be the desired product. Since the affinity of the derivative in peak c in Fig. 1 with hen lysozyme was almost identical to that of tri-*N*-acetyl-β-chitotrioside with hen lysozyme, as judged on fluorescence spectroscopy, we confirmed that the derivative in peak c in Fig. 1 was *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside. The product was lyophilized and then subjected to RP-HPLC

again in order to remove a trace amount of $Na_2S_2O_4$. The lyophilized *p*-aminophenyl-tri-*N*-acetyl-*β*-chitotrioside was immobilized on carboxymethyldextran linked to the detecting surface of the cuvette in the biosensor by EDC/NHS catalysis at pH 3.

Regeneration Conditions for the Detecting Surface of the Cuvette in the Biosensor—Before determining the kinetic parameters of the interaction of lysozymes with the immobilized substrate analogue, we examined the conditions for the removal of lysozymes from the detecting surface of the cuvette. We determined the criteria of complete removal of lysozyme from the cuvette surface as the position of the final baseline returned to that of the initial one before and after the reaction; a proper amount of lysozyme dissolved in acetate buffer, pH 5, was added to the cuvette, the cuvette was refilled with acetate buffer, and then the regeneration solution was added and the cuvette was washed again. The position of the final baseline did not return to that of the initial one when the cuvette was regenerated with a 10 mM HCl solution, which is recommended as a regeneration solution. The position of the final baseline did not return to that the initial one when either the cuvette was regenerated with a 6 M guanidine hydrochloride solution (0.5 M Tris-HCl buffer, pH 8.6, containing 6 M guanidine hydrochloride) or a 8 M urea solution containing 16 mM 2-mercaptoethanol, which is a reductive solution for the disulfide bonds of lysozymes. However, the position of the final baseline did return to that of the initial one when the cuvette was washed, respectively, with 10 mM HCl after regeneration with a 6 M guanidine hydrochloride solution or a 8 M urea solution containing 16 mM 2-mercaptoethanol. The results indicated that solutes such as guanidine and urea must be removed from the matrix on the cuvette surface (carboxymethylated phase) by washing with 10 mM HCl for complete regeneration.

Evaluation of Kinetic Parameters of the Interaction of Hen or Human Lysozyme with the Immobilized Substrate Analogue—On analysis of the response curves obtained under the conditions when various amounts of lysozymes were added to the cuvette using the FAST FIT program, we found that all association or dissociation phases were attributable to monophasic reactions.

In Fig. 2, A and B, plots of the slope (dR/dt *vs.* R) against the concentrations of hen and human lysozyme added to the cuvette are shown, respectively. These plots show good straight lines. The slope gives k_{ass} for the association of a lysozyme with the immobilized substrate analogue. The determined values are shown in Table I. On the other hand, k_{diss}, which can be directly obtained by analyzing the dissociation phase using the FAST FIT program, is also shown in Table I. We examined whether the values of k_{ass} and k_{diss} for the interaction between hen lysozyme and the immobilized substrate analogue depended on R_{max} by using various cuvettes with different R_{max}. Under the conditions employed ($200 < R_{max} < 800$), these values were not affected by different R_{max}. Moreover, the dissociation constants, K_d, of lysozyme-immobilized substrate analogue complex which was obtained as the ratios of k_{ass} to k_{diss}, are also shown in Table I.

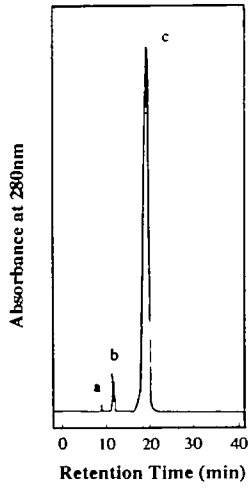

Fig. 1. **RP-HPLC pattern of the reaction mixture of *p*-nitrophenyl-tri-*N*-acetyl-*β*-chitotrioside with $Na_2S_2O_4$ after reaction for 1 min.** The column (Wakosil 5C18, 4.6×250 mm) was isocratically eluted with 1% acetonitrile containing 0.1% HCl. The starting material could not be eluted from the column under the employed conditions.

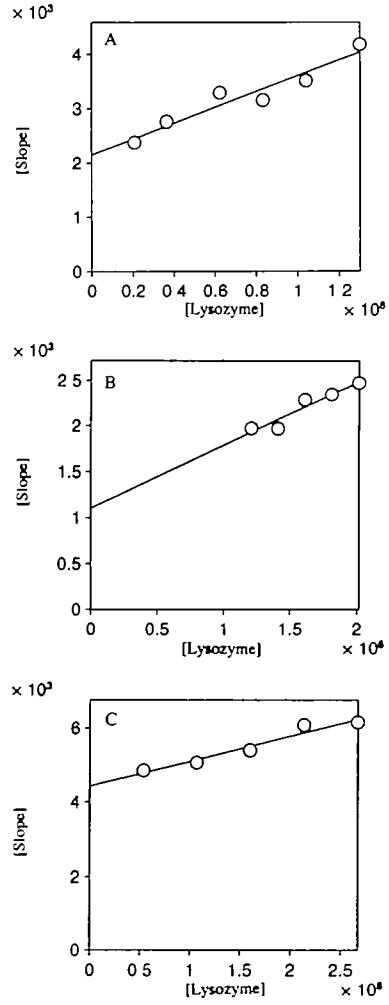

Fig. 2. **Kinetic measurement of the lysozyme-substrate interaction.** A) Hen lysozyme; B) human lysozyme; C) Del 14.15 hen lysozyme. The relationship between the slopes and the concentrations of lysozyme added to the cuvette. The details of the procedure for the kinetic analysis are given under "MATERIALS AND METHODS."

TABLE I. **Kinetic parameters of the interaction of lysozymes with the immobilized substrate analogue at pH 5 and 20°C.**

Lysozyme	k_{ass}[a] ($s^{-1} \cdot M^{-1}$)	k_{diss}[b] (s^{-1})	K_d (M)	K_d[c] (M)	K_m[d] (M)
Hen	1.4×10^3	6.6×10^{-2}	4.7×10^{-5}	6.3×10^{-6}[e]	
Human	6.9×10^2	6.3×10^{-2}	9.1×10^{-5}	3.9×10^{-5}[f]	5.4×10^{-5}
Del 14.15 hen	6.7×10^2	3.8×10^{-1}	5.7×10^{-4}	2.1×10^{-5}[g]	

[a]This value was obtained by using the slope (dR/dt *vs.* R), where the deviation between the slope calculated and that observed is always 0.01%. [b]The deviation between the constant calculated and that observed is always below 0.01%. [c]Dissociation constant for the lysozyme-$(NAG)_3$ complex in solution obtained with an ultraviolet spectrometer. [d]Hydrolyzing reaction of *p*-nitrophenyl-penta-*N*-acetyl-β-chitopentaoside at pH 5 and 37°C (*14*). [e]At pH 5 and 25°C (*15*). [f]At pH 5 and 25°C (*16*). [g]At pH 5.5 and 40°C (*17*).

The Interaction between a Mutant Hen Lysozyme with Higher Activity and the Immobilized Substrate Analogue— We recently found that a mutant hen lysozyme, in which Arg14 and His15, which are located apart from the active site cleft above 20Å, are deleted (Del 14 15 hen lysozyme), exhibits a higher activity toward glycol chitin and *p*-nitrophenyl-penta-*N*-acetyl-β-chitopentaoside than the wild type hen lysozyme (*16*). Therefore, the present method was used to examine the interaction of the mutant hen lysozyme with a substrate analogue. Figure 2C shows plots of the slope (dR/dt *vs.* R) against the concentration of the mutant hen lysozyme added to the cuvette. The plot also shows a good straight line. From the slope, we determined the association rate constant, k_{ass}, for the interaction between the mutant hen lysozyme and the immobilized substrate analogue (Table I). The dissociation rate constant, k_{diss}, and the dissociation constant, K_d, are also shown in Table I.

DISCUSSION

It is not easy to calculate the kinetic parameters of the catalytic reaction of a lysozyme on an oligomer of *N*-acetyl glucosamine because the lysozyme also catalyzes glycosyl transfer to saccharides (*4*). Under these circumstances, it should be meaningful to develop a convenient method for obtaining kinetic parameters, association, and dissociation rate constants, of the interaction between a lysozyme and its substrate analogue. The present method has an advantage in the site-specific immobilization of the substrate analogue of lysozyme. Site-specific immobilization may allow us to analyze the interaction between a lysozyme and an immobilized substrate analogue as simply as possible. On the other hand, *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside is both hydrolyzed by hen and human lysozymes at 37°C, whereas the extent of hydrolysis is low (*12*). Thus, a substrate analogue may be used repeatedly by immobilizing it and by using a low temperature. Moreover, washing of the detecting surface of the cuvette with a 8 M urea solution containing 2-mercaptoethanol, which was not used for the removal of the undissociated complex from the immobilized substrate analogue but for the removal of non-specifically bound lysozymes from the matrix on the cuvette surface, also contributed to the high reproducibility. As a result, the immobilized substrate could be used for more than six months, while the binding efficiency of lysozymes gradually decreased.

As for determination of k_{diss}, we directly obtain it by measuring the dissociation phase. On the other hand, k_{diss} can also be obtained from the vertical intercept in Fig. 2. In the present case, there was a little discrepancy between them (Table I and Fig. 2). We confirmed that the k_{diss} values obtained under two different conditions were identical for the interaction between hen lysozyme and anti-lysozyme monoclonal antibodies, where the association rate is much faster (Ueda *et al.*, unpublished results). Therefore, as the association reaction is much faster, the non-specific reaction, which is relatively slower, may be neglected. On the other hand, in the present case, as the association rate was slower, the non-specific reaction would affect the association reaction. As a result of this, the slopes at the various lysozyme concentrations in Fig. 2 might be each overestimated, resulting in the larger k_{diss} values. In these circumstances, the present k_{diss} values in Table I should be more reliable because they can be obtained from the dissociation phase directly. On the other hand, k_{diss} was shown to be independent of R_{max}.

Before discussing the kinetic parameters, we compared the present dissociation constants for the lysozyme-substrate analogue complex with the previous ones obtained for the interaction between lysozymes and $(NAG)_3$ using spectrophotometer and K_m for the reaction of a lysozyme with *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside (Table I). Since these values were almost the same as each other, the present method was shown to give reasonable values. However, the present K_d values obtained for hen, human and Del 14.15 hen lysozymes were a little larger than the previous ones. The differences may be due to the immobilization of the substrate analogue, as immobilization may cause some steric hindrance. It was found, by analyzing kinetic parameters, that the smaller K_d of the hen lysozyme-immobilized substrate analogue complex than that of the human lysozyme-substrate analogue complex was due to the larger association rate constant for the interaction of the substrate analogue with hen lysozyme than that with human lysozyme. The site of *p*-nitrophenyl-tri-*N*-acetyl-β-chitotrioside hydrolyzed by hen lysozyme was different from that of human lysozyme (*12*). Therefore, we estimated that the immobilized substrate mainly binds to subsites A, B, and C of hen lysozyme, and to subsites B, C, and D of human lysozyme. Thus, we did not consider the difference in the association rate constant further. On the other hand, the glycol chitin activity of hen lysozyme was reported to be similar to that of human lysozyme at pH 5 and 25°C (*18*). The activity should be governed by the slowest step in the reaction of an enzyme. This idea may be supported by the finding that the dissociation rate constant of hen lysozyme was similar to that of human lysozyme for the interaction between lysozymes and the immobilized substrate analogue.

As for Del 14.15 hen lysozyme, it was reported that the activity of this mutant hen lysozyme was higher than that of the wild one because of its higher fluctuation (*17*). For activity measurement, an excess amount of substrate such as glycol chitin or *p*-nitrophenyl-penta-*N*-acetyl-β-chitopentaoside was present. Under these conditions, most hen lysozyme may form the substrate complex. Thus, the amount of the complex (*i.e.* K_d) is not the reason for the difference in the activity. The dissociation rate constant of Del 14.15 hen lysozyme was larger than that of the wild

type hen lysozyme (Table I). This may be the reason why the activity of Del 14.15 hen lysozyme was higher than that of the wild type hen lysozyme, because hen and human lysozymes showed similar dissociation rate constants and activities to each other. On the other hand, we should also mention that the difference in the association rate constant between Del 14.15 hen lysozyme and the wild one (Table I), for the substrate binding modes of these lysozyme should be similar since the mutation site in Del 14.15 hen lysozyme is far from the active site cleft. We have shown that the H-D exchange rate of the Trp residue of the active site cleft in the mutant hen lysozyme is greater than that in the wild one (*17*), in which the timescale of motion in the molecule is millisecond. Therefore, the larger fluctuation in the mutant hen lysozyme molecule may lead to the formation of a stabler and better substrate complex, which also induces the higher activity of the mutant hen lysozyme, resulting in a slower association reaction.

In this study, we developed a method for evaluating the kinetic parameters for the complexes between lysozymes and a substrate analogue, *p*-aminophenyl-tri-*N*-acetyl-β-chitotrioside, linked to the detecting surface of the cuvette in a biosensor site-specifically. Even now, the catalytic mechanism of lysozymes is being investigated (*9, 19, 20*). Since many mutant lysozymes have been prepared, systematically kinetic analyses of the interactions between lysozymes and substrate analogues may provide valuable information on the reaction mechanism of lysozymes. Moreover, *p*-nitrophenyl sugar adducts are often used as ligand analogues for several proteins (such as for the interactions between sugars and lectins). By means of a similar strategy, *i.e.* the site-specific immobilization used here, the kinetic parameters for complexes between proteins and ligand analogues may be evaluated more accurately. In these context, the present results are of general value for obtaining kinetic information on the binding of a wide range of proteins.

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