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SEPARATION PROCESS USING LIGAND-MODIFIED AQUEOUS TWO-PHASE SYSTEM BASED ON STRESS RESPONSIVE BEHAVIORS OF PROTEINS

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The possibility of a heat stress-mediated bioseparation process, which utilizes aqueous two-phase systems modified with functional ligands such as Triton X-405 and Cb-PEG, has been investigated. The change of the surface properties of several proteins caused by their conformational change has been quantified under heat stress conditions. Based on these results, a novel protein separation process has been presented using aqueous two-phase partitioning systems under heat stress conditions. It was found that the partition coefficient of the target protein could be controlled by the combined use of (i) its stress-responsive behavior and (ii) spontaneous binding with the functional ligands under optimal stress conditions. As a case study, a target protein, alcohol dehydrogenase, has been successfully separated from other proteins by using ligand-modified aqueous two-phase systems at a specific temperature of 50 °C.

1. Introduction

Concerning the denaturation and renaturation of proteins, the variation of the conformation and surface properties of proteins has been extensively investigated to clarify many features and functions of proteins[1)2)3)] under various stress conditions such as extreme pH, heat and the addition of denaturants. Among the possible conformations of proteins under such stress conditions, the intermediate state, which is often called the molten globule (MG) state, has been investigated in particular detail because of its importance in clarifying the mechanisms of protein denaturation and renaturation processes.[4)5)] The molten globule state is a compact denatured form of protein molecules, which is thermodynamically stable under mild stress conditions, and has a significantly native-like secondary structure but a largely disordered tertiary structure.[5)6)7)8)] Although many proteins have been classified based on their active three-

dimensional form as well as their amino acid sequence, secondary and tertiary structure, globular or fiber forms, molecular weight, isoelectric point, thermal or denaturant stability and so on,[9] the classification of proteins based on common properties that appear under various stress conditions has not been systematically investigated yet. Understanding the properties of various conformational states of proteins including the MG state during their denaturation or refolding processes is, therefore, important not only for the clarification of protein nature or functions under various stress conditions (what we call stress responsive functions) but also for their utilization in the development of novel stress-mediated bioprocesses.[10)11)]

Among the possible conformations of proteins, the MG state was suggested as participating in the translocation of protein molecules through the phospholipid membrane[12] or in the interaction with natural biopolymers such as heat shock proteins.[13)14)] We have already shown that the variation of the surface properties of proteins during their denaturation / renaturation processes can quantitatively be evaluated by using the aqueous two-phase partitioning method.[15)16)] The observed local hydrophobicity (*LH*) of proteins, which was evaluated by this method, was found to play an important role not only in protein denaturation or aggregate formation processes[16] but also in the interaction with liposomes,[17] heat shock proteins,[15] and hydrophobic ligands.[18] The utilization of (i) the conformational change of proteins and (ii) their complex formation with other functional biomaterials can provide a novel separation process which exploits various environmental stresses. Aqueous two-phase systems, which are composed of noncompatible polymers and water, are effective for such a stress mediated bioseparation process because they can partition the above functional ligands into one phase. It is expected that conventional bioseparation processes using aqueous two-phase systems can be improved by utilizing the (i) stress-responsive behaviors of protein and (ii) stress-induced interaction of protein with the functional synthetic polymers.

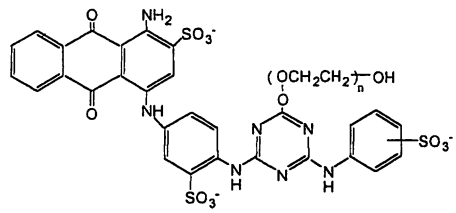
In this study, both of the common and individual properties of proteins as they appear under various stress conditions were firstly evaluated based on the variation of their surface properties related to their stress responsive behaviors. Secondly the surface properties of proteins in the presence of functional ligands (such as Triton and Cb-PEG) under heat stress conditions were characterized. The possibility of an effective stress-mediated separation process for proteins using ligand-modified aqueous two-phase systems was finally presented as an application of (i) the stress responsive behaviors of proteins and (ii) the additional effect of the functional ligands.

2. Experimental

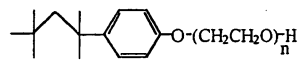
2.1. Materials

Both monomeric proteins, such as bovine carbonic anhydrase (CAB), α-glucosidase from yeast (α-glu), α-amylase from *Bacillus licheniformis* (α-amy), bovine serum albumin (BSA) and α-chymotrypsin from Bovine Pancreas (α-CT), and oligomeric proteins, such as β-galactosidase from *Escherichia coli* (β-gal) and alcohol dehydrogenase from baker's yeast (ADH), used in this study as target proteins were purchased from Sigma Chemical Co. Ltd. (St. Louis, USA). Baker's yeast was purchased from Oriental

Yeast Co. Ltd. (Osaka, Japan). Another oligomeric protein GroELp from *E.coli*, which is classified as a heat shock protein, so called hsp 60, was purified by using aqueous two-phase partitioning combined with PEG fractional precipitation as previously described.[19] The phase organization polymers in the aqueous two-phase systems such as dextran 100k~200k (Dex) (M.W. = 100k~200k Da) and poly(ethylene glycol) (PEG) 1540, 4K, 6K (M. W. = 1.5k, 3k, 7.5k Da) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Triton X-405 was purchased from Sigma. Cibacron Blue F3G-A (Cb), a triazine dye, was purchased from Fluka (Tokyo, Japan). Cibacron Blue F3G-A-PEG (Cb-PEG) (Fig.1) was synthesized as previously described.[20] All other reagents used in this study were of analytical grade.



(a) Cibacron Blue-PEG (n=200)



(b) Triton X-405(n=40)

Fig.1 Structure formulas of the ligands used (a) Cibacron Blue-PEG and (b) Triton X-405.

2.2. Protein Partitioning in Aqueous Two-Phase Systems

The aqueous two-phase systems (ATPS) used here were PEG (9~10.8 %) / Dex (9 %) systems. The preparation methods of ATPS have been described in the previous report.[21] Surface properties of proteins, such as surface net hydrophobicity (*HFS*) and local hydrophobicity (*LH*), under pH, denaturant, and heat stress conditions have been characterized by using the aqueous two-phase partitioning method.[12)13)] Solutions of proteins were prepared in 50 mM Tris-magnesium buffer (TM-buffer, pH = 8.0). The crude extract solution of ADH was also used for ATPS partitioning in the investigation of the ADH separation process. The yeast cells (cell concentration, 0.1 g/ml) were disrupted by using an ultrasonic disrupter (UD-200, Tomy Seiko Co. Ltd., Tokyo, Japan) with an input power of 120 W and an operation volume of 30 ml for 40 min at 4 °C. The supernatant of the disrupted solution after centrifugation (10,000 rpm, 20 min) was used as the crude extract solution. The protein solution and the crude extract solution were exposed to various heat stress conditions (40~75 °C) and, then, the conformational state and surface properties of proteins were analyzed by adding them to the above ATPS under various conditions.

2.3. Measurements

The protein concentration was determined by the Bradford method.[22] The activity of ADH was measured as previously described.[23] Enzyme denaturation and aggregate formation were evaluated by using the denaturation rate constant (k_d) and aggregation velocity (dA_{340}/dt), respectively.[13]

3. Results and Discussion

3.1. Characterization of Surface Properties of Proteins under Heat Stress Conditions

Surface properties of various proteins were firstly characterized under heat stress conditions by varying the temperature in the range of 40~75 °C. The variations of surface net hydrophobicity (*HFS*) and local hydrophobicity (*LH*) of proteins under various heat stresses are shown in Fig.2(a) and 3(a). Under

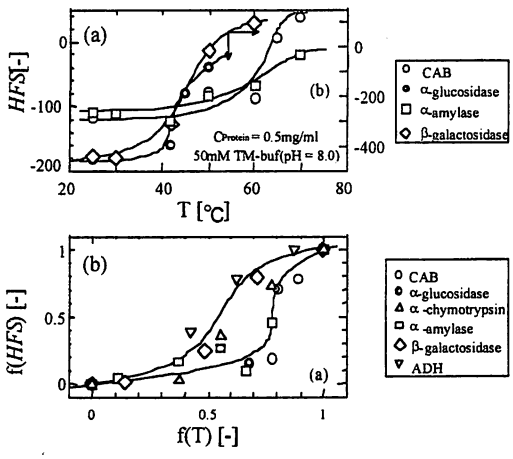


Fig.2 Dependence of surface net hydrophobicity *HFS* on (a) temperature and (b) normalized fractional changes in *HFS* of various proteins under heat stress conditions.

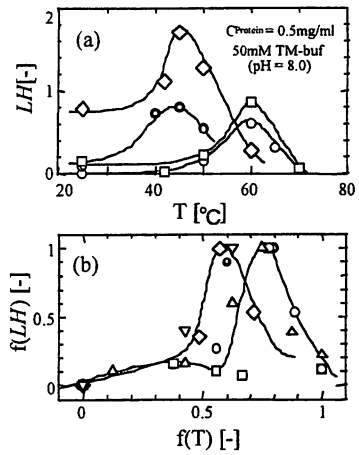


Fig.3 Dependence of local hydrophobicity *LH* on (a) temperature and (b) normalized fractional changes in *LH* of various proteins under heat stress conditions.

normal conditions (25 °C), an oligomeric protein, β-gal, has a very hydrophilic nature (low *HFS* values, Fig.2(a)) and a relatively strong local hydrophobic site (high *LH* value, Fig.3(a)). The *HFS* and *LH* values of β-gal seem to vary in the same manner as the other three monomeric proteins, namely CAB, α-glu, and α-amy, although the absolute values and the stability against stresses are quite different.

Based on the variation of surface properties of CAB caused by pH, GuHCl concentration and heat stress conditions, [12)13)] several simple assumptions can be introduced as follows in order to evaluate the stress responsive behaviors of proteins under various stress conditions.

(i) The molten globule state, formed under pH, GuHCl, and heat stress conditions, can be defined from the maximum point of the *LH* profiles measured by the aqueous two-phase partitioning method.[12)]

(ii) The effect of the strength X of the stresses such as pH, GuHCl concentration (C_{GuHCl}), and temperature on the protein structure can be analyzed by a linear relationship (equation (1)), at least, of the protein denaturation process from the native state to the molten globule one.

$$\text{Normalized Strength of Stress} = f(X_i) = | X_i - X_{iN} | / (X_{iM} - X_{iN}) \quad (1)$$

where $f(X_i)$ is the function of the characteristic variable X_i for each stress i, *i.e.* pH, GuHCl concentration, and heating temperature, T. Subscripts N and M denote the native state and maximum value, respectively

Based on the above assumptions, the stress responsive behavior of CAB under various stress conditions was simply evaluated by using the following methodology. Firstly, the environmental conditions of the native state, which means standard conditions (no stress), are defined as pH = 7, C_{GuHCl} = 0 M, T = 25 °C. Surface properties of protein *LH* and *HFS* can also be normalized by using the following equation (2),

$$\text{Normalized Surface Property} = f(Y_j) = | Y_j - Y_{jN} | / (Y_{jM} - Y_{jN}) \quad (2)$$

where $f(Y_j)$ is the function of surface property Y_j, *i.e.* *LH* and *HFS*, subscript N and M denote the native

state and maximum value, respectively. Consequently, the native state and the molten globule or unfolded state were taken as the standard states and the stress responsive behaviors of proteins under various stress conditions were analyzed by using the above normalized parameters $f(X_i)$ and $f(Y_j)$.

Figures 2(b) and 3(b) show the relationship between the normalized parameter $f(Y_j)$ for *HFS* (Fig.2(a)) and *LH* (Fig.3(a)) of various proteins and that for temperature $f(X_j) = f(T)$. In this case, both the native and unfold states are chosen as the two extreme standard states. The unfold state is defined as the state where the maximum conformational change of a protein against the heat stress has occurred and the strength of stress at that time is estimated from the profiles of *HFS* at which the transition is completed. As shown in Fig.2(b) and 3(b), the proteins can be classified into two groups such as monomeric and oligomeric proteins from the view point of the stress responsive behavior of proteins although the absolute values of surface properties and their heat stability are different (Fig.2(a) and 3(a)). The denaturation / renaturation processes of oligomeric proteins were thought to be rather complicated[24)25)] and, thus, it is difficult to obtain high reactivation yields in their refolding processes even if suitable conditions were chosen.[26)] In this case, the association / dissociation of subunits of oligomeric proteins during the folding / unfolding processes are important in the design and development of such complex processes, which do not exist in the denaturation pathway of monomeric proteins. This is one of the reasons why such a classification could be observed. It is, therefore, intriguing to find and to reclassify such proteins from the viewpoint of their stress responsive behaviors. The above classification should provide a useful method in protein technology for functional modification and so on.

Based on those common stress responsive behaviors of proteins which appear under some stress conditions (Fig.2 and Fig.3), the difference in the individual properties for various proteins can be exploited effectively for application of the stress response of proteins. Figure 4 shows the difference in stress strength from the relationship between the *LH* values in the native state and the molten globule state for various heat-stressed proteins (Fig.4). The variation of the *LH* values of the various proteins from the native state to the molten globule state also seems to be different for each protein (Fig.4). From these results, proteins, which have similar surface properties in the native state, can be easily separated by choosing appropriate stress conditions.

3.2. Modification of Surface Properties of Target Protein (ADH) with Ligands under Heat Stress Condition

The above behaviors of stress response of proteins under stress conditions can be applied to the design of a bioseparation process by the use of functional ligands which selectively bind to the protein surface under stress conditions. In addition, functional ligands, such as t-octylphenyl-PEG (Triton) and Cb-PEG, are preferentially partitioned

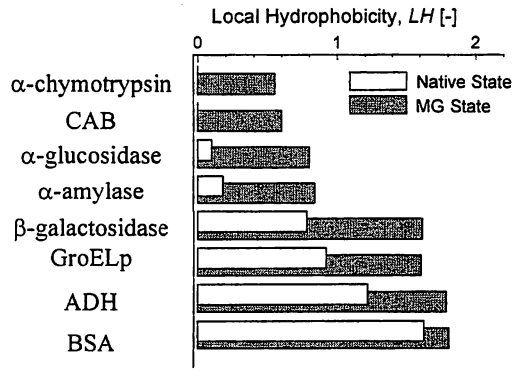


Fig.4 Comparison of *LH* values at native state and MG state of various proteins.

into the top (PEG) phase of the aqueous two-phase systems. As previously reported,[20] these ligands can also bind to the surface of partly-damaged proteins by their recognition of their higher local hydrophobic sites under various stress conditions. The utilization of both stress response functions of proteins and those of the ligands can improve the efficiency and selectivity of the recovery of the target protein in a practical bioprocess using aqueous two-phase systems. The possibility of further improvement in the partitioning behavior of protein in the ligand modified aqueous two-phase systems has been investigated under heat stress conditions by selecting ADH as the target. The partitioning behavior of ADH was determined for several aqueous two-phase systems, which were modified with a functional ligand such as Triton and Cb-PEG. Figure 5 shows the variation of the ADH partition coefficient in ligand-modified aqueous two-phase systems under normal conditions (Fig.5(a)) and under heat stress conditions (Fig.5(b)). As shown in Fig.5(a), the partition coefficients of ADH were increased when Triton or Cb-PEG was added to ATPS. Furthermore, an additional effect on the increase of the partition efficient can be observed in the presence of both ligands. The above results imply that ADH has different surface binding sites for Triton and Cb-PEG and an additional effect can therefore be observed. The partition behavior was thus found to be improved by the addition of the above ligands under appropriate stress conditions.

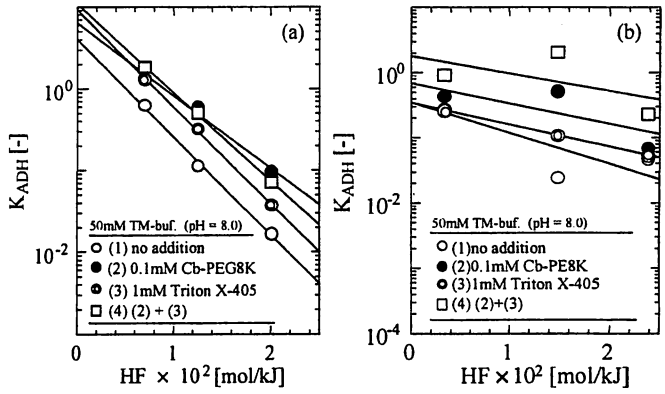


Fig.5 The effect of the addition of various ligands on the partition coefficient of ADH (a) 25 °C and (b) 50 °C.

As shown in the previous section (Result and Discussion 3.1), the partition coefficient of ADH has been improved by exposing it to specific heat stress conditions. The partitioning behavior of ADH was therefore investigated in the ligand modified ATPS under heat stress conditions (50 °C). Here, a similar additional effect can be observed. The value for the partition coefficient of ADH was further improved compared with that at normal temperature (Fig.5(a)). These phenomena can be caused by (i) a conformational change of the ADH from the native to intermediate state, (ii) an increase in binding sites on the ADH surface, and (iii) a further increase in the amounts of ligands bound to the ADH under the specific

Table 1 Variation of surface properties of ADH by the addition of various ligands with and without heat stress conditions.

ligands	25°C		50°C	
	$\Delta \log K_{ADH}$	*HFS*	$\Delta \log K_{ADH}$	*HFS*
none	-	-276	-	-109
(1) 1mM Triton X-405	0.726	-205	0.671	-78.1
(2) 0.1mM Cb-PEG 8K	0.450	-273	1.37	-72.0
(1) + (2)	0.643	-249	1.93	-62.1

Units; $\Delta \log K_{ADH}$: [-], *HFS* : [kJ/mol]

heat condition.

In order to verify the above considerations, the surface properties of ADH in the presence of ligands were evaluated and the results are shown in Table 1. The net and local hydrophobicity of ADH increased with temperature. Especially in the presence of both ligands, the local and net hydrophobicities were distinctly increased, probably because of the modification of the ADH surface by the functional ligands. The partitioning behavior of ADH was thus found to be improved by utilizing the ligand-modified ATPS under heat stress conditions.

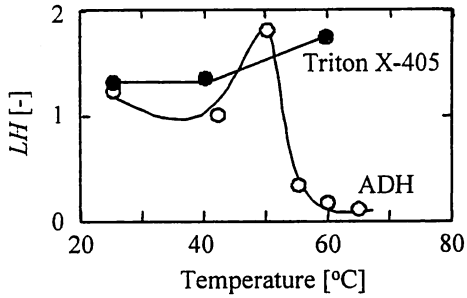


Fig.6 Variation of local hydrophobicity *LH* of ADH and Triton X-405 under heat stress conditions.

3.3. Stress-Mediated Separation of ADH in Ligand-Modified Aqueous Two-Phase System

As a case study, we examined the application of ATPS for (i) the stress responsive behaviors of protein and (ii) the additional effect of functional ligands, even though one of the important strategies of the usual protein separation processes was to ensure minimal stress in such processes and to inhibit protein denaturation as much as possible. An effective stress-mediated separation process of proteins was designed to show the effect of the stress responsive behaviors of protein in the practical bioprocess design. ADH is a tetrameric protein and is known to have a specific affinity for several dye-ligands. The affinity extraction of this protein from crude yeast extract or protein mixture has been carried out using aqueous two-phase systems.[23)27)] The effectiveness of separation was compared between normal non-stressed processes and the stress-mediated process by selecting ADH separation as the model process. Considering the utilization of proteins *in vitro*, heat stress, one of the most popular stresses for proteins, was selected here as a practical stress condition. Figure 6 shows the variation of surface properties of ADH under heat stress conditions. Local hydrophobicity (*LH*) has a maximum value at a temperature of 50 °C, at which temperature the interaction of the protein with a hydrophobic ligand was strongly enhanced.[18)] The variation in the surface property of the ligand, Triton X-405, is also shown in this figure. Throughout the temperature range, the *LH* values of Triton X-405 gave high values (1.3-1.8). In particular, at more than

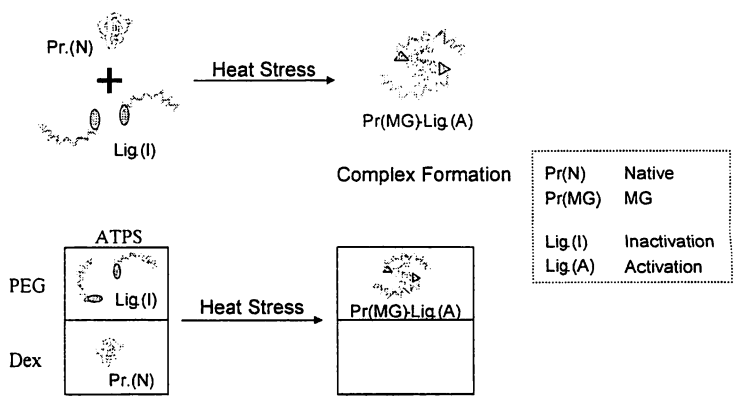


Fig.7 Stress-mediated separation process of proteins by using ligand-modified aqueous two-phase systems.

45 °C, the local hydrophobicity value was slightly increased. If aqueous two-phase systems (ATPS) were modified by hydrophobic ligands which were partitioned selectively to the upper phase, the protein will be also concentrated there by forming the ligand-protein complex under the appropriate stress conditions. In addition, we have already shown that several proteins could be purified effectively by using aqueous two-phase partitioning combined with PEG fractional precipitation.[19)28)] Based on all the above considerations, the stress-mediated separation process for ADH could finally be designed. The scheme of the stress mediated separation process is shown in Fig.7. Conditions in the separation systems are shown as follows.

Table 2 Results of the purification process for ADH contained in the crude extracts of cell.

	(1)	(2)	(3)	(4)
Recovery of activity [%]	1.3	8.3	0.013	20.6
Specific activity [U/mg]	1.5	3.5	1.2	15.6

(1) control (2) ligands (25°C)
(3) heat stress (4) heat stress (50°C) + ligands
※ ligands used were Triton X-405 and Cb-PEG 8K

ATPS; PEG4K (9 wt%) / Dex 100K ~ 200K (9 wt%) + 1 mM Triton X-405 and 0.1 mM Cb-PEG8K

Triton X-405 and Cb-PEG8K were used in this study as representative group affinity ligands. Under normal conditions (25 °C), the recovery of ADH was only 1.3 % without any ligands, which increased to 8.3 % by using the above ligands. In the case of the stress-mediated process, the recovery of ADH was greatly increased (20.6 %) while keeping the activity recovery yield at a similar value to the above non-stress processes in spite of its exposure to heat stress (50 °C, 60 min) (Table 2). Furthermore, both recovered yield and specific activity of ADH were increased under heat stress in the presence of the above ligands. This may be due to (i) the conformational change of ADH from the native state to the intermediate state, (ii) the increase in binding sites on the ADH surface, and (iii) a further increase in the number of ligands bound to the ADH under the specific heat condition. The possibility of a new stress-mediated separation process of proteins was thus presented based on the analysis of the stress responsive behaviors of protein. Effective stress-mediated processes can be designed by evaluating the differences in stress responsive behaviors between the target protein and other impurities *i.e.* the difference in the strength of stress inducing protein denaturation up to the molten globule state (Fig.4), and by choosing the suitable stress conditions.

4. Conclusion

The possibility of a stress-mediated separation process based on the stress-responsive behaviors of proteins has been investigated in ligand-modified aqueous two-phase systems. The variation of the surface properties of proteins (surface net and local hydrophobicity) has been well summarized as a function of normalized stresses. The critical stress condition to change the surface properties of protein has been found to change the surface hydrophobicity. At the specific condition, the protein was found to bind to the hydrophobic ligand, which can be used as a modifier in aqueous two-phase systems. Based on i) the stress-responsive behaviors of proteins and ii) heat-induced interactions between the ligand and the specific

protein, the separation process of target ADH from crude extracts has finally been developed by using ligand-modified aqueous two-phase systems. The above methodology could be applied to the separation process of other proteins by optimizing the exposing stresses.

Nomenclature

HFS	= surface net hydrophobicity	[kJ/mol]
LH	= local hydrophobicity (= $\Delta \ln K_{pr}$)	[-]
MG	= molten globule state	[-]
M.W.	= molecular weight	[Da]
$f(X_i)$	= normalized strength of stress i, X_i	[-]
$f(Y_j)$	= normalized surface properties *LH* and *HFS*	[-]
X_i, Y_j	= characteristic parameters	[-]
X_{iN}, Y_{jN}	= observed value for the characteristic parameter for the native state	[-]
X_{iM}, Y_{jM}	= observed maximum value for the characteristic parameter	[-]

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