Effect of Denaturant and Cosolvents on the Stability of Wheat Germ Lipase†

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Purified wheat germ lipase has a specific activity of 12 ± 1 units, and the addition of urea decreases the activity of the enzyme. Partial specific volume of the enzyme in presence of 8 M urea is 0.723 and 0.717 mL/g for isomolal and isopotential measurements, respectively, and the preferential interaction parameter (ξ_3) value is 0.05 g/g. Ultraviolet and fluorescence emission measurements indicated the exposure of aromatic amino acid residues from the interior of the protein, and thermal denaturation temperature (T_m) measurements indicated a decreased stability of the enzyme by 12 °C from a control value of 56 °C. The addition of specific cosolvents was found to increase the thermal stability of the enzyme to different extents (patent pending). The concentration of the cosolvent (10−40%) used has a direct effect on the retention of activity of the enzyme after being exposed to elevated temperatures in the presence of these cosolvents.

Keywords: *Wheat germ lipase; thermal stabilization; hydration; denaturation; activity*

INTRODUCTION

Lipolytic enzymes are long-chain fatty acid ester hydrolases (Brockerhoff and Jensen, 1974). Among lipases, the mammalian lipases have received more attention because of their medical importance as compared to lipases from other sources such as plants, fungi, and bacteria. Information on lipolytic enzymes in higher plants is important in understanding their physiological roles as well as their action in agricultural products during storage. Among cereal lipases, wheat germ lipase, isolated for the first time by Singer and Hofstee (1948), was actually an esterase, since it hydrolyzes triacetin and tributyrin and is inactive on long-chain triacylglycerols. The various properties of the enzyme and its structural stability were studied to a limited extent (Singer and Hofstee, 1948).

In agriculture, the crushing or storage of seeds or other agricultural products leads to an increase in lipolytic activity which results in the liberation of free fatty acids, making the agricultural products unacceptable (Huang, 1984; Borgstrom and Brockman, 1984). From this point of view, recent studies on isolated wheat germ lipase include characterization of the enzyme from a biophysical point of view and the effect of pH on the mechanism of inactivation of the enzyme (Rajendran *et al*., 1990; Sudhindra Rao *et al*., 1991).

The specific activity of an enzyme has a bearing on the structural stability of the molecule, and this property can be used as a tool to understand the effect of addition of a third component on the extent of inactivation of the enzyme. In this regard, the effect of classical denaturants such as 6 M GuHCl and 3 M GuHSCN on the structural stability of wheat germ lipase has been looked into from the point of view of preferential interactions (Rajeshwara and Prakash, 1994a). Among various other chemical denaturants, urea is a commonly used inexpensive denaturant, the mechanism of action of which has been well worked out (Joly, 1965; Robinson and Jencks, 1965; Tanford, 1968; Jencks, 1969). The effect of chemical denaturants can be interpreted largely in terms of the preferential interaction of the additives with aqueous interfaces and with protein surfaces, which are similar to those interactions that determine protein solubility (Arakawa and Timasheff, 1985). Thus, it is appropriate to obtain the data of interaction of urea with wheat germ lipase and the comparison with GuHCl and GuHSCN data to arrive at the hierarchy of the effectiveness of various denaturants. The interaction of the denaturant can be quantified by partial specific volume measurements supported by other data.

On the other hand, addition of certain cosolvents increases the thermal stability of proteins/enzymes which is attributed to the exclusion of cosolvent molecules resulting in the preferential hydration of the protein (Lee *et al*., 1975; Timasheff *et al*., 1976; Gekko and Timasheff, 1981). In the case of wheat germ lipase also, addition of cosolvents such as glucose, sucrose, glycerol, and DMSO has been shown to induce preferential hydration, as a result of which an increase in the thermal denaturation temperature of the enzyme has been observed (Rajeshwara and Prakash, 1994b). From this point of view, it is necessary to know the extent of retention of activity arising out of preferential hydration. Thus, in the present investigation, the structural stability of the heat-treated enzyme in terms of its activity has been monitored in the presence of various cosolvents to ascertain the extent of protection.

All of the above data would be very useful in determining and comparing the structural stability of the enzyme under the extreme conditions of the presence of a destabilizer or stabilizer.

MATERIALS AND METHODS

Materials. Lipase from wheat germ (type I), triacetin, Sepharose 6B, sucrose, dithiothreitol, and urea were obtained from Sigma Chemical Co., St. Louis, MO. D-Glucose and dimethyl sulfoxide (DMSO) were obtained from E. Merck (India) Ltd. Glycerol was procured from Qualigens Fine Chemicals, Bombay, India,

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and D-sorbitol was obtained from Hi-media Lab Pvt. Ltd., Bombay, India. Buffer salts and calcium chloride dihydrate were procured from Sarabhai M Chemicals, Bombay, India. All other chemicals used were of analytical grade, and glass double-distilled water was used in all experiments.

Purification of Wheat Germ Lipase. Wheat germ lipase obtained from Sigma was purified further to homogeneity according to the method described earlier (Sudhindra Rao *et al.*, 1991). The enzyme was also subjected to Sepharose 6B column chromatography.

Protein Concentration. Protein concentration was determined using a value of 17.5 ± 0.1 as the extinction coefficient ($E^{1\%}_{1cm}$) of the enzyme at 278 nm for wheat germ lipase (Rajendran *et al.*, 1990).

Partial Specific Volume. The partial specific volume of lipase was measured using an Anton Paar DMA 55 or DMA 58 Densitymeter at 20.00 ± 0.05 °C according to the standard procedure (Prakash, 1982; Lee and Timasheff, 1974a,b; Prakash and Timasheff, 1985). The densities of the solvents and of the protein solutions were measured, and the apparent partial specific volume, ϕ, was calculated using the equation (Casassa and Eisenberg, 1961, 1964)

$$\phi = \frac{1}{\rho_0}\left(1 - \frac{(\rho - \rho_0)}{c}\right) \quad (1)$$

where ρ is the density of the solution (in g/mL), ρ_o is that of the solvent (in g/mL), and c is the protein concentration (in g/mL). The values of ϕ were plotted as a function of protein concentration, and the value extrapolated to infinite dilution is the partial specific volume of the protein, $\bar{v}$. In preferential interaction measurements, two types of apparent specific volumes are measured. The first, ϕ_2^0, is measured under the conditions at which the molal concentration, m_3, of diffusible component 3 is kept identical to the solvent and the solutions. The second, $\phi_2'^0$, is measured under the conditions at which it is the chemical potential of component 3, *i.e.*, μ_3, which is kept constant between solution and reference solvent which can be attained to a close approximation by dialyzing the protein solution against the solvent. Here, components 1, 2, and 3 are water, protein, and the denaturant or a stabilizer, respectively, following the standard notations of Scatchard (1946) and Stockmayer (1950).

In such a three-component system, the preferential interaction or exclusion of solvent molecules is determined according to the method described earlier (Rajeshwara and Prakash, 1994a,b). The extent of preferential hydration of the molecule is calculated as described by Rajeshwara and Prakash (1994b), and the volume change occurring upon denaturation is also determined as described earlier (Rajeshwara and Prakash, 1994a).

Ultraviolet Difference Spectra. These were recorded on a Shimadzu 160A double-beam spectrophotometer. The difference spectra were recorded in the ultraviolet region using a 1 cm quartz cell at 27 ± 1 °C. Approximately 1 mg/mL protein concentration was used in all experiments. The difference spectra of lipase in the presence of denaturant was obtained by recording the difference in absorbance between the lipase in denaturant and the solution of equivalent concentration of lipase at neutral pH buffer without the denaturant. The ΔA values obtained from the experiment were later converted into ΔE values.

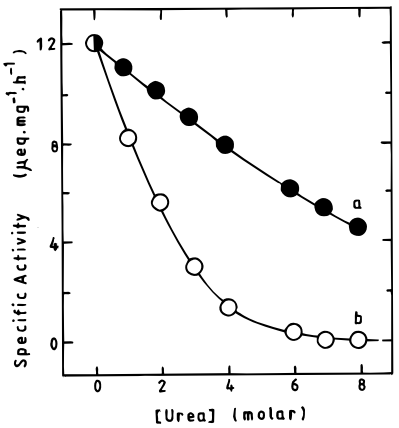

Figure 1. Activity profile of wheat germ lipase as a function of urea concentration: (a) native enzyme; (b) disulfide-reduced enzyme.

Fluorescence Spectra. The fluorescence spectra of wheat germ lipase were monitored at 27 °C using a Shimadzu RF-5000 spectrophotofluorometer. The fluorescence emission spectra were recorded in the range 300−400 nm, keeping the excitation at 284 nm.

Lipase Activity. The activity measurement of the enzyme was carried out using triacetin as the substrate by titrimetry as described by Sudhindra Rao *et al.* (1991).

Thermal Denaturation Studies. The thermal denaturation behavior of wheat germ lipase both in the absence and in the presence of a third component was followed using a Gilford Response II spectrophotometer. The absorbance of the solution was monitored at 287 nm as a function of temperature. Appropriate blanks were also run to get the corrected curves of denaturation. The digital output was converted to van't Hoff plot to obtain the temperature-dependent isotherm.

RESULTS

Purified wheat germ lipase has a specific activity of 12 ± 1 units at neutral pH in its native state (Rajeshwara and Prakash, 1994a). This value is higher as compared to 10 ± 1 units of activity of the enzyme obtained before purification of the enzyme. Activity of the enzyme decreases significantly as the pH is increased or decreased (Rajendran *et al.*, 1990; Sudhindra Rao *et al.*, 1991). What is the effect of addition of a ligand on the activity of the enzyme? This has been probed in the present investigation through the measurement of activity of the enzyme in the presence of various types of added third components such as urea, glucose, sucrose, glycerol, DMSO, and sorbitol.

Effect of Urea on the Activity. In Figure 1 is shown the activity profile of wheat germ lipase as a function of urea concentration. The data were obtained for native wheat germ lipase as well as for disulfide bond-reduced enzyme. From the figure it is clear that the activity of disulfide intact enzyme is retained to the extent of 40% even in the presence of 8 M urea, suggesting stability of the enzyme against urea denaturation. If the disulfide bonds of the enzyme are cleaved in the presence of dithiothreitol, the enzyme gradually loses its activity as a function of urea concentration and the activity becomes zero above 7 M urea concentration (Figure 1).

Effect of Urea on the Partial Specific Volume. The effect of 8 M urea on the partial specific volume of the enzyme was monitored as a measure of hydration.

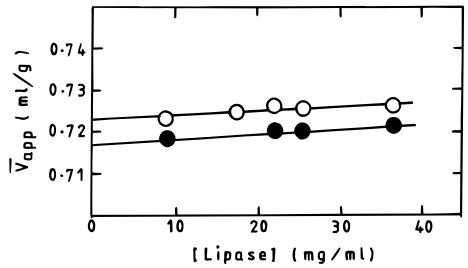

Figure 2. Apparent partial specific volume of wheat germ lipase in 8 M urea as a function of protein concentration: (○) isomolal; (●) isopotential.

Table 1. Partial Specific Volume and Related Parameters of Wheat Germ Lipase in 8 M Urea

parameter	value
ϕ_2^{0} [a] (mL/g)	0.723 ± 0.002
$\phi_2'^{0}$ [b] (mL/g)	0.717 ± 0.001
$(\partial g_3/\partial g_2)_{T,\mu_1,\mu_3}$ (g/g)	0.05 ± 0.01
$(\partial m_3/\partial m_2)_{T,\mu_1,\mu_3}$ (mol/mol)	35 ± 7
A_3 (exptl) (g/g)	0.354 ± 0.010
A_3 (calcd) (g/g)	0.289 ± 0.014
∂v (mL/mol of residue)	−1.077 ± 0.09

[a] Value in native condition is 0.730 ± 0.001 mL/g. [b] Value in native condition is 0.731 ± 0.002 mL/g.

For the sake of comparison, the partial specific volume measurements of the enzyme have been carried out at 8 M urea concentration without breaking the disulfide linkages. In Figure 2 is shown the plot of apparent partial specific volume of wheat germ lipase as a function of protein concentration in the presence of 8 M urea. The extrapolated isomolal and isopotential values are 0.723 and 0.717 mL/g (Table 1). These values are lower as compared to the isomolal and isopotential values of 0.730 and 0.731 mL/g, respectively, in its native state (Rajeshwara and Prakash, 1994a). The decrease in the value of partial specific volume of the enzyme indicates preferential interaction of the enzyme with urea molecules. The preferential interaction parameter (ξ_3) was calculated to be 0.05 g/g and the mole/mole preferential interaction parameter value is 35 (Table 1).

The actual number of urea molecules bound to the enzyme (A_3) was calculated on the basis of the preferential interaction parameter value and the absolute hydration (A_1) of the enzyme calculated from the hydration values of its constituent amino acids according to the method of Kuntz (1971). The value of A_3 thus obtained is 0.354 g/g (Table 1). This has also been obtained by calculations based on the bifunctional nature of the denaturant (Prakash and Timasheff, 1981, 1985). The value obtained by such calculations was 0.289 g/g (Table 1).

Such a process of inactivation of the enzyme in the presence of a denaturant will be reflected in terms of volume changes arising as a result of unfolding. In the presence of 8 M urea, the volume change observed is −336 ± 84 mL/mol (Table 1). The volume change on a residue basis is found to be −1.077 ± 0.09 mL/mol of residue.

Effect of Urea on the Ultraviolet and Fluorescence Spectra. As indicated by $\bar{v}$ results, addition of urea leads to the binding of denaturant molecules to wheat germ lipase. Thus, it is of interest to see the effect of urea binding on the ultraviolet absorption of the enzyme as a function of denaturant concentration. Figure 3 shows the changes in the molar absorptivity values of wheat germ lipase followed at 287 nm. The

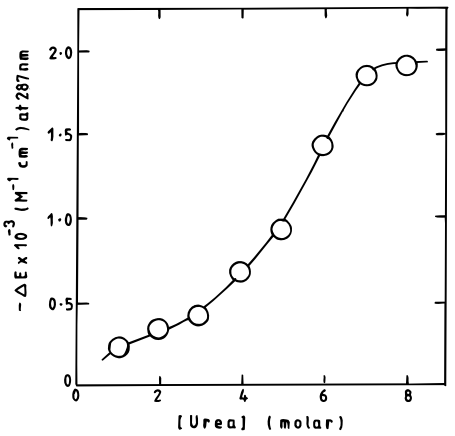

Figure 3. Ultraviolet difference spectral measurements of wheat germ lipase monitored at 287 nm as a function of urea concentration.

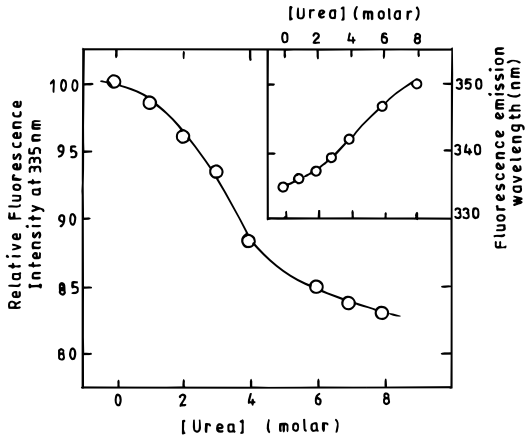

Figure 4. Fluorescence emission measurements of wheat germ lipase as a function of urea concentration. (Inset) Fluorescence emission maximum as a function of urea concentration.

gross conformational changes occur over a wide range of concentration of the denaturant and reach a plateau only at 8 M concentration. A maximum change of −1.9 × 10^3 M^{-1} cm^{-1} has been observed at 8 M urea concentration, which is as a result of change in the environment of the aromatic chromophores. In particular, the conformational status of the tryptophan residues has been probed through the measurement of fluorescence spectra of the enzyme. In Figure 4 is shown the relative fluorescence emission intensity of wheat germ lipase monitored at 335 nm as a function of concentration of urea. The data suggest that the addition of urea results in the quenching of fluorescence emission intensity, which is accompanied by a red shift in the emission maximum (Figure 4 inset). The decrease in the intensity is to the extent of 17% and the red shift is to the extent of 15 nm at 8 M urea concentration, the transition being between 2 and 4 M urea. These data suggest the possibility of exposure of tryptophan residues and microenvironmental changes occurring near the tryptophan residues as a result of urea addition.

Effect of Urea on the Thermal Denaturation. The instability of wheat germ lipase molecule as a result of denaturant action was probed further through measurement of the apparent thermal denaturation temperature of the enzyme. In Figure 5 is shown the plot of log $T_i/T_c - T_i$ as a function of urea concentration. The apparent T_m values have been obtained by the van't Hoff plot which is as shown in the inset in the case of 1.5 M

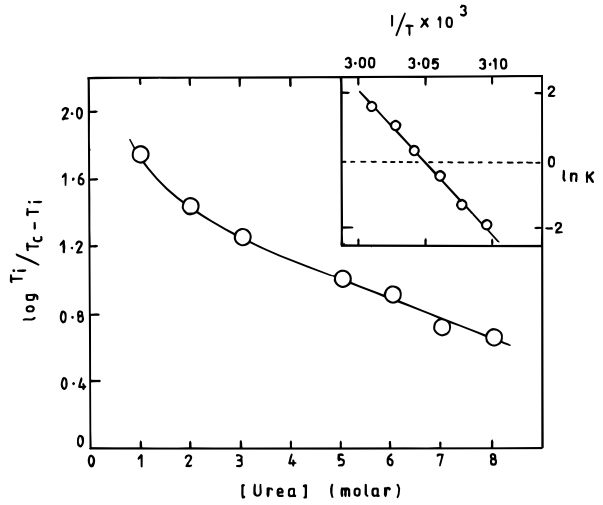

Figure 5. Apparent thermal denaturation temperature measurements of wheat germ lipase in the presence of urea. (Inset) van't Hoff plot of denaturation curve at 1.5 M urea concentration.

Table 2. Specific Activity of Wheat Germ Lipase upon Heating at 70 °C for 10 min in the Presence of Phosphate Buffer[a] Containing Cosolvents

cosolvent	concn (%)	sp act. (μequiv mg^{-1} h^{-1})	% retention	fold increase in stability[b]
buffer[a]				
(a) native (unheated)		12.00 ± 1.00	100.0	
(b) heated		0.30 ± 0.05	2.0*	
glucose	25	3.64 ± 0.30	24.0	12.0 ± 1.4
	40	5.72 ± 0.48	41.9	20.9 ± 2.5
sucrose	25	2.88 ± 0.24	19.0	9.5 ± 1.1
	40	4.40 ± 0.36	32.2	16.1 ± 1.9
glycerol	25	1.34 ± 0.11	8.8	4.4 ± 0.5
	40	2.20 ± 0.18	16.1	8.1 ± 0.9
DMSO	25	1.17 ± 0.10	9.6	4.8 ± 0.6
	40	0.22 ± 0.02	1.8	0.9 ± 0.1
sorbitol	25	3.64 ± 0.30	24.0	12.0 ± 1.4
	40	5.72 ± 0.48	41.9	20.9 ± 2.5

[a] pH 7.0, 0.02 M. [b] Calculated on the basis of the asterisked value, i.e., ratio of percent retention of activity of the enzyme heated in buffer alone as compared to that in different cosolvents.

concentration of urea. The data indicate a progressive decrease in the apparent T_m value of wheat germ lipase as a function of urea concentration. The value of T_m is 44 °C at 8 M urea concentration as against a control value of 56 °C.

Effect of Cosolvents on the Activity. It was also of interest to know the structural stability of wheat germ lipase in terms of its activity in the presence of an added third component which is known to stabilize the protein. The effect of cosolvents such as glucose, sucrose, glycerol, DMSO, and sorbitol on the activity of the enzyme was probed through the measurement of the specific activity of the enzyme. The specific activity of the enzyme in control (0.02 M phosphate buffer) has been compared with the specific activity value of the enzyme exposed to 70 °C for 10 min in the presence of different cosolvents (at both 25% and 40% concentration). In Table 2 is shown the percent retention of activity values of wheat germ lipase in the presence of various cosolvents and the fold increase in thermal stability of the enzyme.

From Table 2 it can be seen that the heat-treated enzyme has only 2% residual activity in phosphate buffer. The maximum retention of activity is 24% or 42% in the presence of glucose or sorbitol at 25% or 40% concentration, respectively (Table 2). The retention of activity is 19% or 32% in the presence of 25% or 40% sucrose, respectively. Similarly, in thepresence of 25% or 40% glycerol, the residual activity value is 8.8% or 16.1%, respectively. In contrast, DMSO is found to stabilize the enzyme only at lower concentrations. Table 2 shows that in the presence of 25% DMSO, the retention of activity is 9.6%, whereas at 40% DMSO concentration the retention of activity is only 1.8%.

DISCUSSION

All of the above data of activity and structural stability measurements indicate the extent of stability of wheat germ lipase in solution in the presence of a denaturant and a stabilizer. Wheat germ lipase exhibits enzymic activity even in the presence of 8 M urea, indicating the rigidity of its structure. Only the disulfide-reduced enzyme loses its activity completely in 8 M urea. However, in the presence of other classical chemical denaturants such as 6 M GuHCl or 3 M GuHSCN, the enzyme is completely inactive (Rajeshwara and Prakash, 1994a). The partial specific volume measurements indicated a less preferential interaction of urea molecules with wheat germ lipase, indicating less effectiveness as a denaturant. Rajeshwara and Prakash (1994a) have shown that wheat germ lipase has higher preferential interaction parameter values with 6 M GuHCl or 3 M GuHSCN, which are equal to 0.08 and 0.14 g/g, respectively. This indicates that there is a critical value of preferential interaction parameter above which the enzyme is completely inactive, the value of which lies between 0.05 and 0.08 g/g. The preferential interaction parameter also depends upon the presence of anion in ionic denaturants such as GuHCl and GuHSCN.

The volume changes occurring to the wheat germ lipase molecule as a result of denaturant action are due to unfolding. This has contributing factors of differences in electrostriction in the two media, difference between the change in the volume of the solvent component and also possible ionization of buried groups (Lee and Timasheff, 1974a). In 8 M urea, the volume change occurring is -336 ± 84 mL/mol, indicating lesser change in the overall structure as against higher volume changes in the presence of other denaturants such as 6 M GuHCl and 3 M GuHSCN (Rajeshwara and Prakash, 1994a). Similarly, ultraviolet difference spectral measurements of the enzyme in the presence of 8 M urea indicate a lesser degree of change in the extinction coefficient of aromatic residues as compared to 6 M GuHCl denaturation (Rajeshwara and Prakash, 1994a). Fluorescence emission spectral measurements and thermal denaturation temperature measurements also substantiate the incomplete inactivation of the enzyme in the presence of 8 M urea.

The activity measurements of heat-treated wheat germ lipase in the presence of cosolvents such as glucose, sucrose, glycerol, DMSO, and sorbitol indicated increased thermal stability of the enzyme as compared to the presence of buffer alone. In a three-component system, preferential hydration is a known phenomenon where the third component is a stabilizer of the structure of the macromolecule as shown for various proteins in the presence of sucrose (Lee and Timasheff, 1981),

glycerol (Gekko and Timasheff, 1981), glucose (Arakawa and Timasheff, 1982a), and some salts (Arakawa and Timasheff, 1982b). Depending upon the cosolvents, the proteins are stabilized by preferential hydration and the protein molecules exist in a more stable form in the presence of a cosolvent. This preferential hydration results in the enhanced thermal stability as observed for ovalbumin in the presence of sucrose (Simpson and Kauzmann, 1953), lysozyme and ribonuclease in the presence of polyhydric alcohols (Gerlsma and Stuur, 1972), and egg white proteins in the presence of sucrose, glucose, sorbitol, and glycerol (Back *et al*., 1979). It has already been shown that wheat germ lipase is preferentially hydrated in the presence of these cosolvents to different extents depending upon their nature and concentration (Rajeshwara and Prakash, 1994b). The resulting more stable form of the molecule has been shown to have a higher apparent thermal denaturation temperature as compared to native enzyme (Rajeshwara and Prakash, 1994b). Thus, the several-fold increase in the activity of the enzyme in the presence of these cosolvents is a result of preferential hydration of the enzyme which restricts the process of thermal inactivation as indicated by both apparent thermal denaturation temperature and activity measurements. The data available for wheat germ lipase in terms of apparent thermal denaturation temperature have been correlated more quantitatively in terms of activity measurements carried out in the present investigation which substantiate the phenomenon of stabilization of proteins and enzymes in general in these cosolvents.

ABBREVIATIONS USED

GuHCl, guanidine hydrochloride; GuHSCN, guanidine thiocyanate; DMSO, dimethyl sulfoxide.

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