Effect of Pressure on Subtilisin Catalysis: Hydrolysis and Peptide Synthesis

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The effects of high pressure on the subtilisin (Carlsberg) catalyzed hydrolysis and aminolysis reactions were investigated.

The pH dependence of the apparent second-order rate constant ($k_{cat}/K_{m(app)}$) for *N*-glutaryl–Ala–Ala–Pro–Leu–*p*NA showed a slightly lowered profile due to increasing pressure. The pressure dependence (up to 150 MPa) of $k_{cat}/K_{m(app)}$ for this substrate or *N*-benzyloxycarbonyl–Gly–Gly–Leu–*p*NA at a constant pH (8.5) showed slightly positive activation volumes: + 2—3 ml mol^{-1}.

The substrate concentration dependence of the hydrolytic rate for *N*-succinyl–Ala–Ala–Pro–Phe–*p*NA showed increased slope and Y-intercept on an Eadie plot at 150 MPa; the reaction volume for the $1/K_m$ was positive and the activation volume for k_{cat} was estimated to be negative. Pressure affected the two parameters in the opposite directions, and as a result, the effect on $k_{cat}/K_{m(app)}$ became rather small.

Pressure promoted the subtilisin-catalyzed transacylation reaction between Fua–Phe–OMe and $GlyNH_2$, but the fraction of the peptide formation (f_a) decreased slightly under high pressure. Pressure seemed to accelerate the hydrolytic process much more, especially the nucleophile-assisted hydrolysis of the acyl-enzyme, than the acyl-transfer to the nucleophile.

The pressure effects on biopolymer structures and functions have been studied for many years.[1—4] An elevation of pressure has been used as an initiator of the higher-order structure changes[5,6] in order to study their refolding and dissociation processes. Such study has recently gained more importance in the fields related to biology and biochemistry, and now pressure is used in several applied fields related to biopolymers.[7—12] These new usages of pressure are essentially based on the structural perturbation of biopolymers, including proteins and protein-lipid composites, caused by an increase in pressure.

We have studied the pressure dependence of several enzymatic reactions[13—21] and the use of pressure in some aspects of enzyme engineering.[22—28] Among them, proteolytic modifications of proteins under pressure-denatured conditions exerted very interesting results,[29] and for such purposes, subtilisin was shown to be highly useful. For example, the limited proteolysis of metallo-proteases by subtilisin was accelerated by pressure and brought about interesting effects on the reaction specificity. Furthermore, subtilisin is known to catalyze some ligation reactions to form peptides by way of transacylation or transpeptidation reactions, and some ligation reactions catalyzed by other proteolytic enzymes were reported to be preferably influenced by applying high hydraulic pressure.[23,24]

However, only a few studies on the fundamental properties of subtilisin under high pressure have been made.[30] Therefore we studied here the effects of pressure on the subtilisin-catalyzed hydrolysis of model peptide substrates and the peptide formation by aminolysis reaction.

Experimental

Materials: Subtilisin Carlsberg (or subtilisin A) was purchased from Sigma (Lot 10F0294). *N*-Benzyloxycarbonyl–Gly–Gly–Leu–*p*-nitroanilide (CbzGGL*p*NA), *N*-Succinyl–Ala–Ala–Pro–Phe–*p*NA (SucAAPF*p*NA), and glutaryl–Ala–Ala–Pro–Leu–*p*NA (GltAAPL*p*NA) were purchased from the Peptide Institute Inc. (Osaka, Japan). Other reagents were commercially available and were used without further purifications.

Methods: Optical absorbance under high pressure was measured using a Drickamer-type high pressure vessel with optical windows made of quartz constructed by Hikari High Pressure Co. (Hiroshima, Japan).[31] This vessel was connected to the light source and the detecting system (PRAS5000, Ohtsuka Electrics, Hirakata, Japan) through optical fibers. Optical absorbance under ambient pressure was measured by a conventional spectrophotometer (Shimadzu UV-2200, Shimadzu Co., Kyoto, Japan). Incubations under elevated pressure were performed using a high pressure vessel HP-1800, constructed by Yamamoto HP Co. (Toyonaka, Japan). Peptide formation reaction was monitored by the reverse-phase liquid chromatography on Shimadzu 10A/Cosmosil 5C-18-P system.

Results

Activity after Incubation at High Pressure: Before extending into high pressure kinetic measurements, we in-

vestigated the effect of pressure-treatment on the residual catalytic activity of subtilisin after the incubations under high pressure. Figure 1 shows the results. By incubating at 150 or 400 MPa, the enzyme showed higher residual activity than that incubated under atmospheric pressure. Subtilisin is known to show autolytic degradation[32] and a prolonged incubation under optimal conditions brought about a decrease in the activity. Pressure seems to prohibit or inhibit the autolysis. This situation is mechanistically similar to the prolonged incubation of this enzyme in the presence of surface active inhibitors.[33]

Besides this autolytic degradation, subtilisin did not seem to suffer from pressure-induced irreversible disactivation or denaturation under the conditions applied here. The intrinsic fluorescence of subtilisin showed a moderate decrease in intensity by increasing pressure, much smaller than thermolysin, but it did not remain irreversibly changed after releasing pressure, up to at least 200 MPa.[34]

Pressure Effect on the Hydrolysis: Figure 2 shows the effects of pressure on the pH dependence of the subtilisin-catalyzed hydrolysis of GltAAPL*p*NA, at constant substrate concentration (0.25 mM, M = mol dm^{-3}). The apparent second-order rate constant changed with the pH, showing an apparent peak at around pH 8.5—9, and this feature did not change significantly under elevated pressure (150 MPa), but the peak became slightly lower. This implies that the pressure dependence of the apparent kinetic constant is rather small.

Then the pressure-dependence of the (apparent) second-order rate for GltAAPL*p*NA was measured from 0.1 to 150 MPa at a pH of 8.5 (Fig. 3). The pressure-induced pH deviation in Tris-HCl buffer under the present conditions

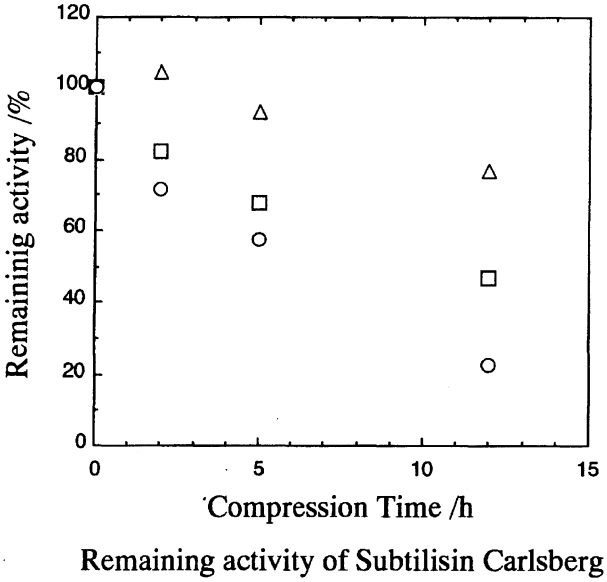

Fig. 1. Catalytic activity of subtilisin Carlsberg after incubations under various pressures. ○, incubated at 0.1 MPa; □, at 150 MPa; △, at 400 MPa. 25 °C and pH 8.5 (Tris-HCl). [E] = 1 μM. Activity was assayed against Suc–Ala–Ala–Pro–Phe–*p*NA.

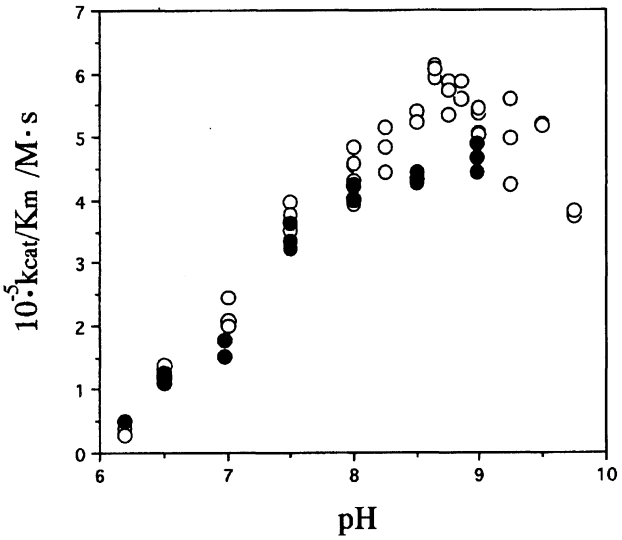

Fig. 2. Effect of pressure on the pH dependence of the subtilisin (carlsberg)-catalyzed hydrolysis of Glt–Ala–Ala–Pro–Leu–*p*NA. [substrate] = 0.25 mM. [enzyme] = 0.25 nM. 0.2 M Mes-NaOH/Hepes-NaOH/Tris-HCl, 25 °C. ○, 0.1 MPa; ●, 150 MPa.

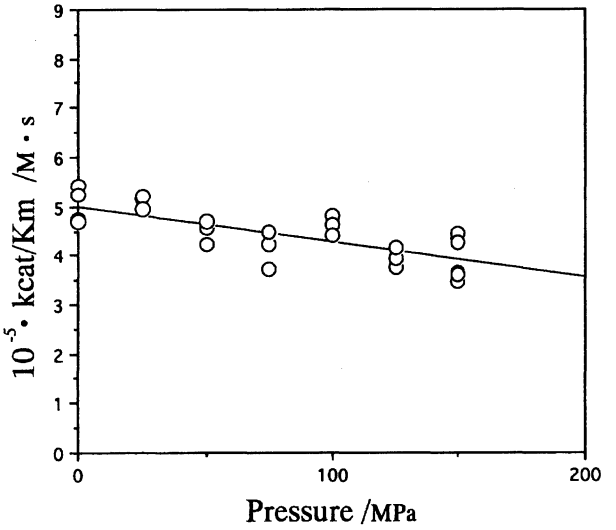

Fig. 3. Pressure-dependence of the (apparent) second-order rate for the hydrolysis of Glt–Ala–Ala–Pro–Leu–*p*NA by subtilisin Carlsberg. [substrate] = 0.25 mM. [enzyme] = 0.25 nM. 0.2 M Tris-HCl (pH 8.5), 25 °C.

was estimated to be about 0.01. The apparent rate constant showed a very slight decrease with increasing pressure, and the evaluated activation volume for $k_{cat}/K_{m(app)}$ (V_{max}/k_m at low substrate level) was 2.3 +/− 0.7 ml mol^{-1}. That for CbzGGL*p*NA (data not shown) was evaluated as 2.7 +/− 0.4 ml mol^{-1}. Since the K_m values for these substrates under atmospheric pressure were evaluated as > 2.5 mM and this value did not seem to decrease by increasing pressure (see below), the very small and positive $\Delta V^{\ddagger}$ values can be considered for k_{cat}/K_m. The substrate concentration dependence of the hydrolytic rate was measured using SucAAPF*p*NA as the substrate, at 150 MPa and 0.1 MPa (Fig. 4), since the K_m

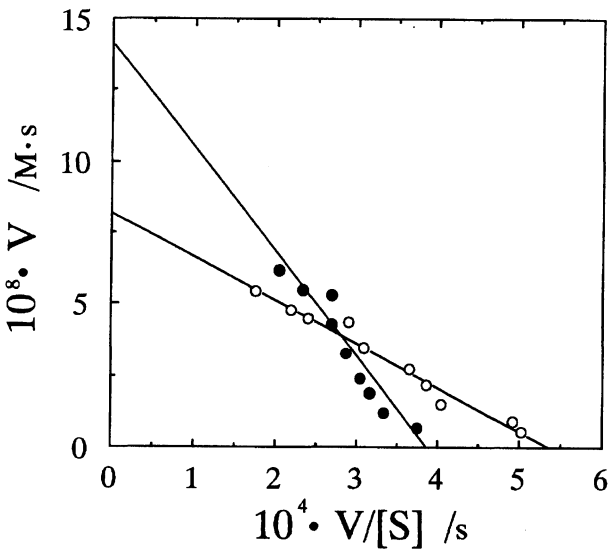

Fig. 4. Substrate concentration dependence of the hydrolytic rate of Suc–Ala–Ala–Pro–Phe–*p*NA by subtilisin Carlsberg, plotted in the Eadie form. ○, at 0.1 MPa; ●, at 150 MPa. [substrate] = 10—300 μM, [enzyme] = 0.25 nM, Tris-HCl (pH 8.5) and 25 °C.

value of this substrate is in the sub mM level, well below the solubility limit and well above the detection limit. At 150 MPa, both the slope and the Y-intercept of the Eadie plot in Fig. 4 were larger than those at 0.1 MPa: both K_m and k_{cat} became larger at higher pressure. The reaction and the activation volumes were estimated from these data as +14 and −8 ml mol^{-1}, respectively (to understand the volume profiles along the reaction coordinate, the volume change related to the K_m parameter is described here as $\Delta V_{(1/K_m)}$, which is $-\Delta V_{(K_m)}$). Pressure affected the two rate parameters so as to force the overall rate in the opposite directions. As a result, the deviation (by pressure) of the specificity ratio became rather small (about 6 ml mol^{-1} in this case) and the result for GltAAPL*p*NA (2—3 ml mol^{-1}) shall also reflect these counteractions.

Kim and Dordick measured the pressure effect on subtilisin in aqueous and organic media up to rather low pressure (35 MPa) and reported the volumetric parameters in aqueous buffer (phosphate) as $\Delta V_{(1/K_m)} = +35.0$ and $\Delta V^{\ddagger}_{(k_{cat})} = -38.5$ ml mol^{-1}. These are larger than our results. This difference might be related to their lower pressure range and their usage of highly pressure-sensitive buffer.[35,36] However the counteracting tendency of these two parameters is essentially similar and their $\Delta V^{\ddagger}$ for the k_{cat}/K_m was also rather small and negative (−3.5 ml mol^{-1}).

Effect on the Aminolysis (Transacylation) Reaction: As the next step, we studied the effect of pressure on the aminolysis or transacylation reaction for Fua–Phe–OMe and $GlyNH_2$. After 2 h reaction at 37 °C, pH 8.5 under various pressures, the reaction mixture contained the components shown in Table 1, where the total degree of reaction (consumption of the ester in %) and % fraction of the aminolysis are also shown. As we observed in a similar aminolysis reaction catalyzed by carboxypeptidase Y,[23,24] the amount of the obtained peptide product was increased at 100 MPa or 200 MPa. At much higher pressure, however, the total consumption of the ester was increased but the fraction of the peptide product formation (Fua–Phe–Gly–NH_2) decreased slightly.

In Figs. 5 and 6, the results of the reaction at 0.1 MPa and 300 MPa with various concentrations of the acyl acceptor (nucleophile) are shown. The % productions of the peptide (○) and the acyl amino acid (●), as well as the total consumption of the ester substrate (100 −□), increased under high pressure. Pressure was much effective for the hydrolysis and, therefore, f_a slightly decreased under elevated pressure. At higher concentration of the nucleophile, ester hydrolysis was promoted but acyl transfer was relatively depressed, and as a result the total consumption of the ester slightly decreased at higher nucleophile concentrations. These were observed both at 0.1 and 300 MPa.

These data indicate that the activation volume for the k_{cat} and/or k_{cat}/K_m of ester hydrolysis by subtilisin is negative and that for the aminolysis reaction, which includes contributions from both the binding of the amine acceptor and the acyltransfer reaction, is also negative.

Discussion

Hydrolysis: We have shown that the nonspecific digestion of casein and bovine serum albumin by subtilisin became optimum at mildly high pressure,[34] and explained the result as follows: The pressure-induced deformation of the higher-order structure of the substrate proteins facilitated the digestions at such pressure. However, extremely high pressure

Table 1. Effect of Pressure on the Subtilisin-Catalyzed Aminolysis (Transacylation) Reaction for FuaPheOMe + $GlyNH_2$[a]

Pressure	Consumption of	% Amount of		f_a[b]
MPa	FuaPheOMe (%)	FuaPhe	FuaPheGlyNH$_2$	%
0.1	83.7	53.3	30.4	36.3
100	93.7	58.1	35.6	38.0
200	96.3	62.7	33.6	35.0
300	97.1	67.4	30.5	31.4
400	99.4	67.6	31.9	32.0

a) [E] = 0.36 μM, [FuaPheOMe] = 1 mM, [$GlyNH_2$] = 0.1 M, pH 8.5, 10% DMSO. After reaction for 2 h at 37 °C. b) f_a = (amount of peptide formed)/(total consumption of the ester).

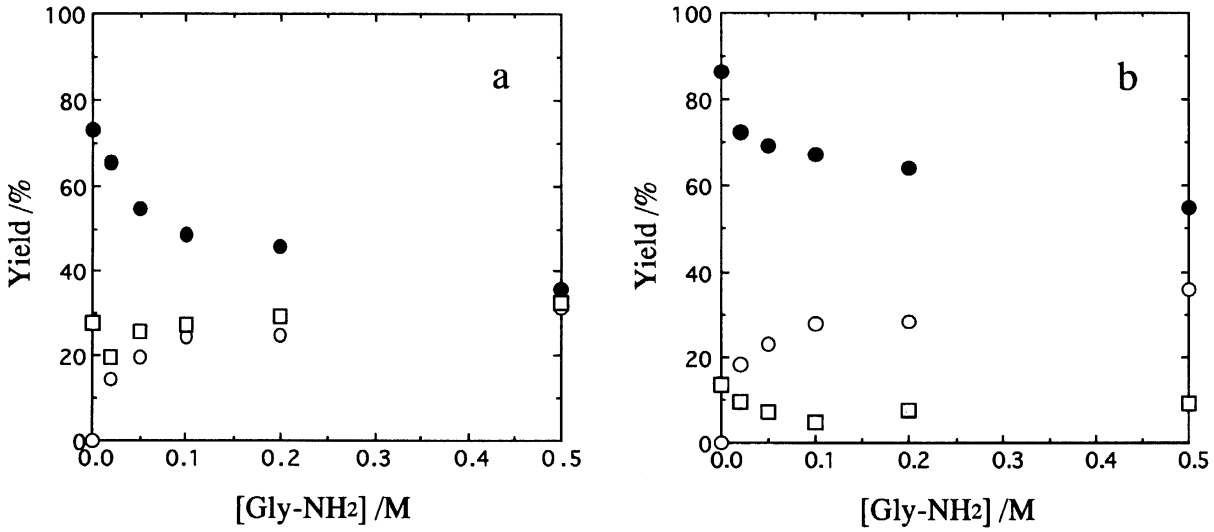

Fig. 5. Effect of pressure on the product distribution of the aminolysis (transacylation) reaction of FuaPheOMe and $GlyNH_2$ catalyzed by subtilisin at various concentrations of $GlyNH_2$. Product distribution after 2 h reaction at pH 8.5 (0.2 M Tris) 37 °C. [FuaPheOMe] = 1 mM, [enzyme] = 0.16 μM. DMSO 10%. ○, % productions of Fua–Phe–Gly–NH_2, ●, that of Fua–Phe; □, that of the remained Fua–Phe–OMe. a, at 0.1 MPa; b, at 300 MPa.

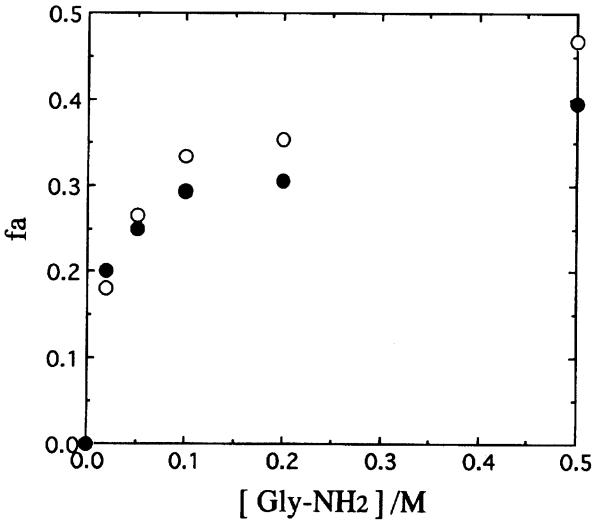

Fig. 6. Effect of pressure on the aminolysis fraction (f_a) of subtilisin-catalyzed reaction of FuaPheOMe and $GlyNH_2$. ○, fraction of aminolysis at 0.1 MPa; ●, that at 300 MPa. Conditions are as in Fig. 5.

(400 MPa or more) hindered the proteolysis. This might occur not only because the pressure-induced (reversible) conformation change of the enzyme reduced the activity, but also due to the intrinsic positive reaction volume for the substrate (polypeptide) binding.

The volumetric parameters for several proteolytic enzymes have been measured. Some of the results are summarized in Table 2, in terms of a classification on the sign of the $\Delta V_{(1/K_m)}$ and $\Delta V^{\ddagger}_{(k_{cat})}$, together with some parameters reported by other investigators.[37,38] The volumetric parameters for subtilisin-catalyzed hydrolysis of peptide substrates obtained in the present study falls in the same class as the ester substrate for trypsin. Though the rate-limiting step in the hydrolysis of normal amide substrate by serine proteases is considered to be in the acylation step, that for the activated anilide substrate such as the present ones is much shifted to deacylation step and has some characteristics similar to the ester substrate, especially in the cases of those with highly specific subsite interactions such as subtilisin. Furthermore, subtilisin shows a rather fast deacylation rate[39] and the rate-limiting character in ester substrates is somehow ambiguous. Activated anilides and normal esters have more or less similar characteristics in the reaction with this enzyme. The roughly estimated activation volume for ester hydrolysis from the study of peptide formation described above also fit this criterion.

Makimoto et al.[40] studied the effects of pressure on the chymotrypsin-catalyzed hydrolysis of Suc–Ala–Ala–Ala–*p*NA and reported $\Delta V_{(1/K_m)}$ and $\Delta V^{\ddagger}_{(k_{cat})}$ values as −5 and +15 ml mol^{-1}, respectively. Both of the parameters showed opposite signs to the present results for the subtilisin reaction. These differences might be related to the different sub-site interactions and the difference in the rate-limiting characters of these two serine proteases. The difference in the specificity level of the substrates, especially the existence of the hydrophobic residue at P_1 site and the Pro residue at P_2 site in our substrate might also influence the result. Matta & Andracki[39] stressed the important contribution of the physical step (change in enzyme conformation) to the rate limiting of subtilisin reaction, especially for specific substrates. Such a conformational contribution in the volumetric profiles of the enzyme reaction was reported in several examples,[34,41,42] though the presently observed volumetric values are smaller than those reported for these instances.

Aminolysis: In the acyltransfer reaction, a promotion of the hydrolytic fraction was observed at higher concentration of the nucleophile. This indicates the existence of some additional hydrolytic processes, in which $GlyNH_2$ can act as a

Table 2. Classification of the Volumetric Parameters for the Reactions of Some Proteolytic Enzymes

Substrate & parameter	Sign of the volumetric parameter		
	negative	near zero	positive
Amide or anilide			
$\Delta V_{(1/K_m)}$	Thermolysin Chymotrypsin	Chymotrypsin Neutral protease from B. sub. Leucine aminopeptidase	Trypsin Subtilisin Carboxypeptidase Y,A,P,W
$\Delta V^{\ddagger}_{(k_{cat})}$	Subtilisin Thermolysin Neutral protease from B. sub. Carboxypeptidase A,P,W Leucine aminopeptidase		Trypsin Chymotrypsin Carboxypeptidase Y
Ester			
$\Delta V_{(1/K_m)}$		Chymotrypsin	Trypsin Carboxypeptidase Y,P,W
$\Delta V^{\ddagger}_{(k_{cat})}$	Chymotrypsin Trypsin Subtilisin[a)] Carboxypeptidase Y,P,W		

a) Estimated for k_{cat}/K_m.

general base or nuclephilic catalysis. In this case the $GlyNH_2$ molecule might be bound on the acylated enzyme to form a ternary complex comprising acyl-enzyme, nucleophile, and water, just as proposed for the reaction catalyzed by carboxypeptidase Y[43] but it must be a kind of nonproductive binding mode. Then the increase of $GlyNH_2$ concentration promotes the hydrolysis and, as a result, an apparent "substrate inhibition" behavior was observed in the aminolysis reaction with respect to $GlyNH_2$.

Even under extremely high pressure, the enzyme can still work well, but the acceleration of the hydrolytic process, especially with respect to this nucleophile assisted hydrolysis, was much larger and hence the fraction of the peptide formation was reduced slightly. For the practical point of view, an elevation of pressure and a depression of the attack by water as well will give a preferable result for peptide formation, which can be realized by introducing organic solvent coupled with a high pressure reactor, as proposed by Kim and Dordick.[30] An attempt to develop such a system is in progress also in our laboratory.

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