Effect of Protease Digestion and Dephosphorylation on High Emulsifying Properties of Hen Egg Yolk Phosvitin

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The emulsifying properties, particularly the emulsion stability, of phosvitin was found to be higher than those of other food proteins. The emulsifying activity and emulsion stability were greatly decreased by protease and phosphatase treatment. The protease digestion of phosvitin resulted in the peptide cleavage of large fragment (a highly phosphorylated core region, 50 to 210 peptide) and small fragments (N-terminal 1 to 49 and C-terminal 211 to 217 peptides). The large fragment lacking the small fragments did not show the excellent emulsifying properties, suggesting that small fragments of protein moiety play an important role in emulsifying properties. On the other hand, the effect of phosphatase treatment showed that electrostatic repulsive force of phosphate in phosvitin has a significant affect on its emulsifying properties and that the protein moiety with abundant phosphorylated residues is also considered to be essential for the high emulsifying properties.

Keywords: *Phosvitin, emulsifying property, chymotrypsin, pepsin, trypsin, phosphatase.*

INTRODUCTION

Phosvitin is a processing product of a much larger hepatic precursor protein, vitellogenin, which has been isolated from the egg yolk of vertebrates (van het Schip et al., 1987; Byrne et al., 1989; Goulas et al., 1996b). This egg yolk phosphoglycoprotein, phosvitin, is a highly phosphorylated serine-rich glycoprotein containing about 10% phosphorus and 6.5% carbohydrates (Taborsky et al., 1967) and comprises ∼7% of the yolk protein and ∼80% of the yolk protein-bound phosphorus (Stadelman and Cotterill, 1977). Phosvitin is one of the major proteins present in the granule of egg yolk, and it comprises 16% of the weight of the egg yolk granules, which constitute 22% of yolk solids (Burley and Vadhera, 1989). Phosvitin has been fractionated into α and β components of different phosphorus contents and molecular weights (Abe et al., 1982). Recent genetic analysis elucidated that the 217 residue phosvitin contains a core region of 99 amino acids consisting of 80 serine residues grouped, of a total of 124 serines, in runs interspersed by arginines, lysines, and asparagines (van het Schip et al., 1987). It is interesting that this highly phosphorylated protein is found in egg yolk. This suggests that phosvitin has an affinity for lipids to form an excellent emulsion during the emulsification (Kato et al., 1987). This protein seems to resemble a polyelectrolyte and has a flexible conformation (Gizzuti et al., 1970). Egg yolk proteins are often used as food ingredients because of their excellent emulsifying properties (Baldwin, 1986). However, it was unclear which proteins were involved in the excellent emulsifying properties. Kato et al. (1987) have reported that phosvitin showed excellent emulsifying properties. However, the molecular mechanism of the high emulsifying properties remains to be solved. In this paper the effects of protease digestion and dephosphorylation of phosvitin were investigated to elucidate the molecular mechanism of high emulsifying properties.

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MATERIALS AND METHODS

Materials. Bovine α-chymotrypsin (52 units/mg), pepsin (2345 units/mg), trypsin (12 600 units/mg), phosphatase (0.8 unit/mg), β-casein, bovine serum albumin, perchloric acid, ammonium molybdate, and amidol were purchased from Sigma Chemical Co. (St. Louis, MO). Unless otherwise stated, all reagents used in this study were of reagent grade.

Preparation of Phosvitin. Phosvitin was prepared from fresh egg yolk according to the method of Mecham and Olcott (1949). The phosvitin was further purified by ion exchange chromatography on a DEAE-Sephadex A-50 column (3.0 × 15 cm) equilibrated with 20 mM Tris-HCl buffer, pH 7, and eluted by a linear salt gradient (0−0.6 M NaCl in the equilibrated buffer). The single peak eluted in a salt concentration of 0.4−0.5 M was collected and lyophilized after dialysis against deionized water.

Preparation of Acid-Precipitated Soy Protein. Acid-precipitated soy protein (APP) was prepared according to the method of Iwabuchi and Yamauchi (1987). A sample of defatted meal (100 g) was extracted once with 2 L of 0.03 M Tris-HCl buffer, pH 8, containing 10 mM 2-mercaptoethanol (2-ME) at 20 °C. After centrifugation, the supernatant was acidified to pH 4.8 with 2 N HCl and then centrifuged. The precipitated protein was dissolved in water at 4 °C and the pH adjusted to 8. After centrifugation (8000*g*), the clear supernatant was dialyzed against distilled water for 24 h at 4 °C and then freeze-dried.

Preparation of Protease-Digested Phosvitin. Freeze-dried phosvitin (180 mg) was suspended in 12 mL of distilled water to a final concentration of 15 mg/mL, and the pH of the solution was adjusted to 8.0 with NaOH prior to the addition of either trypsin or chymotrypsin or to pH 2.5 with HCl prior to the addition of pepsin. Three enzymes were added to the phosvitin at the enzyme/substrate ratio 1:100 (w/w) (Goulas et al., 1996a). Incubating temperatures were maintained at 30 °C for pepsin and trypsin and at 37 °C for α-chymotrypsin digestion. Reactions were allowed to proceed for 24 h; subsequently, digestions were arrested by heating the digested samples at 100 °C for 3 min. The digested mixture was centrifuged (8000*g*) to precipitate a small amount of undigested

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protein. The supernatant was dialyzed against distilled water for 24 h at room temperature.

Phosphatase Treatment of Phosvitin. To 10 mL of 0.1% phosvitin solution in 10 mM acetate buffer, pH 4.8, was added 100 μg of phosphatase (acid type, Sigma Chemical Co., type I). The phosphatase reaction was done at 37 °C for 15 h, and the dephosphorylated phosvitin thus obtained was lyophilized after dialysis against deionized water.

Alkaline Treatment of Phosvitin. Phosvitin solution (0.05%) in 0.25 N NaOH was incubated at 35 °C for 24 h. After the reaction, a sample was dialyzed against deionized water and then lyophilized.

Phosphorus Measurement. An appropriate amount of the material (solid or solution) under examination was placed in a micro-Kjeldahl flask (hard glass), and 2.2 mL of perchloric acid and a small piece of porous pot (to prevent bumping) were added. The flask was heated over a microburner until the contents had become colorless. After the flask was cooled, its contents were thoroughly rinsed into a 25 mL volumetric flask, 2 mL of amidol, 1 mL of molybdate, and water to 25 mL were added, and the extinction coefficient was determined between 5 and 30 min later (Allen et al., 1940).

Gel Filtration by High-Performance Liquid Chromatography (HPLC). Gel filtration was done by HPLC in a TSK gel G-3000 SW column (Tosoh Co., Tokyo, Japan, 0.75 × 30 cm). Each sample solution (0.2% in 200 mM sodium phosphate buffer, pH 6.9) was put into the column at a flow rate of 0.5 mL/min, using the same buffer as eluent. The chromatogram was depicted by monitoring the effluent at 220 nm.

SDS−Polyacrylamide Gel Electrophoresis (SDS−PAGE). SDS−PAGE of samples was done by using the method of Laemmli (1970) with 15% acrylamide separating gel and 5% acrylamide stacking gel containing 0.1% SDS. Samples (20 μL, 0.2%) were prepared in a Tris−glycine buffer at pH 8.8 containing 1% SDS. Electrophoresis was done at a constant current of 10 mA for 4 h in Tris−glycine buffer containing 0.05 M Tris, 0.37 M glycine, and 0.5% SDS. After electrophoresis, the gel sheets were stained for protein staining with 0.05% Coomassie blue in a solution of 0.1 M aluminum nitrate/25% 2-proponol/10% acetic acid/1.0% Triton X-100, prepared according to the method of Hegenauer et al. (1977). The destaining solution was 7% acetic acid. Bromophenol blue was used to monitor the progress of protein separation.

Analysis of Amino Acids for Protease-Digested Phosvitin. Fragments obtained for amino acid analysis were hydrolyzed in 6 N HCl for 22 h at 105 °C, then dried and derivatized by using a Waters Pico Tag work station. Hydolysates were analyzed using a Waters Pico Tag amino acid analysis HPLC system, equipped with a Pico Tag "hydrolysate column" operated with the manufacturer's buffer solutions. Data were evaluated on 805 data station analysis software.

Measurement of Emulsifying Properties. The emulsifying properties of sample solutions were determined according to the method of Pearce and Kinsella (1978). To prepare emulsions, 1.0 mL of corn oil and 3.0 mL of protein solution (0.2%) in 0.1 M phosphate buffer, pH 7.0, were shaken together and homogenized in an Ultra Turrax homogenizer (Hansen and Co., Germany) at 12 000 rpm for 1 min at 20 °C. A 50 μL sample of emulsion was taken from the bottom of the container at different time intervals and diluted with 5 mL of 0.1% SDS solution. The absorbance of the diluted emulsion was then determined at 500 nm. The relative emulsifying activity was determined from the absorbance immediately measured after the emulsion formation. The emulsion stability was expressed as the half-time of the initial turbidity of the emulsion (Kato et al., 1991).

RESULTS AND DISCUSSION

Figure 1 shows the elution pattern of phosvitin by ion exchange chromatography on DEAE-Sephadex A-50. The fractionated phosvitin (fraction 65−85) was collected. Further purification seems to be difficult, because of some heterogeneity resulting from different phosphorylation (Kato et al., 1987). Therefore, the pooled fractions (65−85) were used in the experiments.

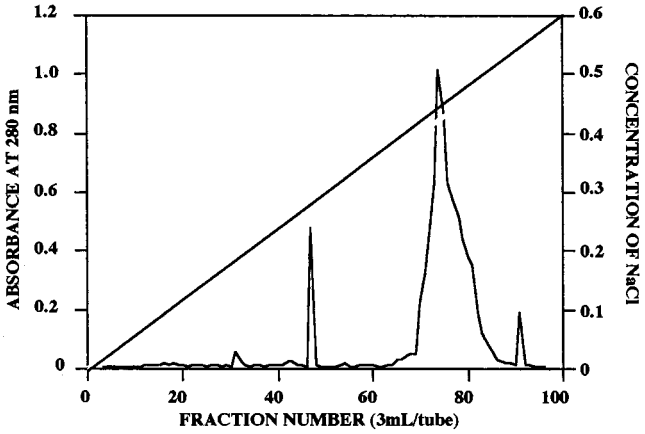

Figure 1. Elution pattern of phosvitin on DEAE-Sephadex A-50 column.

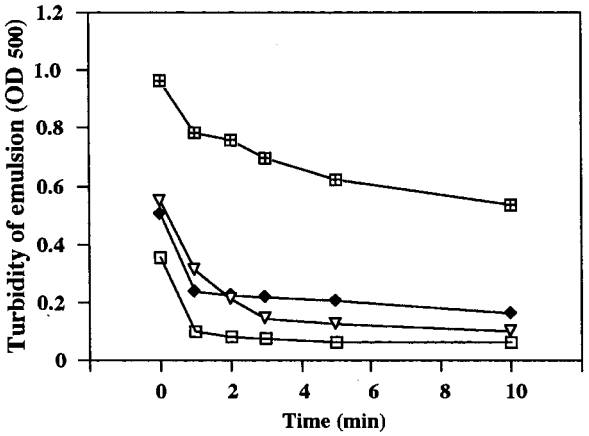

Figure 2. Comparison of emulsifying properties of native phosvitin (⊞), bovine serum albumin (▽), β-casein (◆), and soy protein (□).

Table 1. Comparison of Emulsifying Activities and Emulsion Stabilities of Various Food Proteins[a]

protein	emulsifying activity (OD_{500})	emulsion stability (min)
phosvitin	0.979 ± 0.054	>10
bovine serum albumin	0.535 ± 0.011	1.3 ± 0.10
β-casein	0.500 ± 0.002	0.6 ± 0.11
soy protein	0.352 ± 0.001	0.5 ± 0.10

[a] Values are mean ± standard deviation, $n = 3$.

Comparison of the emulsifying properties of various food proteins, β-casein, serum albumin, and soy protein, with phosvitin was done as shown in Figure 2. The results are summarized in Table 1. As shown in Figure 2 and Table 1, phosvitin showed the highest emulsifying properties, especially emulsion stability. To estimate the effect of protein moiety of phosvitin on the excellent emulsifying properties, digestions with α-chymotrypsin, pepsin, and trypsin were attempted. The gel filtration pattern by HPLC showed that three protease digests of phosvitin commonly gave peaks with a slightly slower retention time (large fragments) and much slower retention time (small fragments) than those of native phosvitin, as shown in Figure 3. When standard proteins (ovalbumin, β-lactoglobulin, and lysozyme) were used as markers, they showed the inconsistency of the elution on the HPLC column with phosvitin and

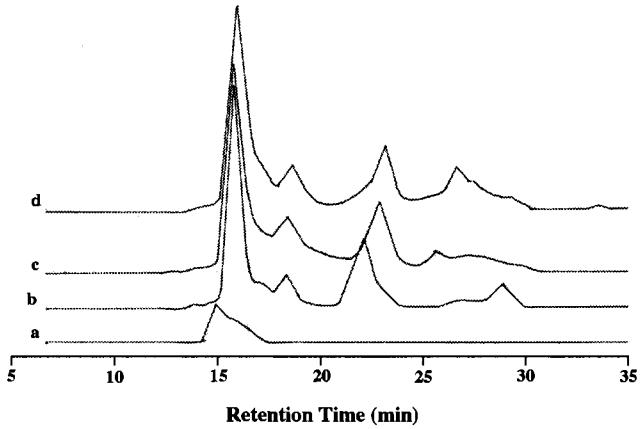

Figure 3. HPLC pattern of (a) native phosvitin, (b) trypsin, (c) pepsin, and (d) α-chymotrypsin digests.

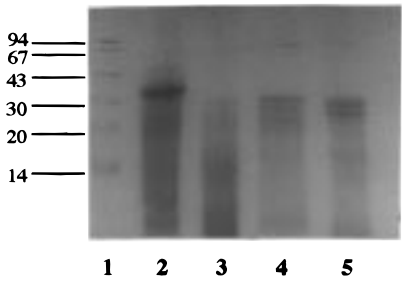

Figure 4. SDS−PAGE patterns of protease-digested phosvitin: (lane 1) molecular marker; (lane 2) native phosvitin; (lane 3) α-chymotrypsin digest; (lane 4) pepsin digest; (lane 5) trypsin digest.

its protease-digested peptides, which might be due to the conformational differences: the markers have globular conformation, whereas phosvitin has expanded conformation.

A large number of negative charges on the phosphate residues are contained in phosvitin, which cause the phosvitin to expand its peptide chain, thereby eluting with faster retention time. The native phosvitin was not eluted to give the height of peak in proportion to the amount of the protein injected to the column. It would seem that a considerable amount of the protein was adsorbed to the column.

To confirm the molecular size of the digested phosvitin, SDS−PAGE analysis was done as shown in Figure 4. The molecular size of the phosvitin ranges from 35 000 to 40 000 (Mok et al., 1961; Cook, 1968), whereas phosvitin was cleaved by protease into two distinct peptides that had molecular weights of ∼29 000 and ∼28 000. The former may be the 50−217 and the latter may be the 50−210 peptide. To determine the amino acid composition of the protease (α-chymotrypsin)-digested fragments, the gel filtration was carried out on a G-75 Sephadex column (data not shown) and then two fragments, the large and small fragments, were collected. Subsequently, amino acid analysis of protease-digested phosvitin was done as shown in Table 2.

It has also been recently reported that chymotryptic digestion of phosvitin resulted in a large peptide fragment, Ala-50−Trp-210, and the smaller N-terminal fragment Gly-4−Gln-49 (Goulas et al., 1996a). The data obtained here show that the large fragment (core region) is the 50−210 peptide and the smaller fragment (N-terminal) is the 1−49 peptide. We could not analyze the smallest fragment (C-terminal), 211−217, because of the difficulty of fractionation.

Table 2. Amino Acid Analysis of α-Chymotrypsin-Digested Phosvitin Fragments[a]

	large fragment		small fragment	
amino acid	standard mol %	analyzed mol %	standard mol %	analyzed mol %
Asx	(4.97)	**6.55**	(10.20)	**9.65**
Glx	(1.24)	**3.67**	(14.29)	**16.58**
Ser	(66.46)	**59.51**	(32.65)	**31.38**
Gly	(1.86)	**2.90**	(2.04)	**3.26**
His	(7.45)	**6.97**	(0.00)	**2.66**
Arg	(5.59)	**5.21**	(2.04)	**4.53**
Thr	(0.00)	**0.90**	(8.16)	**6.80**
Ala	(1.24)	**2.16**	(10.20)	**10.02**
Pro	(0.00)	**1.27**	(6.12)	**4.49**
Tyr	(0.00)	**0.15**	(0.00)	**0.56**
Val	(1.24)	**1.51**	(2.04)	**3.29**
Met	(0.00)	**0.64**	(2.04)	**0.59**
Ile	(0.62)	**0.52**	(0.00)	**0.95**
Leu	(1.86)	**1.49**	(0.00)	**0.84**
Phe	(0.00)	**0.36**	(2.04)	**1.61**
Lys	(6.83)	**5.81**	(8.16)	**6.40**
Trp	(0.62)	**ND**	(0.00)	**ND**

[a] Standard values of mol % of amino acid residues in phosvitin are given in parentheses. Analyzed values of mol % of amino acid residues in the large and small fragments are given in boldface. ND indicates that tryptophan was not determined. Large fragment indicates Ala50−Trp210 peptide and small fragment indicates Ala1−Gln49 peptide.

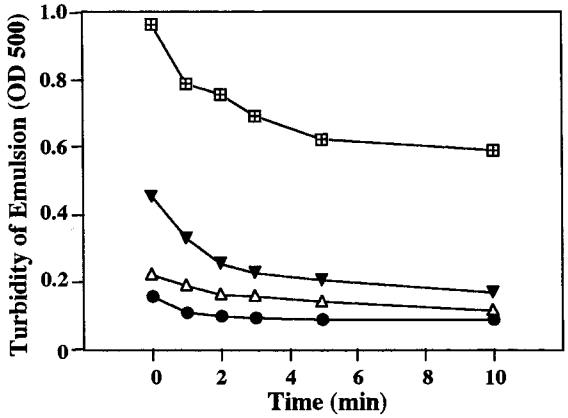

Figure 5. Emulsifying properties of native phosvitin (⊞), pepsin (▼), trypsin (△), and α-chymotrypsin (●) digests.

The emulsifying properties of the protease-digested phosvitin were greatly decreased as shown in Figure 5. The peptide cleavage caused dramatic decrease in the emulsifying properties, suggesting that the protein moiety is important for the emulsifying properties. The smaller fragments of protease-digested phosvitin at the N- and C-terminal regions were found to be rich in hydrophobic residues and behaved as anchors to oil droplets. The N- and C-terminal parts of the protein moiety may be essential for the high emulsifying property of the protein. A composition rich in alanine is indicative of N-terminal origin because alanine residues are found exclusively in the N-terminal part of the protein (Goulas et al., 1996a). If it is supposed that the N-terminal fragment (1−49) forms an α-helix structure, five alanine residues are localized on the same plane, thereby forming a strong hydrophobic plane. In addition, the C-terminal fragment forms a β-strand comprising four hydrophobic residues. On the other hand, the highly phosphorylated core region is hydrophilic. Thus, phosvitin has an amphiphilic structure to express surface activity.

Table 3. Determination of Surface Tension and Interfacial Tension of Native Phosvitin, α-Chymotrypsin (CDP), Pepsin (PDP), and Trypsin (TDP)-Digested Phosvitin[a]

surface tension	dyn/cm	interfacial tension	dyn/cm
air−water	72.6 ± 0.23	oil−water	36.1 ± 0.07
phosvitin	64.1 ± 0.03	phosvitin	30.8 ± 0.05
CDP	67.4 ± 0.21	CDP	33.6 ± 0.14
PDP	68.1 ± 0.12	PDP	32.4 ± 0.09
TDP	67.4 ± 0.27	TDP	33.5 ± 0.11

[a] Values are mean ± standard deviation, $n = 3$.

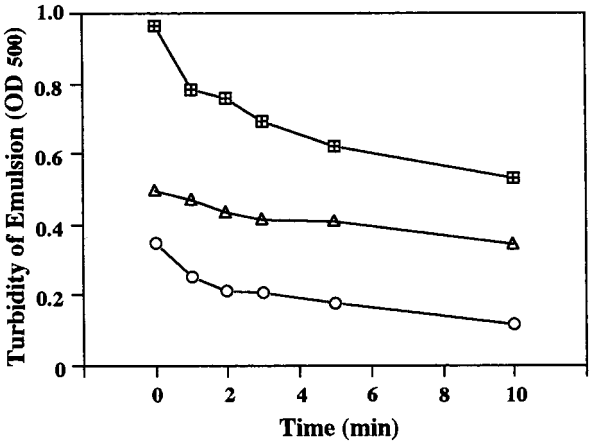

Figure 6. Emulsifying properties of phosvitin (⊞), phosphatase-digested phosvitin (△), and alkali-treated phosvitin (○). Phosphatase digest was done for 10 h, and alkaline treatment was done in 0.25 N NaOH for 24 h at 30 °C.

Table 3 shows the air−water surface tension and oil−water interfacial tension of native phosvitin and protease-digested phosvitin. The increase in surface and interfacial tensions of protease-digested phosvitin was observed as a result of loss of amphiphilic property, because of the absence of hydrophobic N and C termini, which probably orient to the air and oil interfaces. Damodaran et al. (1996) suggested that the C-terminal stretch of ∼17 amino acid residues in phosvitin has the possibility to attach itself to the interface.

To further investigate the structural factors governing the excellent emulsifying properties of phosvitin, the effects of the dephosphorylation were studied. As shown in Figure 6, the emulsifying activity and emulsion stability were also decreased by enzymatic and alkaline dephosphorylation to 48 and 100%, respectively. These data suggest that the electrostatic repulsive force of phosphate in phosvitin significantly affects its emulsifying properties by stabilizing the emulsion droplet (Petrowski, 1976).

The amphiphilic properties and the flexibility of the protein molecule are governing factors of emulsifying properties (Kato et al., 1995). The phosphate groups take part in giving the amphiphilic properties to the molecule in the emulsion system (Seguro and Motoki, 1990). In addition, the repulsion of negative charges brings about the extended conformation of protein, resulting in a suitable form to entrap oil droplets. Phosvitin has 124 serine residues and most are esterified to phosphate (Goulas et al., 1996a,b; Byrne et al., 1989). The relationship between the degree of dephosphorylation and emulsifying properties was determined to explain the importance of phosphate residues in the emulsion system.

After phosphatase digestion of phosvitin, the phosphate groups released for 1, 5, and 10 h were 21, 32, and 48%, respectively as shown in Figure 7. The maximum release of phosphate residues was found to be 40% by phosphatase treatment for 10 h. These 40% phosphate residues may be exposed at the surface of the molecule, whereas other phosphate residues may be buried in the interior of molecule. The dramatic decrease in the emulsifying properties was observed by the removal of negatively charged phosphate residues. It seems that the polyanion on the surface of oil droplets in an emulsion inhibits the coalescence of oil droplets by their repulsive electrostatic force, thus stabilizing the emulsion (Kato et al., 1987).

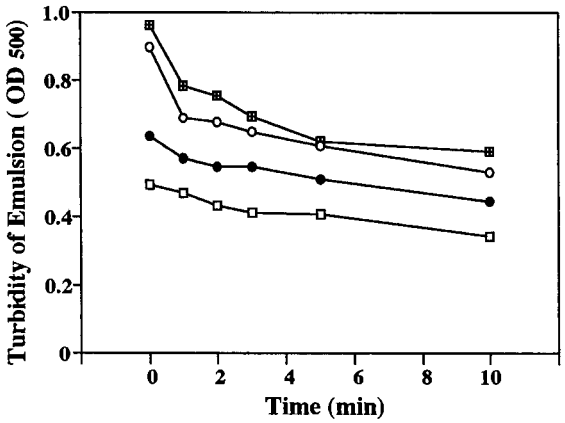

Figure 7. Relationship between the degree of dephosphorylation and emulsifying properties: native phosvitin (⊞) digested by phosphatase for 1 h (○), 5 h (●), and 10 h (□). The phosphate residues released during dephosphorylation by phosphatase digestion for 1, 5, and 10 h were 21, 32, and 48%, respectively.

In conclusion, the emulsifying properties of phosvitin are the best among other food proteins. Modification of the phosvitin by protease and phosphatase indicated that the N- and C-terminal peptide portions and phosphate residues are essential in the excellent emulsifying properties of phosvitin.

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