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Reduction of Antigenicity of Cry j 1, a Major Allergen of Japanese Cedar Pollen, by Thermal Denaturation

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The soluble aggregates of Cry j 1, a major allergen of Japanese cedar pollen, were formed without any coagulates during heat treatment at acidic pH 5, as shown in HPLC and SDS−PAGE patterns. A remarkable change in the CD spectrum was observed between native and heat-denatured Cry j 1 at a linear rate of 1 °C/min from 40 to 90 °C. The negative peak of native Cry j 1 at 222 nm was moved to 218 nm, suggesting the transition of an α-helix to β-structure during heat denaturation. The increase in β-structure was also observed during heat denaturation by monitoring the fluorescence with Thioflavin T. These results suggest that Cry j 1 forms intermolecular cross-β-structure between denatured proteins during heating at 90 °C. The antigenicity of Cry j 1 detected by dot-blotting was greatly diminished during heating at a linear rate of 1 °C/min from 40 to 90 °C without any coagulates. These results suggest that IgE epitopes exposed on the molecular surface of Cry j 1 was buried inside soluble aggregates through intermolecular β-structure formed by heating.

KEYWORDS: Allergen of Japanese cedar pollen; Cry j 1; antigenicity; heat induced soluble aggregate; cross-β-sheet

INTRODUCTION

More than 20% of the Japanese population suffers from Japanese cedar pollinosis in early spring, which causes a serious social problem in Japan. Cry j 1 is one of the major immunoglobulin E (IgE)-binding allergens in Japanese cedar pollen. Cry j 1 consists of 353 amino acids and four possible N-linked glycosylation sites having the characteristic Asn-X-Ser/Thr motif (*1*, *2*). Recently, it has been reported that Jun a 1, an allergen of mountain cedar, similar to Cry j 1, is about 40 kDa in size, has an N-terminal amino acid sequence identical to that of Cry j 1 (*3*), and has four IgE epitope sites that have sequences similar to those of Cry j 1 (*1*). The high degree of sequence identity between Jun a 1 and Cry j 1 may explain the cross-reactivity among these plants. Despite this basic information, efficient therapies for cedar pollinosis have not established. We have reported that IgE epitopes of the major allergen of Japanese cedar pollen, Cry j 1, can be masked by the attachment of galactomannan using the Maillard reaction in dry heating at 60 °C in controlled humidity at 65% without the use of any chemicals between the ε-amino group in protein and the reducing-end carbonyl group in polysaccharide (*4*). Since the allergen−polysaccharide conjugates remarkably reduced the antigenicity of the cedar pollen allergen, it may provide the possibility to be used as an oral immune tolerance agent without any anaphylaxis (*5*).

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The alternative masking method of IgE epitopes of Cry j 1 should be investigated because it may be used as another immune tolerance agent without chemical modification. The most promising method to mask the IgE epitopes of Cry j 1 is the thermal denaturation without the production of coagulates. We have reported that ovalbumin forms easily soluble aggregates during heat denaturation through intermolecular cross-β-structure (*6*). Ovalbumin is a glycoprotein with N-glycosylation sites at Asn-292 and Asn-311 (*7*). The protein is found in the monoglycosylated form with a carbohydrate chain attached to Asn-292 only in egg whites (*7*). The cedar allergen Cry j 1 is also a glycoprotein with N-glycosylation sites at Asn-137, Asn-170, Asn-273, and Asn-333 (*1*). Therefore, the allergen protein seems to form soluble aggregates during heating in a manner similar to that of ovalbumin. Thus, this article describes the changes in Cry j 1 during heat denaturation. The soluble aggregates of Cry j 1 were formed without any coagula during controlled heat denaturation in a behavior similar to that of ovalbumin.

As previously reported (*6*), soluble protein aggregates may be formed by a common mechanism during heating. That is, the soluble aggregates are formed by cross-β-structure, where unfolded protein molecules are assembled through β-structure in a manner similar to that in amyloidogenic proteins. The β-structure of proteins buried inside is exposed by heat denaturation, and then the exposed β-structure is reconstituted between unfolded protein molecules during cooling. Thus, the soluble aggregate of cedar allergen, Cry j 1, also may be formed in a controlled heating state. It is well known that allergenic

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epitopes are exposed in the molecular surface. Therefore, the epitope region may be masked by the molecular assembly such as the soluble aggregate induced by heat denaturation. This article describes the masking of the allergenic structure of Japanese cedar allergen, Cry j 1, by controlled heat denaturation.

MATERIALS AND METHODS

Isolation of Major Allergen Cry j 1 from Japanese Cedar Pollen. Crude extracts of cedar pollen allergen were extracted from 100 g of cedar pollen in 2 L of 0.1 M phosphate buffer, pH 8.0, containing 0.14 M NaCl and 0.01 M KCl. The mixtures of cedar pollen and extract solution were stirred gently for 1 h. The pollen extract solution was separated by centrifugation (15,000*g*, 30 min). The supernatant was further filtered to remove contaminant pollen. Then solid ammonium sulfate was added to the extracts with stirring to attain 80% saturation (561 g/L). After stirring overnight at 4 °C, the precipitate was collected by centrifugation (18,500*g*, 40 min). The precipitate was dissolved in distilled water and then dialyzed against distilled water. The dialyzates were lyophilized and stored as crude extracts of Cry j 1. The crude extracts were dissolved in 0.01 M acetate buffer (pH 5.0) and then diluted to five times with the same buffer. The sample solution was put on CM-Toyopearl equilibrated with the same buffer, and the column was washed with the same buffer until the washing solution was free from proteins. The adsorbed proteins were eluted gradually with a linear gradient from 0.05 to 0.3 M NaCl in the same acetate buffer. The main fraction detected by absorbance at 280 nm was collected and dialyzed against distilled water, then lyophilized.

Preparation of the Soluble Aggregate of Heat-Denatured Cry j 1. A 0.1% Cry j 1 solution in 10 mM acetate buffer (pH 5.0) was heated at a linear rate of 1 °C /min from 40 to 90 °C. A transparent soluble aggregate was observed in the controlled conditions without any coagula. After cooling at room temperature, the soluble aggregate was used in the experiments.

HPLC of Soluble Aggregate of Heat-Denatured Cry j 1. The amount of soluble aggregate of heat-denatured Cry j 1 was followed by HPLC using a TSK gel G-3000 SW column (Tosoh Co., Tokyo, 0.75 × 30 cm). A 0.1% Cry j 1 solution in 10 mM acetate buffer (pH 5.0) was heated at a linear rate of 1 °C /min from 40 to 90 °C. Each Cry j 1 solution was put into a HPLC column at a flow rate of 0.5 mL/min, using 100 mM sodium phosphate buffer (pH 7.0). The HPLC patterns were monitored using the absorbance at 280 nm at a given temperature. The peak of the higher molecular size emerged during the heating of the Cry j 1 solution.

Sodium Dodecyl Sulfate−Polyacrylamid Gel Electrophoresis (SDS−PAGE). SDS−PAGE was carried out using the method of Laemmli (*8*) with a 12.5% acrylamide separating gel and a 5% acrylamide stacking gel containing 0.1% SDS. The formation of the soluble aggregate of heat-denatured Cry j 1 was followed by SDS−PAGE. A 0.1% Cry j 1 solution in 10 mM acetate buffer (pH 5.0) was heated at a linear rate of 1 °C/min from 40 to 90 °C, and then a 0.1% sample (10 μL) was prepared in Tris-glycine buffer at pH 8.8 containing 1% SDS. Heat-denatured Cry j 1 was treated in the absence and presence of 2-mercaptoethanol, and SDS−PAGE carried out. Electrophoresis was done at a current of 10 mA for 0.5 h on stacking gel and 20 mA for 1 h on separating gel in electrophoretic Tris-glycine buffer (pH 8.8) containing 0.1% SDS. After electrophoresis, the gel sheets were stained with 0.2% Coomassie brilliant blue R250 and destained with 10% acetic acid containing 20% methanol.

Circular Dichroism (CD) Analysis. CD measurements were carried out with a Jasco spectropolarimer (J-600, Tokyo) at 25 °C for a final concentration of 1 mg/mL in 10 mM acetate buffer (pH 5.0) in the cell with 1.0 mm path length using the ellipcíticity at 200−260 nm. An average of two to four consecutive scans was taken for each sample. Each spectrum was normalized for protein concentration and represented as the mean residue ellipticity (degree cm^2/dmol). The protein concentration was estimated from the absorption at 280 nm using $E_{1\,\mathrm{cm}}^{1\%}$ = 13.7. Thermal unfolding curves were automatically monitored by the ellipticity at 222 nm from 40 to 90 °C.

Fluorescence Measurement of Heat-Denatured Cry j 1 with Thioflavin T. Thermal denaturation of Cry j 1 was carried out in 10 mM acetate buffer, pH 5.0. Soluble heat-induced Cry j 1 aggregates were prepared as follows. A 0.2 mL 0.1% Cry j 1 solution was placed in a microtube of 1.5 mL and immersed in a water bath. The Cry j 1 solution was heated from 25 to 90 °C for 5 min at 5 °C intervals, the samples were placed into microtubes in an ice bath, and then fluorescence measurement were carried out at room temperature near 25 °C after the addition of 1.8 mL of thioflavin T (65 μM). Fluorescence measurements were carried out using a JASCO FP-6300 spectorofluorometer (Tokyo) in a ETC-273 temperature-controlled cell holder (Tokyo). Thioflavin T binding assay was performed by using a modification of the method of Levine (*9*). The fluorescence of thioflavin T was excited at 440 nm with a slit width of 5 nm, and the emission was measured at 482 nm with a slit width of 5 nm. To 0.2 mL of heat-denatured Cry j 1 solution was added to 1.8 mL of thioflavin T (65 μM) solution freshly prepared through a 0.2 μm filter before use to remove insoluble particles.

Human Sera of Cedar Pollinosis Patients. The antisera from four Japanese cedar pollinosis patients were provided by Dr. M. Muto, Medical School of Yamaguchi University, Japan.

Measurement of IgE Binding of Heat-Denatured Cry j 1 Soluble Aggregate. The binding of patient IgE antibody with heated Cry j 1 was measured by dot blotting. Dot blotting was done using the biotin−avidin method. Native Cry j 1 and soluble aggregates heated at a linear rate of 1 °C/min from 40 to 90 °C were diluted to 0.0005%, and then 10 μL of native and heated Cry j 1 was dotted onto the PVDF membrane, which was washed with methanol. Detection of IgE binding to Cry j 1 was carried out using biotin−avidin interactions. After the membrane was dried, it was incubated with blocking solution (0.5% BSA in PBS-Tween) for 1 h. Then the membrane was washed 3 times with PBS-Tween to remove excess BSA. The membrane was incubated overnight with patients' sera diluted to 1:20 in PBS-Tween. After the membrane was washed with 4 times PBS-Tween, 1/10,000 dilution of biotinylated antihuman IgE antibody was added to the membrane and incubated for 1 h. After the membrane was washed with 4 times PBS-Tween20, bound IgE antibodies were detected using 1:5,000 dilution of HRP-streptavidin and an ECL-Western blotting.

RESULTS AND DISCUSSION

The major allergen of Japanese cedar pollen, Cry j 1, formed soluble aggregates without any coagulates during the heat-treatment in acidic pH 5.0. The turbidity of heat-denatured Cry j 1 was not observed by the absorbance at 500 nm, although most protein are turbid during heating. Therefore, the formation of the soluble aggregate was followed by HPLC and SDS−PAGE. The HPLC patterns of native and heat-treated Cry j 1 are shown in **Figure 1**. The peak of unheated Cry j 1 eluted at the elution times of 24.5 and 25 min as an overlapped peak. The difference in molecular size is attributed to the degree of N-linked glycosylation (*10*). However, heating the Cry j 1 solution led to the formation of aggregates, as detected by HPLC. The lowering of the peak area may be due to partial adsorption to the column. **Figure 2** shows the SDS−PAGE patterns of native and heat-denatured Cry j 1 without 2-mercapto ethanol. The molecular size of native Cry j 1 is distributed 42−45 kDa, reflecting the degree of N-glycosylation of protein. However, the presence of higher molecular aggregates in the heated Cry j 1 was observed at the boundary between the separation and concentration gel as shown in **Figure 2**, suggesting the formation of the soluble aggregate. Furthermore, in **Figure 3**, the higher molecular weight aggregate in heat-denatured Cry j 1 was dissociated by reducing agent treatment to the same molecular mass bands as native Cry j 1. Therefore, it seems likely that the formation of the intermolecular disulfide bound is involved in the polymerization of Cry j 1 by heating.

To further investigate the molecular mechanism of the formation of soluble aggregates of heat-denatured Cry j 1, the structural

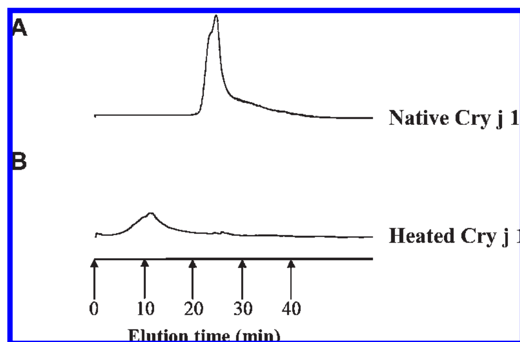

Figure 1. Gel filtration patterns by HPLC of Cry j 1 (**A**) and heated Cry j 1 (**B**). A 0.1% Cry j 1 native and heated solution was applied to the HPLC column. The peaks were detected by the absorbance at 280 nm.

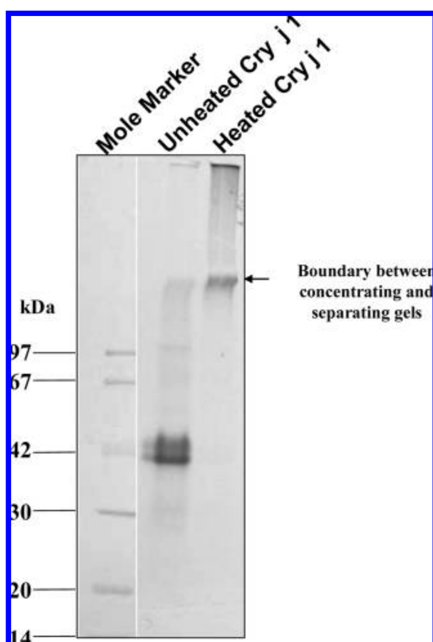

Figure 2. SDS−PAGE pattern of Cry j 1 heated at a linear rate of 1 °C/min from 40 to 90 °C. The SDS−PAGE pattern of Cry j 1 before and after heating.

changes were followed by using CD analysis during heat treatment at a linear rate of 1 °C/min from 40 to 90 °C. **Figure 4** shows the CD spectra of native and heated Cry j 1 at a linear rate of 1 °C/min from 40 to 90 °C. The heated Cry j 1 indicated characteristic CD curves, unlike general protein denaturation curves. The CD spectra of most proteins are generally shifted to the lower wavelength of the peak, less than 210 nm, during their heat denaturation. However, the CD curves of heat-denatured Cry j 1 showed the characteristic minimum absorption at 218 nm, suggesting increases in β-structure during heat denaturation. The tendency was further strengthened by cooling the heat-denatured Cry j 1 solution, suggesting a further increase in β-structure during cooling of the heat-denatured Cry j 1. These results reflect the increase in intermolecular β-sheet structure. **Figure 5** shows changes in the CD spectra of heat-denatured Cry j 1 in the presence of denaturant 4 M guanidine HCl. The minimal absorption at 218 nm in the CD spectrum of heat-denatured Cry j 1 was greatly diminished in the presence of chemical denaturant guanidine HCl, suggesting the destruction of intermolecular cross-β-structure.

These results suggest that Cry j 1 can form the soluble aggregate with the regulatory assemble structure via intermolecular

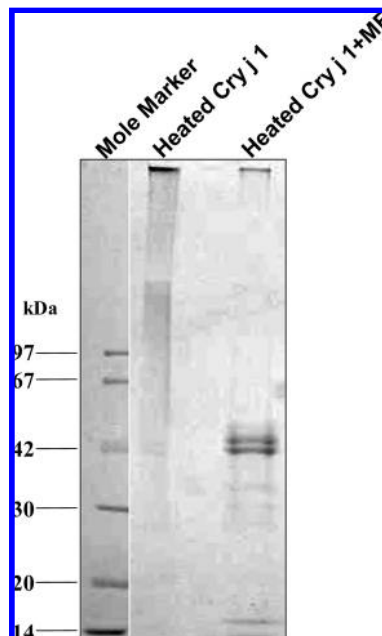

Figure 3. Changes in the SDS−PAGE pattern of heated Cry j 1 in the presence of mercaptoethanol. The heating was carried out at a linear rate of 1 °C /min from 40 to 90 °C. The reducing agent (mercaptoethanol) was added after heating.

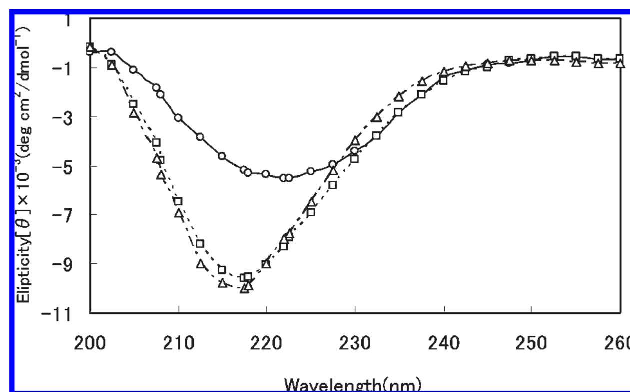

Figure 4. CD spectra of Cry j 1 on heating at 90 °C and the CD spectra between 200 and 260 nm of Cry j 1 before and after heating. The CD spectra were also measured for samples cooled to 40 °C immediately after heating to 90 °C. Room temperature (○-○), heating at 90 °C (□ - □), and cooling down after heating up (△ - △).

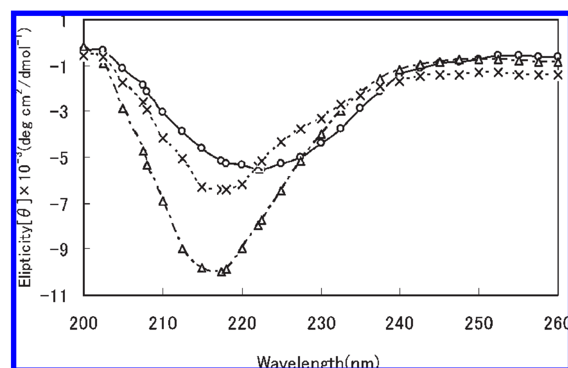

Figure 5. Changes in the CD spectra of Cry j 1 by heating at 90 °C and treating with guanidine hydrochloride after heating. Native Cry j 1 (○-○), heating at 90 °C (△ - △), and treated by guanidine hydrochloride after heating at 90 °C (× - ×). Heated Cry j 1 was treated with 4 M guanidine HCl after heating.

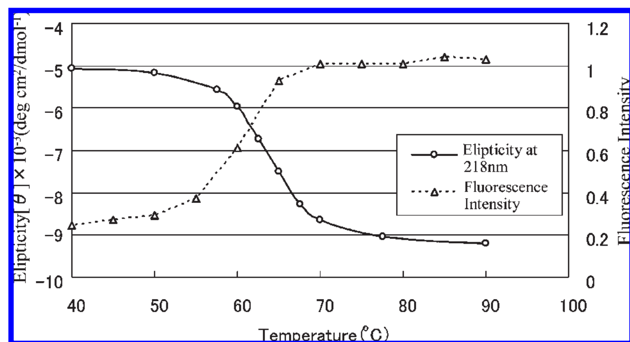

Figure 6. Thermal denaturation curves followed by CD at 218 nm and fluorescence intensity with thioflavin T during heat denaturation of Cry j 1. CD analysis of Cry j 1 was carried out during heating. Denaturation curves were obtained by following ellipticity at 218 nm from 40 to 90 °C. The fluorescence intensity of 0.1% Cry j 1 solution (0.2 mL) heated at a given temperature was diluted into 1.8 mL of 10 mM acetate buffer (pH 5.0) containing at 65 μM thioflavin T, and the fluorescence intensity was measured at 482 nm with excitation at 440 nm.

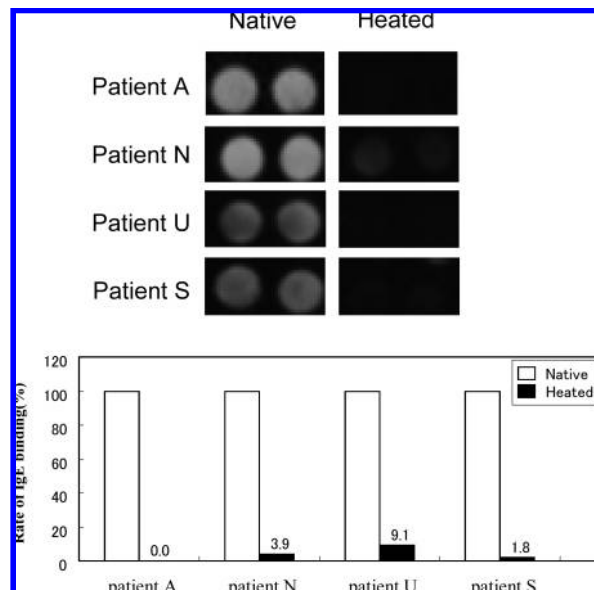

Figure 7. Reduction of antigenicity of heated Cry j 1. The binding of patient IgE with native and heated Cry j 1 was measured by dot blotting (above). The density in the shade of two spots was measured by densitometer and the average density was expressed as the rate of IgE binding (below). The IgE binding of native Cry j 1 was fixed at 100% and the rate of heated Cry j 1 and IgE antibody was calculated.

β-structure during heat denaturation. In order to investigate the formation of cross-β-structure in the soluble aggregate, fluorescence methodology using thioflavin T was carried out. It has been reported that the soluble forms of the 20 or so proteins are involved in the well-defined amyloidogenic diseases. The aggregation forms of amyloidogenic proteins have characteristics in common. The amyloid fibril shows specific optical behavior on binding with dye molecules such as Congo red and thioflavin T (*11*, *12*). The fibrillar structures typical of many amyloidogenic proteins have similar morphorogic and characteristic cross-β-structure. The ability of proteins to form an amyloid structure is not restricted to specific amyloidogenic proteins (*13*). Therefore, Cry j 1 may also form the cross-β-structure during heat denaturation. It has been reported that thioflavin T specifically interacts with amyloid β-sheet structures and that the fluorescence intensity with thioflavin T was proportional to the formations of cross-β-structures. As shown in **Figure 6**, the fluorescence intensity of thioflavin T in heated Cry j 1 was increased in proportion to the increase of heating temperature. The result suggests that Cry j 1 also forms cross β-structures via intermolecular interactions during heat denaturation. Thus, it seems likely that the formation of the intermolecular disulfide bond and cross-β-structure is involved in the polymerization of Cry j 1 by heating.

Since Cry j 1 forms cross-β-structures and intermolecular disulfide bonds in soluble aggregates during heating, the IgE epitopes of Cry j 1 exposed in the molecular surface may be buried inside the soluble aggregate to induce the reduction of antigenicity. To elucidate the possibility, dot blotting analysis was attempted to investigate the masking of IgE epitopes. As shown in **Figure 7**, IgE antibody recognition was greatly decreased during the heating of Cry j 1. Thus, four main IgE epitopes of Cry j 1 (*1*) can be masked by the intermolecular β-structure and the intermolecular disulfide bond of soluble aggregates to reduce antigenicity.

We have reported that the antigenicity of Cry j 1 can be masked by the attachment of a polysaccharide (galactomannan) through the naturally occurring Maillard reaction (*4*). In addition to this method, the heat-induced soluble aggregate of Cry j 1 also can be used to mask antigenicity. The four epitopes of Japanese cedar pollen are exposed on the molecular surface(*1*). These epitopes are easily masked by the formation of soluble aggregates during heating. Therefore, the soluble aggregate of Cry j 1 may be used as an effective oral immune tolerance agent without the risk of anaphylaxis. It is possible that the soluble aggregate of Cry j 1 may be digested in the stomach and intestine. Therefore, the IgE epitope of Cry j 1 can be exposed in the digestive tract. The effect of Cry j 1 digests on patients should be elucidated in clinical trials. In addition, the T-cell reactivity of this therapeutic material also should be investigated.

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