Monitoring the Irradiation-induced Conformational Changes of Ovalbumin by Using Monoclonal Antibodies and Surface Plasmon Resonance

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Two types of conformationally specific anti-irradiated ovalbumin monoclonal antibodies were prepared in order to study and monitor irradiation-induced structural changes in the ovalbumin molecule. Surface plasmon resonance (SPR) detection was used to investigate the kinetic parameters of the reaction between antibodies and ovalbumin which had been administered with different doses of irradiation (0, 1.5, 2.0, 5.0, 10, 20, 50, and 100 kGy). The results demonstrate that the combination of monoclonal antibodies and the SPR method can be used to characterize the irradiation-induced conformational change with an unlabelled reagent.

Key words: monoclonal antibody; surface plasmon resonance; antigen-antibody interaction; ovalbumin; irradiation

Food-poisoning caused by pathogenic microorganisms such as Salmonella enteritidis1 and Escherichia coli O1572-3 have posed serious problems in many countries.4,5 Food irradiation is first and foremost a preservation technique6-8 and shows considerable advantages.9-12 It is well known that heating prevents the growth of organisms and causes an irreversible structural change in protein. The structural change in ovalbumin caused by heating have been well investigated by Rumbo et al.13 and Ikura et al.14 However, there is a little information about the effects of irradiation treatment on the protein molecule; it remains to be elucidated to what extent food constituents are damaged by irradiation, whether an irradiation-specific conformational or compositional change in protein occurs, and especially which types of antigenic site can be newly generated. Kume et al.15 have investigated the properties of irradiated egg-white proteins and succeeded in detecting the degraded fragments specific to irradiation. However, no further detailed studies have been performed to identify the irradiation-specific products and to detect any differences from the irradiation dose.

In recent years, a considerable number of studies by surface plasmon resonance (SPR) have been performed to detect and determine differences in the functional and conformational properties of proteins, including the kinetic parameters and equilibrium affinity constants regarding the biomolecular aspects of antigen-antibody interaction.16-18 These biosensor systems provide a quantitative analysis of molecular interaction, therefore allowing the epitope map of a large panel of antibodies to be readily and rapidly analyzed19-21 with substantial time-saving compared with such conventional methods as radioimmunoassay, fluorescence, and absorption. In addition, SPR can detect changes in the refractive index of the bound-species mass close to the sensor surface as a function of time, so that no labelling of reagents is required. This avoids changes in the binding properties caused by the labelling process or immobilization of the microtiter plate.

In this study, we used a surface plasmon resonance biosensor to study and monitor the structural change in protein molecules caused by irradiation. Two types of conformational-sensitive monoclonal antibodies (one reacting with both non-irradiated and irradiated ovalbumin, and the other reacting only with irradiated ovalbumin) were used to examine the reactivity of ovalbumin to different doses of irradiation ranging from none to 100 kGy. We finally evaluated the kinetic parameters obtained by the SPR technique and attempted to test the new detection methods against irradiated samples tested by ELISA.

Materials and Methods

Materials. EDC (N-ethyl-N′-(3-dimethylamino-propyl) carbodiimide hydrochloride), NHS (N-hydroxy-succinimide), a solution of surfactant P 20 (10% Tween 20), and an ethanolamine-HCl packaged amine coupling kit were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). Sensor Chip

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CM5 was also purchased from Pharmacia. All other chemicals were of guaranteed reagent grade.

**Preparation of irradiated ovalbumin (I-ova).** Ovalbumin was purified from fresh egg white by crystallization in an ammonium sulfate solution, and this procedure was repeated three times. The purified ovalbumin was dialyzed against H$_2$O and lyophilized. This sample was then irradiated in a Gamma cell 220 (Nordion International Inc., Canada) with Cobalt-60 gamma-rays at a rate of 7.5 kGy/h with a dose of 100 kGy. In this study, we obtained 0, 1.5, 2.0, 5.0, 10, 20, 50, and 100 kGy irradiated ovalbumin.

**Preparation of anti-irradiated ovalbumin monoclonal antibodies.** Monoclonal antibodies against irradiated ovalbumin were obtained according to the conventional method of Köhler and Milstein. A detailed characterization of these antibodies has been provided by Masuda et al. (submitted for publication). Antibody-secreting hybridomas were produced in a serum-free culture or in mouse ascites. These crude antibodies were purified by Protein G affinity chromatography (Pharmacia LKB).

**Biosensor instrumentation.** The BIAcore system (BIAcore 2000, Pharmacia, Uppsala, Sweden) was used to analyze molecular interaction. Briefly, one of the reactants was fixed to the hydrophilic dextran matrix which was coupled to a thin gold film, and the second interreactant, called the analyte, was injected into the solution flowing over the sensor surface. The reaction was continuously monitored, and the binding event was directly observed on a computer screen. A detailed description of the BIAcore system and its detection principle has been provided by Karlsson et al.

**Immobilization of antibodies on the sensor surface.** The standard amine coupling procedure was used for immobilization. First, the sensor chip surface was activated with a mixture of 0.2 mM EDC (N-ethyl-N′-3-dimethylaminopropyl) carbodiimide hydrochloride and 0.05 mM NHS (N-hydroxy-succinimide), and then the antibody solution (approximately 7 µg/ml) prepared with a 10 mM acetate buffer at pH 5.0 was injected. The remaining activated groups were blocked by the injection of 0.1 M ethanolamine-HCl at pH 8.5. The flow rate for this procedure was maintained at 5 µl/min. The activation time, antibody coupling time, and deactivation time were all 4 min. We used an HBS buffer (10 mM Hepes (2-[4-(2-hydroxyethyl)-1-piperazine] ethanesulfonic acid), 0.15 M NaCl, 3.4 mM EDTA, and 0.05% surfactant P20) at pH 7.4 as standard, and the sensor surface was equilibrated with this buffer after immobilization.

**Theory of the kinetic analysis.**

**A. Linear regression method.** The association and dissociation rate constants were calculated by fitting the primary sensorgram curve by BLAevaluation 2.1 software. To briefly describe the system’s theoretical background, the general rate equation for binary complex formation \([A] + [B] = [AB]\) is written as

\[
\frac{d[AB]}{dt} = k_{a,0} [A][B] - k_{d,0} [AB]
\]

where \(k_{a,0}\) is the association rate constant and \(k_{d,0}\) is the dissociation rate constant. In the biosensor, the formation of the complex is directly monitored as the change in response \((R)\) over time \((t)\), so equation 1 can be written as

\[
\frac{dR}{dt} = k_{a,0} C (R_{\text{max}} - R) - k_{d,0} R
\]

and also as

\[
\frac{dR}{dt} = k_{a,0} C R_{\text{max}} - (k_{a,0} C + k_{d,0}) R
\]

where \(dR/dt\) is the rate of surface complex formation, \(R\) is the resonance unit reflecting the amount of analyte bound to the immobilized ligand on the sensor chip, \(R_{\text{max}}\) is the resonance unit value when the binding sites of the immobilized ligand have been saturated by the analyte, and \(C\) is the concentration of the analyte in a free solution. \(R_{\text{max}} - R\) corresponds to area of the ligand’s remaining free binding site. A plot of \(dR/dt\) versus \(R\) for different concentrations of the analyte yields the relationship

\[
S = k_{a,0} C + k_{d,0}
\]

where \(S\) is the slope of the straight line of \(dR/dt\) versus \(R\). From equation 4, \(k_{a,0}\) can be readily obtained from the slope of this plot. Since \(k_{d,0}\) is usually very low compared to \(k_{a,0}\), \(k_{a,0}\) cannot be reliably measured from the intercept on the \(y\) axis. When the analyte flowing over the chip’s surface is replaced by the buffer, the concentration of the analyte reaches zero and equation 2 can be simplified as

\[
\frac{dR}{dt} = -k_{d,0} R
\]

When the complex begins to dissociate at time \(t_l\) from response level \(R_{l1}\), \(k_{d,0}\) can also be obtained from the deviation of the response curve at the dissociation phase as follows

\[
\ln \left( \frac{R_{l1}}{R_{n}} \right) = k_{d,0} (tn - t_l)
\]

where \(R_{n}\) and \(tn\) are values obtained along the dissociation curve. The slope of the plot of \(\ln \left( \frac{R_{l1}}{R_{n}} \right)\) versus \((tn - t_l)\) provides \(k_{d,0}\).

**B. Non-linear regression method.** O’Shannessy et al. have demonstrated how to analyze the integrated rate equations

\[
R_t = CK_{a,0} R_{\text{max}} (1 - e^{-(Ck_{a,0} + k_{d,0}t)}) / CK_{a,0} + k_{d,0}
\]

and

\[
R_t = R_e e^{-k_{a,0}t}
\]
where $R_o$ is defined as the amplitude of the dissociation process. The use of the foregoing integrated form of the equations allows the direct determination of the association and dissociation rate constants for each binding experiment.

**Measurement of the kinetic parameters of ovalbumin at varying doses of irradiation in relation to immobilized monoclonal antibodies.** Each analyte (0, 0.5, 1.5, 5.0, 10, 20, 50 or 100 kGy-irradiated ovalbumin) diluted in the HBS buffer at a concentration ranging from 200 to 500 nM was allowed to interact with sensor surfaces on which specific antibodies had been immobilized. Association and dissociation were both allowed for 3 min by using the KINJECT injection command. Each run was performed at 25°C and at a flow rate of 20 μl/min. At the end of the run, the sensor surfaces were regenerated with 100 mM glycine-HCl containing 200 mM NaCl at pH 2.7.

**Characterization of the monoclonal antibodies by ELISA.** Different concentrations of the antigen solution (N-OVA or I-OVA; from 1 to 1000 μg/ml) were put together with the antibody at a fixed concentration (10 μg/ml or 1 μg/ml) and incubated overnight at 4°C. One hundred μl of this incubated solution was removed, transferred to an OVA-precoated microtiter plate and then incubated overnight at 4°C. The wells of the plate were washed with PBS containing 0.05% (v/v) Tween 20 (T-PBS). After washing, 100 μl of horseradish peroxidase (HRP)-conjugated goat anti-mouse (H+L) at a final dilution of 1:3000 was added, and the culture incubated for 1.5 hr at 20°C. After discarding the solution, the bound secondary antibodies were quantified by the addition of a substrate solution (10 ml of a 50 mM sodium citrate buffer at pH 5.0 containing 4 mg of o-phenylenediamine and 2 μl of 30% H$_2$O$_2$), and the enzyme reaction was terminated by the addition of 50 μl of 2 N H$_2$SO$_4$. The absorbance was measured at 492 nm with an immunoreader MPR 4i (TOSOH, Japan).

**Determination of Kd by the Scatchard equation.** To elucidate the dissociation constant ($K_d$), we used the Scatchard equation

$$\frac{[\text{Ab} \cdot \text{Ag}]}{[\text{Ag}]} = \frac{([\text{Ab}] - [\text{Ab} \cdot \text{Ag}])}{K_d}$$

(9)

where $[\text{Ab}]$ indicates the total antibody concentrations, and $[\text{Ab} \cdot \text{Ag}]$ the antigen-antibody complex concentration. The antigen-antibody complex concentration can be written as

$$[\text{Ab} \cdot \text{Ag}] = [\text{Ab}] - [\text{Ab}][A_o]/A_o$$

$$= [\text{Ab}][A_o - A_o]/A_o$$

(10)

where, $A_o$ is the absorbance measured for the total antibody concentration ([Ab]) in the absence of the antigen, $A_o$ is the absorbance measured in the presence of the antigen, and $[\text{Ab} \cdot \text{Ag}]$ is the non-binding antibody concentration. Therefore, the antigen-antibody complex concentration ($[\text{Ab} \cdot \text{Ag}]$) can be replaced by $[\text{Ab}][A_o - A_o]/A_o$. Equation 9 can be written as

$$\frac{(A_o - A_o)}{A_o} \times [\text{Ag}] = \frac{1}{K_d} \times (1 - \frac{(A_o - A_o)}{A_o})$$

(11)

**Results**

**Kinetic measurement by surface plasmon resonance**

The optimal conditions for kinetic measurements require the presence of a low concentration of the antigen on the matrix. About 900 RU to 2700 RU of antibody corresponding to approximately 0.02 to 0.06 pmol ligand/mm$^2$ was covalently coupled to the dextran surface. Fig. 1 shows a typical sum of kinetic runs at various antigen concentrations ranging from 200 nM to 500 nM at 25°C, while Fig. 2 indicates a plot of the slope ($dR/dt$ vs. $R$) against the concent-

![Fig. 1. Typical Kinetic Runs for the BIAcore Measurement.](image)

(A) Overlay plot illustrating the OVA-02 antibody binding at different concentrations (200 to 500 nM) to 0 kGy-irradiated ovalbumin. (B) and (C) are typical examples of the curve fit and residual plots for the association and dissociation phases, respectively.
Irradiation-induced Structural Changes in the Ovalbumin Molecule Detected by Surface Plasmon Resonance

Table 1. Rate and Affinity Constants for Different doses of Irradiation to Ovalbumin for the OVA-02 Antibody Immobilized on a Dextran Matrix

<table>
<thead>
<tr>
<th>Dose (kGy)</th>
<th>$k_{on}$ (10$^9$ M$^{-1}$·s$^{-1}$)</th>
<th>$k_{off}$ (10$^{-4}$ s$^{-1}$)</th>
<th>$K_d$ (10$^{-7}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.0 ± 0.1</td>
<td>3.1 ± 0.1</td>
<td>3.1</td>
</tr>
<tr>
<td></td>
<td>1.3 ± 0.1</td>
<td>3.1 ± 0.3</td>
<td>2.4$^{**}$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>4.7$^{**}$</td>
</tr>
<tr>
<td>1.5</td>
<td>1.2 ± 0.1</td>
<td>3.7 ± 0.1</td>
<td>3.1</td>
</tr>
<tr>
<td>2.0</td>
<td>1.2 ± 0.1</td>
<td>3.7 ± 0.2</td>
<td>3.1</td>
</tr>
<tr>
<td>5.0</td>
<td>1.2 ± 0.1</td>
<td>3.8 ± 0.4</td>
<td>2.7</td>
</tr>
<tr>
<td>10.0</td>
<td>1.2 ± 0.1</td>
<td>4.0 ± 0.3</td>
<td>3.3</td>
</tr>
<tr>
<td>20.0</td>
<td>1.1 ± 0.1</td>
<td>4.4 ± 0.4</td>
<td>4.0</td>
</tr>
<tr>
<td>50.0</td>
<td>0.7 ± 0.1</td>
<td>6.5 ± 0.4</td>
<td>9.3</td>
</tr>
<tr>
<td>100</td>
<td>0.6 ± 0.1</td>
<td>9.0 ± 0.7</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>26$^{**}$</td>
</tr>
</tbody>
</table>

All measurements were performed with SPR apparatus as shown in the text. Parameters except *a* and *b* were determined by a linear regression method. *a* and *b* were determined by a non-linear regression method and ELISA method, respectively.

Table 2. Rate and Affinity Constants for Different doses of Irradiation to Ovalbumin for the OVA-03 Antibody Immobilized on a Dextran Matrix

<table>
<thead>
<tr>
<th>Dose (kGy)</th>
<th>$k_{on}$ (10$^9$ M$^{-1}$·s$^{-1}$)</th>
<th>$k_{off}$ (10$^{-3}$ s$^{-1}$)</th>
<th>$K_d$ (10$^{-7}$ M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.$^{**}$</td>
</tr>
<tr>
<td>1.5</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>2.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>5.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>10.0</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>20.0</td>
<td>3.7 ± 0.8</td>
<td>0.8 ± 0.1</td>
<td>2.2</td>
</tr>
<tr>
<td>50.0</td>
<td>5.3 ± 0.5</td>
<td>0.9 ± 0.1</td>
<td>1.7</td>
</tr>
<tr>
<td>100</td>
<td>8.7 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>1.3$^{**}$</td>
</tr>
<tr>
<td></td>
<td>8.8 ± 0.3</td>
<td>1.1 ± 0.1</td>
<td>2.3$^{**}$</td>
</tr>
</tbody>
</table>

All measurements were performed with SPR apparatus as shown in the text. Parameters except *a* and *b* were determined by a linear regression method, and *a* and *b* were determined by a non-linear regression method and ELISA, respectively.

Fig. 2. Kinetic Measurements for the Binding between Antibodies and N-OVA or I-OVA.

The slope was plotted against the OVA concentration (equation 14). (A) OVA-02 vs. N-OVA, (B) OVA-02 vs. I-OVA, and (C) OVA-03 vs. I-OVA. Details of the procedure for analysis are given in the Materials and Methods section.

An analysis of the association and dissociation phases was performed by a linear fitting or non-linear fitting method. The kinetic constants of OVA-02 and OVA-03 against different irradiation doses are shown in Tables 1 and 2, respectively. In OVA-02, the $K_d$ value for 0 kGy was 3.1 × 10$^{-8}$ M, and no significant difference in $K_d$ value was observed up to 10 kGy irradiation (low irradiation level). However, above 20 kGy (medium to high irradiation level), $K_d$ dramatically increased with increasing dose of irradiation, the $K_d$ value for 100 kGy (1.5 × 10$^{-7}$ M) being approximately five times higher than that for 0 kGy. This difference was due to the lower $k_{on}$ values and higher $k_{off}$ values. In contrast, OVA-03 didn’t react with non-irradiated ovalbumin or ovalbumin irradiated with a low dose. However, $K_d$ dramatically decreased with increasing dose of irradiation, and the $K_d$ value for 100 kGy being 1.3 × 10$^{-7}$ M.

Kinetic measurement by ELISA

A Scatchard plot of binding between the antibody and N-OVA and I-OVA is shown Fig. 3. The $K_d$ values are also shown in Tables 1 and 2. The $K_d$ values for OVA-02 against non-irradiated ovalbumin and 100 kGy-irradiated ovalbumin were 4.7 × 10$^{-8}$ M and 2.6 × 10$^{-7}$ M, respectively, and that of OVA-03...
against 100 kGy-irradiated ovalbumin was $2.3 \times 10^{-7}$ M.

**Discussion**

The Food and Agriculture Organization (FAO), the International Atomic Energy Agency (IAEA) and the World Health Organization (WHO) convened the Joint Expert Committee on Food Irradiation (JECFI) to evaluate existing information on food irradiation. In 1980, JECFI concluded that the irradiation of foodstuffs at a dose not exceeding 10 kGy presented no toxicological hazard to human health. Since this conclusion, a number of investigations concerning the establishment of methods for detecting irradiated products have been performed. Most of these studies have concentrated on using physicochemical methods, including thermoluminescence (TL),\(^{25}\) electron spin resonance (ESR)\(^{26,27}\) and gas chromatography-mass spectrometry (GC-MS).\(^{28}\) It is necessary to evaluate the detection methods used for irradiated products from several view points: for example, biological, microbiological, and immunochemical. However, these methods have not given satisfactory results for detecting irradiated products. Monoclonal antibodies are useful not only for detecting and characterizing a specific antigen, but also for investigating and monitoring the structural changes involved in a denaturation process.\(^{29,30}\) Antibodies produced against irradiated products would provide valuable insight to evaluate the detection methods. In the previous study, we produced monoclonal antibodies to irradiated ovalbumin and successfully obtained two types of conformationally sensitive monoclonal antibodies. One reacted to both native and irradiated-denatured ovalbumin, while the other recognized only irradiated ovalbumin. These antibodies were used as probes in an attempt to detect irradiated ovalbumin by employing ELISA. However, native ovalbumin is known to be susceptible to surface denaturation by interaction with the surface of a plastic plate, thus immobilizing ovalbumin on the microtiter plates.\(^{31}\) Therefore, we always had to take into the consideration the denaturation caused by this immobilization. In fact, the irradiated ovalbumin-specific antibody, OVA-03, reacted to non-irradiated ovalbumin coated on the microtiter plate (data not shown). Consequently, we first immobilized the antibody on the microtiter plate, before adding an ovalbumin sample to accurately measure the binding affinity. The amount of ovalbumin was quantified by another biotinylated antibody. We have named this assay sandwich ELISA and established it as a system for discriminating between non-irradiated and irradiated ovalbumin. However, since this method employs ELISA and also requires biotinylation of the antibody, it takes a lot of time and would be unsuitable for dealing with many samples. It takes more than a week with the ELISA method to collect the same results that could be obtained in several days by an SPR analysis. The reagent consumption by the SPR analysis is also less than that by ELISA. The SPR technique allows for rapid quantitative and qualitative analyses of such molecular interaction as that between the antigen and antibody in real time, and no molecule labelling is required. Furthermore, with the SPR system, since kinetics parameters (the association constant and dissociation constant) are simultaneously obtained, information regarding the binding efficiency is available for a standard determination of the denaturation process. We used two types of monoclonal antibodies and immobilized them on the sensor surface. With the OVA-02 antibody, which reacted to both non-irradiated and irradiated ovalbumin, no significant difference in $K_d$ value was apparent up to 10 kGy. However, above 20 kGy, $K_d$ dramatically increased with increasing dose of irradiation. The association constant for OVA-02 against ovalbumin irradiated by a low dose was larger than that obtained by a high dose, and dissociation of the bound antigen (ovalbumin irradiated by a low dose) was slower than that obtained by a high dose. These results suggest that OVA-02 would exhibit a stable binding to ovalbumin molecules irradiated by a low dose. On the other hand, OVA-03 reacted only to 20, 50, and 100 kGy-irradiated ovalbumin. The larger the association constant, the larger the dose of irradiation, while the dissociation constant was not significantly changed. This result suggests that the binding mode of OVA-03 would be dependent on the association phase which can be a function of the exposure of the
epitope of OVA-03 with increasing dose of irradiation. In fact, the epitope of OVA-03 includes the central β-strands 3A (172-MVLVNAIVFKGLWEK-186) which represent the spine of the molecule.32 The residues in this area are abundant with alpha- and hydrophobic amino acids, and are positioned internally in the molecule. Additionally, strand 3A is covered by both helix F (132Ala-Gln152) and a turn segment between helix F and strand 3A in native ovalbumin. Asn149 and Thr156 in helix F form hydrogen bonds with the subsequent turn segment and play a crucial role in the stabilization of this segment.32 We have demonstrated from these facts that irradiation-induced denaturation was dependent on the irradiation dose, and that a low irradiation level (below 10 kGy) caused negligible structural change to ovalbumin, while a high level of irradiation (20, 50 or 100 kGy) induced conformational change to helix F and the subsequent turn covering the epitope of OVA-03. By destabilizing the hydrogen bonds between helix F and the turn segment, strand 3A would be readily exposed to the molecular surface, and newly antigenic sites would be produced. These results are consistent with not only the results obtained by differential scanning calorimetry (DSC), showing that 10 kGy-irradiated ovalbumin had a native-like structure, while 100 kGy-irradiated ovalbumin was completely denatured (Yasumoto et al., unpublished results), but also with the conclusion and recommendation declared by JECCI in 1980 that the irradiation of any commodity up to an average dose of 10 kGy presented no toxicological hazard. It seems entirely reasonable to suppose that the SPR system that uses specific monoclonal antibodies can detect conformational changes in the denaturation process like a DSC analysis can. In addition, the Kd value determined by ELISA was almost the same as that by using the SPR technique. Comparing the two methods, the SPR system requires a lesser amount of sample than the ELISA system does. We have demonstrated in this study that the SPR system is applicable to and convenient for not only measuring antigen-antibody interaction in real-time, but also for monitoring the change in protein conformation induced by such a physical treatment as irradiation.

References


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