

Investigation of the IgE-Binding Proteins in Soybeans by Immunoblotting with the Sera of the Soybean-Sensitive Patients with Atopic Dermatitis

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Summary The IgE-binding proteins in soybeans were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the fractionated soybean proteins probed with the sera of the patients with atopic dermatitis. About 20% of the patients examined were shown to have specific IgE antibodies to soybean proteins. At least 16 soybean proteins with molecular weights ranging from about 70,000 to 14,000 were recognized by the sera of the patients: 10 major IgE-binding components were found in the 7S-globulin fraction, and the others mainly in the 2S-globulin and whey fractions. The IgE antibodies of the patients bound most strongly and frequently to a unique protein with molecular weight of about 30,000 in the 7S-globulin fraction, which appeared to be the major allergen in soybeans and was named as *Gly m Bd 30 K*. The proteins in the 11S-globulin fraction were scarcely recognized by the patients' sera and assumed to be less allergenic for the patients with atopic dermatitis.
Key Words soybeans, soybean proteins, soybean allergens, IgE-binding proteins, atopic dermatitis, soybean-sensitive patients, food allergy

Food allergy is one of the serious nutritional problems both in children and adults, since a number of patients with atopic dermatitis are considered to be suffering from food hypersensitivity mediated by allergen-specific IgE antibodies

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and the incidence of atopic dermatitis is increasing mainly among children. Of many foods that elicit such adverse reactions, soybeans are known as an allergenic food in common with other protein-rich foods such as milk and eggs (1). The allergenicity of soybeans are known to reside in the protein fractions (2), not in soybean oil itself (3), whereas oxidized soybean oil has been shown to enhance the IgE-binding ability of soybean or other food proteins (4).

Avoidance of food responsible for allergy is recommended as a conventional therapy. However, if there are sensitivities to multiple food or if nutritionally-fundamental or essential foods are incriminated, the elimination of all the corresponding foods may lead to malnutrition for an extended period of the treatment. It is also required that the patients have much knowledge about food compositions, especially processed foods, in which allergenic residues are incorporated as processing aids. Since soybeans or soybean products have been increasingly used in a number of processed foods as a nutritional and economical protein source, the safety choice of the processed foods free from soybean products may become practically impossible.

The diagnosis of a confirmed adverse reaction to soybeans mediated by IgE has been generally made by the skin prick test or radioallergosorbent test (RAST) using the whole extract from soybeans or the commercial allergen disk of soybeans (1). However, these screening methods could not clarify what constituents are responsible for the allergic reactions. It is, therefore, important to get more precise information about the individual allergenic components in order to develop hypo-allergenic soybean products for the soybean-sensitive patients. Although several studies have been done on the allergenicity of soybean proteins (5–11) after the first report of soybean allergy (12), they are still insufficient to understand the detailed properties of allergenic components which can be recognized by IgE antibodies of the soybean-sensitive patients. The present work was undertaken to define the allergenic importance of soybean proteins by a combination of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting methods, using the sera of the soybean-sensitive patients with atopic dermatitis. This technique also enables identification of the individual allergen recognition spectra of a large number of the patients (13).

MATERIALS AND METHODS

Materials. Kits of Phadebas radioallergosorbent test (RAST) and radioimmunosorbent test (RIST) (^{125}I -labeled antihuman IgE antibodies, 1.7×10^5 Bq/5 ml) were obtained from Pharmacia Diagnostics AB (Uppsala, Sweden). Defatted soybean flakes (*Glycine max*, mixture of IMO varieties) were generously supplied from Fuji Oil Co. (Izumisano, Osaka). The molecular weight marker proteins, Nonidet P-40, Kunitz soybean trypsin inhibitor (KSTI) and soybean lectin were obtained from Sigma Chemicals Co. (St. Louis, MO). Other reagents used were of analytical grade.

Patients and serum. Serum samples were prepared from 361 patients with atopic dermatitis (mean age: 6.1 years old, ranging from 3 months to 23 years) who were asked to donate blood samples for testing at Tokushima Kensei Hospital and National Kagawa Children's Hospital. The control sera were obtained from 5 healthy adult volunteers who have no clinical history of atopic or allergic diseases.

RAST and RIST. RAST and RIST were carried out according to the instructions of the supplier. RAST was performed using the commercial allergen disks, such as soybeans, egg white, milk, wheat, rice in addition to house dust and *Dermatophagoides* sp., and the disks of birch pollen allergen as a reference standard. The results were recorded as RAST scores in comparison with four reference sera supplied in Phadebas kit: 1 (0.35 Phadebas RAST Unit (PRU)/ml), 2 (0.70 PRU/ml), 3 (3.50 PRU/ml) and 4 (17.50 PRU/ml). The patients giving the RAST score more than 2 were diagnosed as positive subjects according to the manual of the supplier. One unit of RIST value is equivalent to about 2 ng of IgE antibodies.

Fractionation of soybean proteins. The whole extract was prepared from defatted soybean flakes. Soybean flakes (100 g) were homogenized with 2,000 ml of 30 mM Tris-HCl buffer, pH 8.0, at 4°C and the homogenate was centrifuged at $10,000 \times g$ for 20 min at 20°C. The supernatant was dialyzed against deionized water at 4°C for 20 h to obtain the whole extract. Soybean proteins in the whole extract were fractionated into four fractions (2S-globulin, 7S-globulin, 11S-globulin and whey fractions). The 7S- and 11S-globulin fractions were prepared according to the procedure of Thanh and Shibasaki (14). The 7S- and 11S-globulin fractions were also obtained as a reference by ultracentrifugation at $233,000 \times g$ for 20 h at 20°C on a linear gradient of sucrose (10 to 30%) in 35 mM potassium phosphate buffer, pH 7.6, containing 0.4 M NaCl according to the method of Mori and Utsumi (15). The 2S-globulin fraction was separately prepared by the method of Koshiyama *et al.* (16) and purified by a gel filtration chromatography on Sephacryl S-300. In this report, the whey fraction used was the protein fraction free from 7S- and 11S-globulin and most part of 2S-globulin proteins.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Immunoblotting. SDS-PAGE of soybean proteins was performed on the 12.5% polyacrylamide gel (or 17% polyacrylamide gel for KSTI) according to the method of Laemmli (17). The proteins on the gels were transferred electrophoretically onto a nitrocellulose membrane as described previously (18). One part of the membrane was stained with Coomassie brilliant blue and the other part was cut into strips (0.5 cm width) and the strips were blocked with 3% bovine serum albumin in blocking buffer containing 10 mM Tris-HCl, pH 8.0, and 0.9% NaCl (Tris-buffered saline: TBS) at 25°C for 30 min. The strips were washed with TBS. Each strip was incubated with 1 ml of a 5-fold diluted solution of patient's serum at 4°C for 20 h with gentle stirring. Then, the membrane was three times washed with TBS, twice with TBS containing 0.5% Nonidet P-40 and finally with TBS. The strip was incubated with 0.6 ml of ^{125}I -labeled antihuman IgE (1.7×10^4 Bq/ml) for 20 h at 20°C. After being washed with TBS, the membrane was wrapped with polyethylene

film. Autoradiography of the IgE-binding proteins was taken on Fuji AIF New RX medical film (Fuji Photo Film Co., Kanagawa) in a cassette equipped with Kodak intensifying screen by exposure for several days at -80°C . Non-specific binding of IgE antibodies or antihuman IgE antibodies to soybean proteins was determined by using the strips treated without patient's serum or with control serum.

RESULTS

Incidence of soybean allergy among patients with atopic dermatitis

When the randomly-selected 86 patients (about 25% of 361 patients who donated the blood samples) were examined simultaneously by RAST or RIST using various commercial allergen disks and by the immunoblotting method using the whole extract of soybeans, the frequency of the positive response to the individual food disks (RAST) was estimated as follows: soybean (14.0%), milk (11.6%), egg white (26.7%), wheat (12.8%), and rice (8.1%). About 19% (16/86) of the patients tested showed the positive response against the multiple food disks. Some of the patients also showed strong response to the disk of house dust or *Dermatophagoides* sp. similarly to the food disks. The RIST values varied ranging from 6 to 7,300 units/ml (average 610 units/ml), which did not correspond to the RAST scores for foods because of the presence of excess amounts of specific IgE antibodies for house dust or *Dermatophagoides* sp. By the immunoblotting method, the sera of the 11 patients who did not give the positive RAST response (<2) on the soybean disk were shown to carry specific IgE antibodies for soybean proteins, whereas the 4 RAST-positive patients (≥ 2) gave no response by the immunoblotting method. This fact indicated that the screening of specific IgE antibodies by the immunoblotting technique was more sensitive than that by RAST, and the incidence of soybean allergy among the above patients was estimated to be about 22% (19/86) by the SDS-PAGE and immunoblotting method.

Soybean proteins

Figure 1 shows the SDS-PAGE patterns of the proteins in the whole extract and whey and 11S-, 7S-, and 2S-globulin fractions. Both the 11S- and 7S-globulin fractions prepared by the ultracentrifugation (15) gave the same SDS-PAGE patterns of subunit compositions as reported in the literature (14), while the 7S-globulin fraction prepared by the method of Thanh and Shibasaki (14) was found to contain some unidentified minor proteins: this fraction was nevertheless used for the immunoblotting analysis in order to examine the possible allergenicity of these minor components because these minor components could not be detected in other fractions.

Immunoblotting

The SDS-PAGE and immunoblotting analyses of the IgE-binding proteins of soybeans were carried out using the sera from 361 patients with atopic dermatitis.

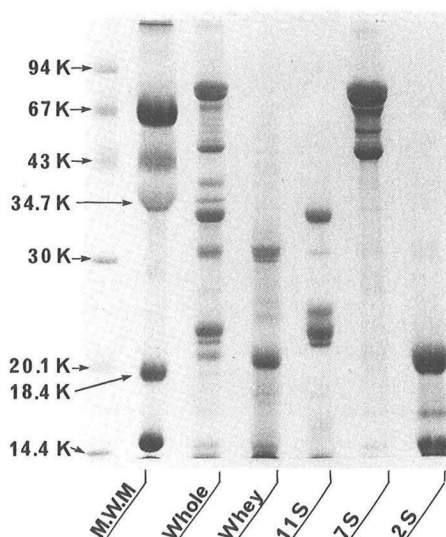


Fig. 1. SDS-PAGE patterns of soybean proteins. M.W.M. means molecular marker proteins with relative molecular mass in kDa, K. 2S, 7S, 11S and whey represent the 2S-globulin fraction, 7S-globulin fraction, 11S-globulin fraction and whey fraction, respectively.

It was shown that about 20% (69/361) of the patients had the specific IgE antibodies binding to soybean proteins. Figure 2 shows the autoradiograms of the immunoblot of the whole extract with the sera of 20 different patients, by which almost all types of the blotting patterns detected in the patients can be covered. As the results, the multiple protein components which bound IgE antibodies were recognized by the IgE antibodies of the soybean-sensitive patients (Nos. 4–20). No IgE-binding could be observed on the autoradiograms of the blank (C-1), control (C-2) and the soybean-insensitive patients (Nos. 1–3), who presented the high RIST values (about 800 units/ml), whereas a weakly-radiostained band was detected at the position corresponding to the basic subunit of 11S-globulin on all the autoradiograms. The sera of the soybean-sensitive patients (Nos. 4–20) gave the multiple radiostained bands corresponding to the proteins with the molecular weights ranging from 70,000 to 14,000. Radiostained protein bands with molecular weights between 70,000 and 40,000 (see patients Nos. 5, 7, 10, 13, 15, 16 and 20) were attributed to the major components of the 7S-globulin fraction. The patients Nos. 4, 6, 8, 10, 11, 14, 15 and 16–19 gave the characteristic radioactive bands corresponding to 2 minor proteins with molecular weights of about 30,000 and 28,000, respectively, of which the former was recognized most strongly and frequently by the sera of the soybean-sensitive patients. The sera of the patients Nos. 4, 6, 9, 14 and 15 also perceived the components with relatively low molecular weights of about 20,000–14,000. The serum of the patient No. 15 strongly

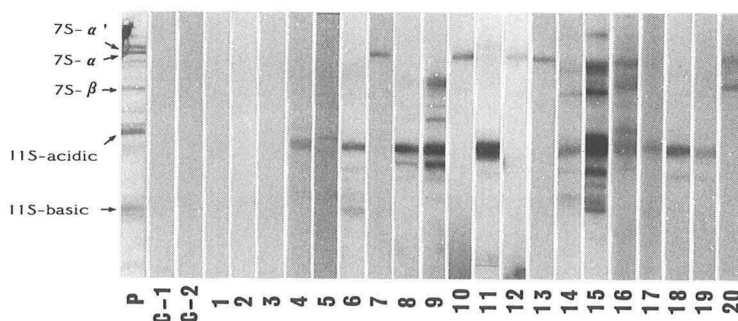


Fig. 2. Autoradiograms of soybean proteins after immunoblotting with the sera of the patients with atopic dermatitis. P, proteins stained with Coomassie brilliant blue; C-1, blank (— serum); C-2, control serum; Nos. 1–3, the sera of the soybean-insensitive patients with atopic dermatitis; Nos. 4–20, the sera of the soybean-sensitive patients with atopic dermatitis.

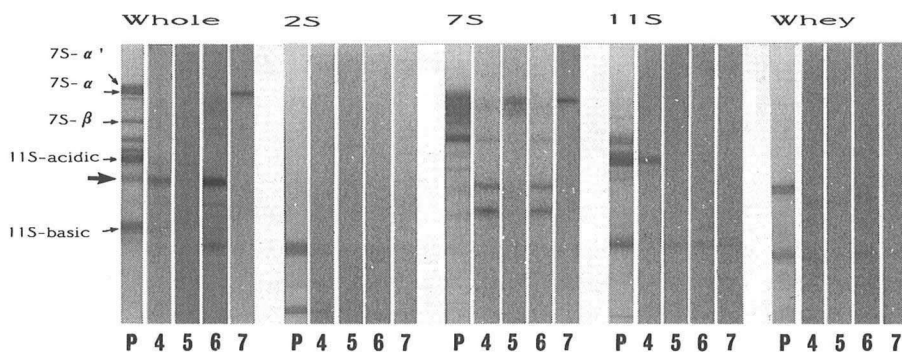


Fig. 3. Autoradiograms of the fractionated soybean proteins after immunoblotting with the sera of the soybean-sensitive patients. Whole, 2S, 7S, 11S and whey represent the whole extract, 2S-globulin fraction, 7S-globulin fraction, 11S-globulin fraction and whey fraction, respectively. Arrow indicates the position of the major allergen, *Gly m Bd 30 K*, in the 7S-globulin fraction (see the text). P, proteins stained with Coomassie brilliant blue; Nos. 4–7, the sera of the soybean-sensitive patients presented in Fig. 2.

immunostained various protein components including all the characteristic radio-stained bands observed in other patients. Figure 3 shows the representative immunoblotting patterns of the fractionated soybean proteins with the sera containing the specific IgE antibodies most frequently recognized in the patients Nos. 4–7 (Fig. 2). Most of the radiostained bands were assigned to the protein components of the 7S-globulin fraction. The IgE-binding protein with molecular weight of about 30,000 was due to the minor constituent in the 7S-globulin fraction. This protein could be detected neither in the 2S- nor 11S-globulin nor whey

Table 1. Major IgE-binding proteins in soybeans and the frequency among the patients with atopic dermatitis.

IgE-binding protein (Molecular weight)		Assignment of protein fraction and component	Frequency ^a (%)
1	70,000–68,000	7S-globulin fraction (α subunit)	23.2
2	67,000–63,000	7S-globulin fraction	18.8
3	55,000–52,000	7S-globulin fraction	14.5
4	50,000–47,000	7S-globulin fraction	13.0
5	45,000–43,000	7S-globulin fraction (β subunit)	10.1
6	41,000–40,000	7S-globulin fraction	10.1
7	38,000–35,000	7S-globulin fraction	7.2
8	35,000	11S-globulin fraction (acidic subunit)	1.4
9	35,000–33,000	7S-globulin fraction	15.9
10	31,000–29,000	Whey fraction (HMW ^b group)	4.3
11	30,000	7S-globulin fraction (<i>Gly m Bd 30 K</i>)	65.2
12	28,000	7S-globulin fraction	23.2
13	21,000–18,000	Whey fraction (LMW ^b group)	7.2
14	20,000	2S-globulin fraction (KSTI)	2.9
15	17,000	2S-globulin fraction	1.4
16	15,000–14,000	2S-globulin fraction	2.9

^aDetection frequency among the 69 soybean-sensitive patients with atopic dermatitis.^bHMW, high molecular weight; LMW, low molecular weight.

fractions. The serum of the patient No. 4 also perceived the protein(s) corresponding to the acidic subunit of 11S-globulin. The patients Nos. 4, 5 and 6 had the IgE antibodies weakly binding to the components with low molecular weights fractionated into the 2S-globulin and whey fractions. The protein components immunoblotted by the sera of all the patients tested and their detection frequency are summarized in Table 1. At least 16 protein components were recognized by IgE antibodies in the sera of the patients with atopic dermatitis. Of these components, about 10 major components with the molecular weights of about 70,000–68,000 (α subunit of β -conglycinin), 67,000–63,000, 55,000–52,000, 50,000–47,000, 45,000–43,000 (β subunit of β -conglycinin), 41,000–40,000, 38,000–35,000, 35,000–33,000, 30,000, 28,000 were found in the 7S-globulin fraction and other 3 components with molecular weights of about 20,000 (KSTI), 17,000, and 15,000 in the 2S-globulin fraction, 2 components with molecular weights of about 31,000–29,000 and 21,000–18,000 in the whey fraction and one component with the molecular weight of about 35,000 (basic subunit of glycinin) in the 11S-globulin fraction. These molecular weights were estimated by SDS-PAGE as shown in Fig. 1 and some of the IgE-binding proteins were assigned to the known protein components according to the electrophoretic behavior reported in the literature (19). The immunoblotting of the fractionated soybean proteins also revealed that the sera of the patients recognizing the proteins in the 7S-globulin fraction did not react with any components in other fractions. This suggests that there is no allergenic cross-reactivity between the

proteins in the 7S-globulin fraction and those in the 2S- and 11S-globulin fractions or in the whey fraction. The cross-reactivity found between the components with high molecular weight of about 20,000 in the 2S-globulin fraction and those with low molecular weight of about 21,000–18,000 in the whey fraction (see Fig. 3) was assumed to arise from contamination of KSTI, *i.e.* the major component of the 2S-globulin fraction (16). Among the multiple protein components recognized by the patients' sera, the protein with molecular weight of about 30,000 in the 7S-globulin fraction was corresponding to the major allergen in soybeans and therefore was named *Gly m Bd 30K* according to the nomenclature system recommended by Marsh *et al.* (20). One of the major storage proteins, 11S-globulin or glycinin consisting of the acidic and basic subunits, could be scarcely recognized by IgE antibodies in the sera of almost all the patients with atopic dermatitis, except that one patient (patient No. 4 in Fig. 3) gave the radiostained band corresponding to the acidic subunit of glycinin. The basic subunit of glycinin bound weakly and non-specifically to ¹²⁵I-labeled antihuman IgE antibodies (IgG) used as a second antibody (Fig. 2).

DISCUSSION

The SDS-PAGE and immunoblotting analyses revealed that 69 out of 361 patients with atopic dermatitis possessed the IgE antibodies specific for soybean proteins. This means was more sensitive than the RAST using commercial soybean allergen disk. The immunoblotting analyses of the fractionated soybean proteins with the patients' sera demonstrated that at least 16 distinct IgE-binding components occur in soybeans. Most of the IgE-binding proteins in soybeans were assigned to the components in the 7S-globulin fraction. The bulk of the soybean-sensitive patients (about 65%) were shown to have the IgE antibodies specific for a unique protein component, *Gly m Bd 30K*, which was a minor constituent in the 7S-globulin fraction and was assumed to be the major allergen in soybeans against the patients with atopic dermatitis. The behavior of this protein on SDS-PAGE was similar to that of the known component, *i.e.* soybean lectin with molecular weight of about 30,000 or to the subunits of basic 7S-globulin with molecular weight of about 28,000 (21), but our preliminary experiments showed that the authentic lectin did not bind to the patient's IgE antibodies and the electrophoretic properties were not identical with those of the basic 7S-globulin subunit. Moreover, many of the soybean-sensitive patients were shown to have IgE antibodies specific for the major protein components in the 7S-globulin fraction such as the α - and β -subunits of β -conglycinin, suggesting that these proteins might become allergenic against the patients. On the other hand, protein components in the 11S-globulin fraction were shown to be less allergenic; nevertheless the acidic and basic subunits of glycinin in the 11-globulin fraction account for about 40 to 50% of the total storage proteins in soybeans (22). In addition, our results showed that the protein components in the 2S-globulin and whey fractions probably containing KSTI as a major constit-

uent (14) had only weak reactivity with the patients' sera and low frequency of the IgE-binding among our patients. Immunoblotting of authentic KSTI with the patients' sera also showed only weak binding to the patients' IgE. Although there is only one clinical report that KSTI is a strong allergen against a soybean-allergic patient (7), Burks *et al.* (8) showed that allergenic proteins in soybeans predominated in the 7S- or 11S-globulin fractions rather than in the 2S-globulin fraction as a result of immunochemical analysis using the sera from the soybean-sensitive patients with atopic dermatitis. Recently, Herian *et al.* (10) reported that the sera of the patients sensitive to both peanuts and soybeans bound to several protein components with molecular weights ranging from 60,000 to 50,000 (probably the subunits of β -conglycinin) and that the component with molecular weight of 20,000, not identical with KSTI, was strongly recognized by the IgE from the patients allergic only to soybeans. Moreover, Shibasaki *et al.* (6) suggested that various allergenic protein components occurred in all the soybean protein fractions and the IgE antibodies in the sera of the soybean-sensitive patients showed the cross-reactivity among the 2S-, 7S- and 11S-globulin fractions by the RAST-inhibition analyses (6), but the RAST-inhibition method could not characterize the individual protein components responsible for the cross-reactivity. Rodrigo *et al.* (11) showed that the patients with asthma in Barcelona raised against soybean dust had specific IgE antibodies for the glycoproteins with molecular weights lower than 14,000, which were assumed to be degradation products of β -conglycinin or unique protein species occurring in soybean hulls.

Herian *et al.* (10) described that different soybean-allergic subjects were sensitive to quite different proteins and could be classified into three categories according to the immunoblotting patterns. Our results also showed that the IgE-binding proteins varied among the patients but that the patients could not be classified into distinct groups according to their immunoblotting patterns. This discrepancy may depend on the difference in complicated conditions including (i) different ages and symptomatic situations, (ii) history of the sensitization of the individual patients, including an inhalation through airways, mucous membrane and gastrointestinal tract, and/or (iii) the preparation of the fractionated soybean proteins used for the experiments. Moreover, it may be related to the differences in the patient immune responses, probably to the genetic basis responsible for the IgE production against the different allergens. Our patients examined showed no severe anaphylactic responses (e.g. nausea, diarrhea and respiratory distress) against soybeans or soybean products, but were diagnosed as having general atopic dermatitis on their symptomatic situations. Therefore, it may be an interesting problem to examine whether the allergenicity of soybean proteins depends on the sensitization process of the patients, where the proteins may undergo their structural changes. In fact, we found that the strongest allergenic protein in soybeans was KSTI in the model experiment with mice sensitized by a subcutaneous injection of the whole extract (Ogawa, Tsuji, Bando and Sasaoka, unpublished), although the problem is still unclear regarding the species specificity between mice and

human-beings as pointed out by Shibasaki *et al.* (6). In the present experiment, the IgE antibodies in the sera of almost all the patients were shown to lack the reactivity with the proteins in the 11S-globulin fraction. This observation proposes further study on the possibility that the low or marginal specific IgE in the sera may have been counteracted with the simultaneously raised specific IgG antibodies, since recent investigations report that the comparative specificity and relative abundance of IgE and IgG in the patient sera may have fundamental importance in explaining the allergic reactivity (23,24). In either case, it gives useful information that the acidic and basic subunits of glycinin, the most abundant protein components in soybeans, may be one of the most possible candidates to fabricate the hypoallergenic soybean products for the soybean-sensitive patients with atopic dermatitis. However, the effects of physical and chemical treatments during the cooking or processing of soybeans on the allergenicity of the individual soybean proteins remain to be established subsequently.

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