

Class I chitinases, the panallergens responsible for the latex-fruit syndrome, are induced by ethylene treatment and inactivated by heating

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Background: Class I chitinases have been identified as the major panallergens in fruits associated with the latex-fruit syndrome, such as avocado, banana, and chestnut. However, other plant foods containing these enzymes have not been related to this syndrome.

Objective: We sought out class I chitinases in the green bean, a legume that is known to express chitinases but is not associated with latex allergy, and examined whether the content or allergenic activity of chitinases can be modified by physical or chemical treatments.

Methods: IgE-binding proteins in untreated bean samples, as well as in ethylene- and heat-treated samples, were detected by using a pool of sera from patients with latex-fruit allergy. Putative allergens were purified by cation-exchange chromatography and characterized by N-terminal sequencing, enzymatic activity assays, immunodetection with sera and antichitinase antibodies, and immunoblot inhibition tests. Skin prick tests with untreated and heated purified allergens were also carried out.

Results: An IgE-binding protein of 32 kd that was also recognized by antichitinase antibodies was detected in green bean extracts. This reactive component was strongly induced by ethylene treatment. The protein, designated PvChI, was identified as a class I chitinase closely related to the major avocado allergen Prs a 1. Immunoblot inhibition assays demonstrated cross-reactivity between both allergens. Purified PvChI induced positive skin prick test responses in 7 of 8 patients with latex-fruit allergy. Heat treatment of both Prs a 1 and PvChI produced a full loss of their allergenic capacities both in vitro and in vivo. No IgE-binding component was detected in the white mature bean in which the main isolated 32-kd protein corresponded to a nonreactive phytohemagglutinin.

Conclusions: Ethylene treatment induces the expression of plant class I chitinases. The allergenic activity of plant class I chitinases seems to be lost by heating. This fact could explain why plant foods containing these putative allergens that are consumed after cooking are not usually associated with the latex-fruit syndrome. (*J Allergy Clin Immunol* 2000;106:190-5.)

Key words: Class I chitinases, fruit panallergens, green bean, latex-fruit syndrome, ethylene induction, allergen heat inactivation

Allergy to latex proteins has been recognized as a major occupational problem in several glove-wearing populations, especially among health care workers.^{1,2} Besides other allergens, hevein (Hev b 6.02), a 43 amino acid chitin-binding polypeptide, has been identified as a major allergen for patients allergic to latex.^{3,4} Around 50% of these patients also show hypersensitivity to some plant foods, especially fruits, leading to the proposal of the so-called latex-fruit syndrome.^{5,6} An increasing number of plant sources has been associated with this syndrome, but avocado, banana, chestnut, and kiwi are those most frequently reported.⁵⁻⁷

Recently, class I chitinases with an N-terminal hevein-like domain have been identified in avocado,⁸⁻¹⁰ chestnut,^{8,10} and banana^{11,12} as the main cross-reacting allergens with latex. Both their in vitro and in vivo reactivity have been demonstrated in patients with latex-fruit allergy. Furthermore, full inhibition of IgE binding by these chitinases is achieved by using latex hevein as an inhibitor,^{7,12} and neither IgE recognition nor positive skin prick test (SPT) responses are found when homologous class II chitinases lacking the hevein domain are tested.^{8,10} With regard to other plant sources, a survey of 20 different food extracts indicates that putative class I chitinases seem to also be relevant cross-reacting proteins linked to latex-fruit allergy in cherimoya, passion fruit, kiwi, papaya, mango, tomato, and wheat.¹³

The above-mentioned data point to a central role of the class I chitinases, particularly their N-terminal hevein-like domains, as the main allergens responsible for the cosensitizations between latex and fruits. However, these enzymes are widely distributed throughout the plant kingdom¹⁴ and are present in several foods, such as cereals and legumes, that have not been truly associated with the latex-fruit syndrome. This is the case of green beans (*Phaseolus vulgaris*), an extensively consumed legume

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Abbreviations used

PVDF: Polyvinylidene difluoride

SPT: Skin prick test

that has been proven to have and express genes encoding class I chitinases.¹⁵ We sought out these enzymes in green beans and examined whether their content or allergenic activity can be modified by physical or chemical treatments.

METHODS

Plant material, treatments, and crude extract preparations

Beans (*P vulgaris* L, cultivar Iluro) were grown in an experimental field and collected at 8 weeks (green bean pods) and at maturity (14 weeks; white bean seeds). Ethylene treatment was achieved by spraying green beans with a solution of ethephon (2-chloroethyl-phosphonic acid, Sigma; 1 mg/mL) or with water (control) on being collected and enclosing the pods in sealed cellophane bags for 24, 48, or 72 hours. Heat treatment was defined as boiling green beans at 100°C for 15 minutes. In the case of purified proteins, dissolved samples were transferred to Eppendorf tubes, heated in a boiling water bath (100°C for 15 minutes), freeze-dried in the same Eppendorf tubes, resuspended in the appropriate buffer, and then used for immunodetection or SPT.

Treated, control, and untreated green bean pods and white bean seeds were freeze-dried and ground. Plant material was defatted with ethanol/ether (1:3 [vol/vol]; 3× 1:5 [wt/vol] for 1 hour at 4°C), and, after drying, dissolved with PBS buffer (0.1 mol/L sodium phosphate [pH 7.0] and 0.15 mol/L NaCl; 1× 1:5 [wt/vol] for 1 hour at 4°C). After centrifugation (10,000 rpm for 30 minutes at 4°C), the supernatants were dialyzed against H₂O and freeze-dried. Protein concentration was quantified according to the method of Bradford.¹⁶

Patients and sera

Patients were selected from a population with latex allergy (70 subjects) during a prospective study carried out by the Allergy Section of Hospital de Gran Canaria Dr. Negrin (Canary Islands, Spain).¹⁷ The hospital institutional review board approved the study, and informed consent was obtained from all participants. Patient selection was based on the following criteria: (1) a clinical history of immediate reactions to banana, chestnut, and/or avocado, suggesting an IgE-mediated hypersensitivity; (2) a positive prick by prick test response to the corresponding foods; and (3) a specific IgE level higher than 3.50 kU/L to the reactive fruits, as determined by using the Pharmacia CAP system FEIA (Pharmacia Diagnostics). A pool of sera from 8 selected patients was used for immunodetection studies. Eight volunteers not allergic to fruits or latex paired for age and sex with the selected patients were included as a control group.

Isolation and characterization of allergens and bean phytohemagglutinin

Crude extracts (5 mg of protein) from untreated and 72-hour ethylene-treated green beans, as well as untreated white beans, were fractionated by using cation-exchange chromatography on a Mono S HR 5/5 column (Pharmacia; 1.0 mL total volume) with a fast-performance liquid chromatography system (Pharmacia). The column was equilibrated with 20 mmol/L formic acid-NaOH, pH 4.0, and eluted with a salt linear gradient of 0% to 100% of 750 mmol/L

NaCl in the same buffer (140 minutes; 0.5 mL/min). The fractions obtained were concentrated, desalted, and freeze-dried. Protein content was determined by using the bicinchoninic acid method.¹⁸ Major peaks corresponding to putative allergens (class I chitinases) were identified by SDS-PAGE and immunodetection with rabbit monospecific polyclonal antibodies to plant chitinases. A chitinase-enriched protein preparation from avocado pear, as well as Prs a 1, the class I chitinase and major allergen of this fruit,^{8,9} were obtained according to the methods of Diaz-Perales et al.⁸

SDS-PAGE was performed on Bio-Rad Miniprotein II system gels, according to the method of Laemmli¹⁹ but excluding reducing agents and heat treatment of samples.

N-terminal amino acid sequences were determined by using standard methods with an Applied Biosystems 477A gas-phase sequenator.

Chitinase activity was tested by using a colorimetric microassay with carboxymethyl-chitin-Remazol Brilliant Violet 5R as the substrate. Procedure I of Wirth and Wolf²⁰ was followed for 30 minutes at 37°C per assay. Several protein amounts of each sample were tested to fit the Δ absorbance per assay between 0 and 250×10^{-3} units.

Immunodetection and immunoblot inhibition assays

Both assays were carried out according to methods previously described.^{8,13} Briefly, after SDS-PAGE, gels were electrotransferred to polyvinylidene difluoride (PVDF) membranes. Blocking membranes were incubated with the appropriate pool of sera (1:3 dilutions) and then with alkaline phosphatase-conjugated monoclonal anti-human IgE (clone GE-1, Sigma; 1:500 dilution) and revealed by adding 5-bromo-4 chloro-3 indolyl phosphate/nitro blue tetrazolium solution.

Replica blocked membranes were also immunodetected with previously developed rabbit monospecific polyclonal antibodies (1:100 dilution) to a class II chitinase from chestnut seed, which recognizes both class I and II chitinases from different plant sources.^{8,13} Membranes were then incubated with an alkaline phosphatase-conjugated anti-rabbit IgG (Sigma; 1:500 dilution) and a 5-bromo-4 chloro-3 indolyl phosphate/nitro blue tetrazolium solution.

Purified class I chitinases (allergens) from green bean and avocado, as well as BSA (negative control), were used as inhibitors of the specific IgE binding to the corresponding crude extracts fractionated by SDS-PAGE and electrotransferred to PVDF membranes. Sera were incubated with each inhibitor (3 hours at room temperature) before being used for immunodetection.

Skin prick tests

SPTs were performed with bean and avocado crude extracts (0.5 mg/mL), as well as with purified proteins (allergens; 50 μ g/mL). Puncture with a Morrow-Brown needle (Bayer) was carried out in the volar side of the forearm according to standard methods.²¹ A sterile device was used in each test. Duplicates of each sample were dissolved in PBS buffer, and one of them was heated (100°C during 15 minutes). After reaching room temperature, glycerine (50% vol/vol) was added, and both heated and nonheated samples were stored at 4°C until used. Histamine phosphate (10 mg/mL) and PBS were used as positive and negative controls, respectively. A mean wheal area of 7 mm² or greater (15 minutes after puncture) compared with the negative control was considered a positive response.

RESULTS

Crude extracts from untreated beans, as well as ethylene- and heat-treated green beans, were fractionated by

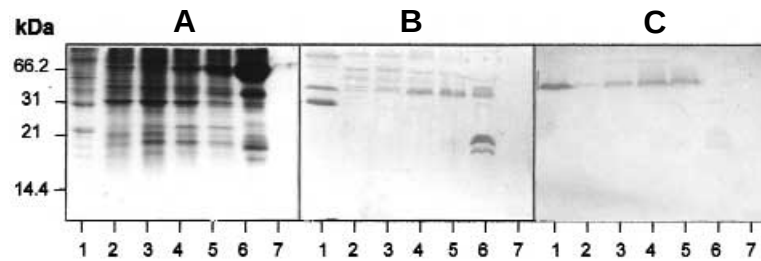


FIG 1. SDS-PAGE separation of crude extracts (15 μ g) from avocado (lane 1); untreated green beans (lane 2); 24-hour (lane 3), 48-hour (lane 4), and 72-hour (lane 5) ethylene-treated green beans; heat-treated (lane 7) green beans; and untreated white beans (lane 6). Replica gels were stained with Coomassie blue (A) or electrotransferred to PVDF membranes and immunoblotted with monospecific polyclonal antibodies to plant chitinases (B) or a serum pool from patients with latex-fruit allergy (C).

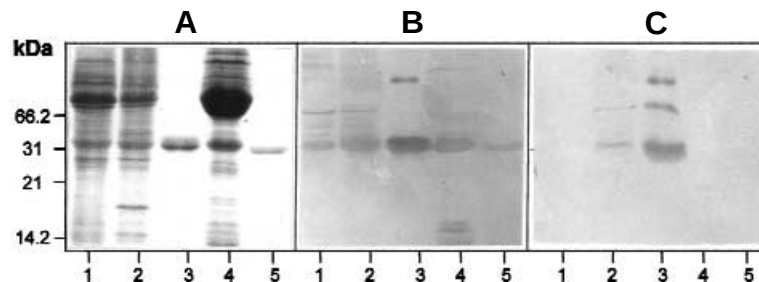


FIG 2. SDS-PAGE separation of crude extracts from untreated green beans (lane 1), 72-hour ethylene-treated green beans (lane 2), white beans (lane 4), and 32-kd purified proteins from ethylene-treated green beans (lane 3) and white beans (lane 5). Replica gels were stained with Coomassie blue (A) or electrotransferred to PVDF membranes and immunodetected with monospecific polyclonal antibodies to plant chitinases (B) or a serum pool from patients with latex-fruit allergy (C).

using SDS-PAGE (Fig 1). A complex pattern of bands from around 20 to 70 kd was obtained in all extracts, except extracts from heated green beans, when protein staining was performed (Fig 1, A). The boiled bean extract showed no detectable bands, even when the same protein amount as in all the other extracts tested (as quantified by the method of Bradford¹⁶) was loaded into the gel. The same results were observed when PBS extracts from commercial canned green beans were analyzed.

Monospecific polyclonal antibodies to class I and II chitinases were used to detect these enzymes in the bean preparations (Fig 1, B). An avocado extract, used as positive control, showed the two main previously characterized bands⁸ with apparent molecular weights of 25 (class II) and 32 kd (class I and major avocado allergen Prs a 1). Besides other reactive components, putative 32-kd class I chitinases were also recognized in all the bean extracts, except the extracts from boiled green beans. Interestingly, this 32-kd band clearly increased in the ethylene-treated samples of green beans.

A pool of sera from 8 patients with latex-fruit syndrome was used to identify IgE-binding proteins. Only a single band of 32 kd was recognized both in the avocado and in the different green bean extracts, whereas this component was not detected in white bean or in boiled green bean preparations. Negative control sera did not react with any band when all the extracts shown in Fig 1 were tested.

Crude extracts from untreated and 72-hour ethylene-treated green beans, as well as from mature seeds (white beans), were separated by means of cation-exchange chromatography (not shown) to isolate the major 32-kd component (putative class I chitinases) from both beans. Proteins included in the main peaks of the 72-hour treated bean and the white bean profiles were analyzed by using SDS-PAGE and immunodetected with the antichitinase serum and the pool of sera from allergic patients. A 32-kd protein that behaved as a single band in SDS-PAGE and was recognized by the antichitinase serum was purified in both green and white beans after this procedure (Fig 2, A and B). By contrast, the green bean isolated protein (hereafter designated *P vulgaris* class I chitinase or PvChI), but not the white bean protein (named *P vulgaris* phytohemagglutinin or PvPhy; see below), showed specific IgE-binding capacity (Fig 2, C).

PvChI exhibited a strong chitinase activity when tested with a specific colorimetric assay (Table I). Determination of its N-terminal amino acid sequence (Fig 3) demonstrated an N-terminus (14 residues) fully identical to that of the N-terminal hevein-like domain of Prs a 1, the major allergen and class I chitinase from avocado pear.⁸ Furthermore, an ethylene-induced endochitinase previously described in bean leaf¹⁵ also showed the same N-terminus, thus suggesting that this enzyme and PvChI are probably the same protein. Comparison of the full amino acid sequences deduced from the corresponding

complementary DNA clones of Prs a 1⁹ and the leaf bean class I chitinase¹⁵ rendered a 73.9% identity. Immunoblot and in vivo tests further supported the structural and allergenic similarity of both allergens. Prs a 1 exerted a full IgE-binding inhibition against the 72-hour ethylene-treated green bean extract and therefore against PvChI, which was the major IgE-binding protein in this preparation (Fig 4). PvChI elicited a similar effect when assayed against the avocado extract. SPTs carried out in a group of 8 patients allergic to latex and fruits (Table II) showed the high and similar activity of both purified chitinases in vivo; Prs a 1 induced 8 of 8 and PvChI induced 7 of 8 positive responses. Neither of these proteins elicited positive responses in a control group of nonallergic volunteers.

The purified PvPhy protein from white bean showed neither significant chitinase activity (Table I) nor the typical N-terminal hevein-like domain of class I chitinases (Fig 3). By contrast, its determined N-terminus was identical to that of PHA-L,²² a phytohemagglutinin identified in bean cotyledons. The recognition of PvPhy by monospecific antibodies to plant chitinases must be due to the partial homology found between PHA-L and the catalytic domain of plant chitinases, such as those from chestnut and barley (not shown). SPTs (Table II), eliciting no positive responses in any of the 8 allergic patients tested, further confirmed the lack of relevance of PvPhy in bean allergenicity.

To investigate the effect of heating on the allergenic properties of avocado and bean class I chitinases, immunoblots and SPT assays were carried out. Comparison of the immunoblots of untreated and heated avocado protein preparations enriched in chitinases (Fig 5, lanes AvF) showed a full loss of IgE-binding capacity of the 32-kD band corresponding to Prs a 1 when heated. These data were confirmed after analyzing the effects on the purified bean and avocado allergens. The heat-treated PvChI sample was undetectable by protein staining and nonrecognized by both monospecific antibodies to chitinases and by specific IgE from allergic patients (Fig 5, lanes PvChI). Similar results were obtained when isolated Prs a 1 was tested. Both purified allergens also lost their enzymatic activity and changed their HPLC profile with the heat treatment (not shown). Two other unrelated proteins used as controls, BSA and a peach allergen belonging to the lipid-transfer protein family,²³ did not show apparent changes in their electrophoretic behaviors after heating (Fig 5, D). The inactivation by heat treatment of the bean and avocado allergens was fully supported by SPTs in vivo (Table II), showing that both heated samples elicited negative responses in the same patients who reacted to the untreated proteins.

DISCUSSION

Plant chitinases are defense proteins widely distributed throughout the plant kingdom.¹⁴ Those belonging to class I, which harbor a hevein-like domain in their N-termini, have been identified as the major panallergens in those fruits most closely associated with the latex-fruit

Protein	N-terminal amino acid sequence
PvChI	E Q C G R Q A G G A L C P G
PvPhy	S N D I Y F N F Q R F X E T
PHA-L	S N D I Y F N F Q R F N E T

FIG 3. N-terminal amino acid sequences of green bean class I chitinase PvChI and white bean phytohemagglutinin PvPhy aligned with that previously reported for *P vulgaris* PHA-L.²⁵ X, Undetermined residue; PHA, phytohemagglutinin.

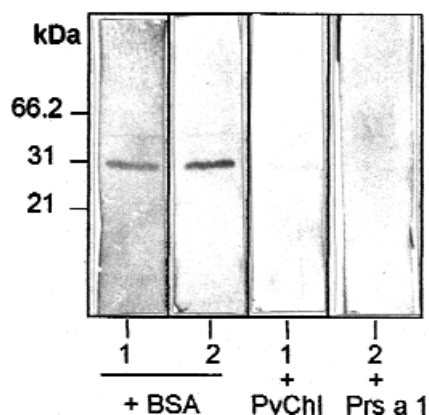


FIG 4. Immunoblot inhibition assays with the purified class I chitinases Prs a 1 and PvChI as inhibitors. PBS extracts (15 µg) from avocado (lanes 1) or 72-hour ethylene-treated green beans (lanes 2) were fractionated by means of SDS-PAGE and electrotransferred to PVDF membranes. Immunodetection was performed with a pool of sera from patients with latex-fruit allergy preincubated with BSA (+BSA; negative control) or with the major avocado allergen (+Prs a 1) and green bean chitinase (+PvChI). Five micrograms of each inhibitor was tested.

TABLE I. Chitinase activity of crude PBS extracts from green bean, 72-hour ethylene-treated green bean, white bean, and the purified proteins PvChI and PvPhy

Sample	ng/assay	Δ Absorbance × 10 ⁻³ (550 nm)
GbE	500	145 ± 5
GbE72	500	622 ± 3
WbE	500	118 ± 2
PvChI	100	636 ± 4
	10	207 ± 8
PvPhy	500	25 ± 2

Values are given as means ± SD (n = 3).

GbE, Green bean; GbE72, 72-hour ethylene-treated green bean; WbE, white bean.

syndrome.⁷⁻¹³ Considering the high degree of structural similarity among chitinases from different plant species, it could be guessed that patients with this syndrome must be sensitized to most foods containing class I enzymes. However, this is not the case for several plant sources, such as legumes and cereal flours, that are rarely related with latex allergy.^{5,6} Thus no immediate hypersensitivity has been described to the green bean, a legume that pos-

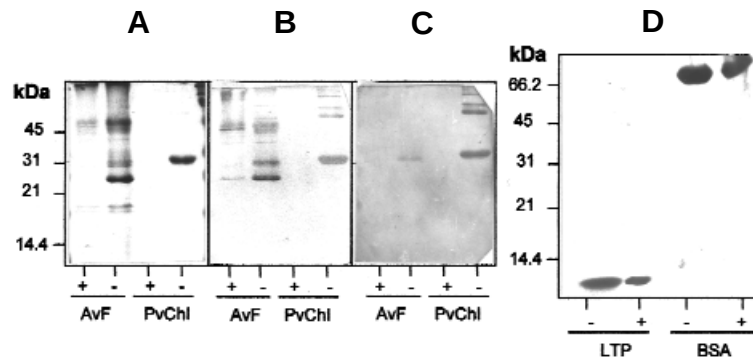


FIG 5. SDS-PAGE separation of untreated (–) and heat-treated (+) purified green bean class I chitinase (PvChI), peach lipid-transfer protein (LTP), BSA, and an avocado protein preparation enriched in chitinases (AvF). Gels were stained with Coomassie blue (A and D) or electrotransferred to PVDF membranes and immunoblotted with monospecific polyclonal antibodies to plant chitinases (B) or a serum pool from patients with latex-fruit allergy (C).

TABLE II. SPT results expressed in square millimeters of wheal area

Patient No.	SPT							
	Prs a 1	Heated Prs a 1	GbE	Heated GbE	PvChI	Heated PvChI	WbE	PvPhy
1	24	–	9	–	9	–	12	–
2	9	–	9	–	9	–	–	–
3	9	–	12	–	16	–	–	–
4	9	–	12	–	20	–	–	–
5	16	–	12	–	–	–	–	–
6	15	–	9	–	9	–	–	–
7	9	–	9	–	9	–	–	–
8	9	–	15	–	12	–	–	–

Testing agents used were PBS extract from green bean (GbE) and white bean (WbE), purified class I chitinases from avocado (Prs a 1) and green bean (PvChI), and white bean phytohemagglutinin (PvPhy). Heated samples were heated at 100°C for 15 minutes. Samples were tested at 500 µg/mL (PBS extracts) or 50 µg/mL (purified proteins).

–, Negative response.

sesses a class I chitinase¹⁵ and the crude extract of which provoked positive SPT responses in patients with latex-fruit allergy (Table II).

A single IgE-binding protein band of around 32 kd, also recognized by antichitinase antibodies, has been detected in crude green bean extracts (Fig 1). The reactive protein, named PvChI, was identified as a class I chitinase (Figs 2 and 3 and Table I) closely related to the major avocado allergen Prs a 1.^{8,9,15} Cross-reaction between both allergens was supported by immunoblot inhibition assays (Fig 4). Purified PvChI is almost as active in vivo as Prs a 1 (Table II), inducing positive SPT responses in 7 of 8 allergic patients tested. Joint consideration of these data demonstrates that crude green beans contain an allergen homologous to those described in avocado, banana, and chestnut.⁷⁻¹³

Interestingly, the amount of PvChI is strongly increased by ethylene treatment. This fact can be relevant in relation to fruit allergy because this plant hormone is used to hasten fruit ripening after storage of several climacteric fruits, such as apple, banana, avocado, or tomato. Thus the treatment can substantially change the levels of allergens in the mature fruits. On the other hand, no

IgE-binding protein was detected in mature white bean seeds, suggesting that very low levels of PvChI are present in this plant source. In this case the main component of the 32-kd band recognized by the antichitinase antibodies is not a chitinase but a phytohemagglutinin (Figs 1-3). This purified component was not reactive either in vitro or in vivo.

A potentially important property of the avocado and bean allergens is their inactivation by boiling. Both purified Prs a 1 and PvChI fully lose their IgE-binding capacity, as well as their in vivo reactivity, after heating (Fig 5 and Table II). These losses are joined by a lack of detection by protein staining after SDS-PAGE, which could probably be explained by denaturation and aggregation of the heated samples. The inactivation has also been observed in extracts from boiled green beans and in commercial canned and frozen green beans, which are usually heat treated during the industrial processing. All these data, together with the fact that beans are consumed cooked, can explain why green beans are not an issue for latex-sensitive patients. If this behavior is common to other class I chitinases with allergic capacity, it seems most probably that cooking inactivates the putative aller-

gens. This fact could explain why fresh fruits are the main foods associated with the latex-fruit syndrome, whereas plant materials that are heated before being consumed, even those possessing class I chitinases, are rarely related with latex allergy.

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