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Lima bean (*Phaseolus lunatus*) seed coat phaseolin is detrimental to the cowpea weevil (*Callosobruchus maculatus*)

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Abstract

The presence of phaseolin (a vicilin-like 7S storage globulin) peptides in the seed coat of the legume *Phaseolus lunatus* L. (lima bean) was demonstrated by N-terminal amino acid sequencing. Utilizing an artificial seed system assay we showed that phaseolin, isolated from both cotyledon and testa tissues of *P. lunatus*, is detrimental to the nonhost bruchid *Callosobruchus maculatus* (F) (cowpea weevil) with ED_{50} of 1.7 and 3.5%, respectively. The level of phaseolin in the seed coat (16.7%) was found to be sufficient to deter larval development of this bruchid. The expression of a *C. maculatus*-detrimental protein in the testa of nonhost seeds suggests that the protein may have played a significant role in the evolutionary adaptation of bruchids to legume seeds.

Key words
- Lima bean
- Phaseolin (vicilin)
- 7S storage globulin
- Seed coat
- Cowpea weevil
- Resistance to bruchids

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Introduction

Phaseolus lunatus (lima bean) is a New World legume that has been domesticated in areas corresponding to present day Peru and Mexico (1) and is currently cultivated in many tropical regions of the World (2). It is attacked by the stored product insect *Zabrotes subfasciatus* (Boh.) (Coleoptera: Bruchidae), both in the field and during storage. This bruchid also attacks the wild relatives of *P. lunatus*, suggesting that the two species have been associated through evolutionary processes (3,4). Lima bean has long been cultivated in the Brazilian Northeast alongside the Old World legume cowpea (*Vigna unguiculata* (L.) Walp.) in regions where both grow well. In many places the seeds of both legumes are stored together and, while *Callosobruchus maculatus* F. (Coleoptera: Bruchidae), which is associated with *V. unguiculata*, completely destroys cowpea seeds within a very short time after infestation, it does not attack lima bean seeds (Xavier-Filho J, personal observations; 4).

An explanation for this apparent specificity was given by Janzen (5), who suggested that *C. maculatus* larvae die shortly after passing through the testa of wild *P. lunatus* due to the high levels of HCN origi-

nating from the hydrolysis of the glucoside linamarin. Subsequently, Simmonds et al. (4), working with both wild and cultivated *P. lunatus*, showed that *C. maculatus* larvae die on entering the cotyledons and suggested that this happens due to the delayed consequences of the ingestion of toxins present in the testa. These authors gave no suggestion of what these toxins might be. Twenty years ago Janzen (5) demonstrated that the seed coat can be a barrier to bruchid infestation and suggested that this could complicate the analysis of the toxicity of seed contents, emphasizing the need to analyze seed contents separately from seed coats. Here we describe the isolation and characterization of protein fractions which are expressed in the testa of *P. lunatus* seeds and which are detrimental to *C. maculatus* larvae. We suggest that these proteins may have played an evolutionary role in establishing the basis for bruchid infestation of legume seeds (6,7).

Material and Methods

Seeds

P. lunatus seeds ("Ceará" landrace) were obtained commercially. Cowpea (*V. unguiculata*) seeds (cultivar EPACE-10) were supplied by Centro de Ciências Agrárias, Universidade Federal do Ceará, Fortaleza, CE, Brazil. Seeds were separated into cotyledons and testa with a common razor blade; seed coats are easily separated from cotyledon tissues with no contamination of one tissue with the other. Seed coat thickness was measured with a caliper. The concentrations (as % fresh weight) of phenols and cyanide in testa and cotyledons were measured according to published methods (8,9).

Insects

C. maculatus (cowpea weevil) were provided by J.H. Ribeiro dos Santos, Centro de Ciências Agrárias, Universidade Federal do Ceará, and were maintained in culture (28-30°C and 65-75% relative humidity, RH) in the Laboratório de Química e Função de Proteínas e Peptídeos, Centro de Biociências e Biotecnologia, Universidade Estadual do Norte Fluminense (Campos dos Goytacazes, RJ, Brazil). Development of *C. maculatus* on *P. lunatus* seeds was observed by placing 2-day-old females in glass vials containing three seeds at 28°C and 70% RH. After 2 days the insects were discarded and the number of laid and hatched eggs was counted. Seeds were observed daily for the emergence of adults until the end of the experiment (70 days). Six replicates of each measurement were carried out. For each replicate one seed was chosen for counting holes made in the testa by larvae.

Protein extraction and phaseolin purification

The fine powdered meal of cotyledons was extracted (1:10 ratio) with water adjusted to pH 8.0 (with 0.1 M NaOH) for 1 h at 4°C and then centrifuged at 10,000 *g* for 30 min. The residue was dialyzed against water using a membrane with a 6-kDa cutoff and freeze-dried. The supernatant was dialyzed against water and the dialysate containing low molecular weight substances was freeze-dried. The suspension containing albumins and globulins was centrifuged at 10,000 *g* for 30 min and the clear supernatant containing albumins and the precipitate containing globulins were recovered by freeze-drying. Testa tissue was fractionated by extraction with 50 mM sodium phosphate buffer, pH 7.6, 1:10 ratio, for 3 h at 4°C, followed by centrifugation at 10,000 *g* for 30 min. The insoluble fraction was re-extracted with the same buffer and freeze-dried. The supernatants were combined, dialyzed against water and freeze-dried. The soluble fraction obtained from the testa was submitted to gel filtration through a Sephadex G-100 column (2.2 x 46 cm) equilibrated and eluted with 50

mM sodium phosphate buffer, pH 8.0 (equilibrating buffer). The column was calibrated with protein standards. One hundred milligrams of soluble material from the testa, dissolved in 3 ml of equilibrating buffer, was applied to the column, and fractions of 2.2 ml were collected. Fractions containing protein (280 nm) and corresponding to materials of molecular masses 90 kDa (F1), 80 kDa (F2), 25 kDa (F3), and <10 kDa (F4) were pooled and recovered by dialysis against water and by freeze drying. Vicilin (phaseolin) from cotyledons of seeds of both cowpea and lima beans was prepared according to published methods (10,11). The same procedure was used for the preparation of phaseolin from *P. lunatus* testa. The concentrations of both phaseolin and vicilin in seed tissues were measured by ELISA. We employed an antibody against cowpea vicilins previously prepared by us and an antibody against *Phaseolus vulgaris* phaseolin kindly provided by Dr. Maarten Chrispeels (Department of Biology, University of California, San Diego, CA, USA). Protein was determined by the method of Bradford (12).

Effect of protein fractions on *C. maculatus* development

The deleterious effects of the various *P. lunatus* seed fractions on *C. maculatus* development were assessed using an artificial seed system previously developed by us (13). Artificial seeds (400 mg) were prepared with finely ground cowpea seed meal mixed with the various fractions described above, all at 1, 2, 4, 8 and 16% (by weight). Protein fractions recovered from the gel filtration experiments were incorporated into artificial seeds at 0.25, 0.5, 1.0 and 2.0% (by weight). Artificial seeds were infested by placing 2-day-old female insects in glass vials containing two seeds for 24 h at 28°C and 70% RH. The excess eggs laid were removed from the seeds, leaving 3 eggs per seed. After 20 days at 28°C and 70% RH infested seeds were opened and the weight and number of larvae were determined. Dose-response curves were drawn and the effective doses for a 50% (weight) response (ED_{50}, %) and lethal-doses (LD_{50}, %) were calculated for each fraction. Control artificial seeds were prepared with the meal of cowpea seeds without additions. All experiments were carried out in duplicate and the data shown are the average of these.

Phaseolin analysis

Vicilins were analyzed by SDS polyacrylamide (PAA) gel electrophoresis using 7.5% polyacrylamide, according to Laemmli (14). The running gel was overlaid with 3.5% PAA as stacking gel. Samples (10-20 µg) were solubilized by heating at 100°C for 5 min in 50 mM sodium phosphate buffer, pH 7.6, containing 5% (w/v) SDS, 10% (w/v) sucrose, without any reducing agent. Gels were stained with 0.25% (w/v) Coomassie brilliant blue R in methanol/acetic acid/water (5/1/4) and destained in the same solution. Proteins utilized as molecular mass standards were bovine serum albumin (66 kDa), ovalbumin (45 kDa), glyceraldehyde-3-phosphate dehydrogenase (36 kDa), soybean trypsin inhibitor (20 kDa), and ß-lactoglobulin (14.2 kDa). Carbohydrate was detected by employing a "glycoprotein detection kit" based on the periodic acid-Schiff method from Sigma Chemical Co. (St. Louis, MO, USA). N-terminal amino acid sequences were analyzed on a Shimadzu PPSQ-10 Automated Protein Sequencer performing Edman degradation. PTH-amino acids were detected at 269 nm after separation on a reverse-phase C18 column (4.6 mm x 2.5 mm) under isocratic conditions, according to the manufacturer's instructions. The phaseolin sequences were compared to amino acid sequence data banks. The sequences selected were submitted to automatic alignment, which was performed by using the Genetic Computer Group (GCG) Sequence Analysis Software Package.

Results

Behavior of *C. maculatus* on lima bean seeds

Large white lima type seeds of *P. lunatus* ("Ceará" landrace) with an average testa thickness of 0.14 ± 0.10 mm (Table 1) permitted oviposition but not the full development of *C. maculatus* larvae. A high proportion (80.5 ± 12.8%) of the eggs laid hatched 24 h after oviposition but did not give rise to adult insects. Furthermore, only 31.3 ± 13.3% of the corresponding larvae perforated the testa and fed on cotyledon tissues. A high proportion of the larvae (100-31.3% = 68.7%) died in the testa presumably as a result of ingesting this tissue and the deleterious compounds present therein. For cowpea, the principal host seed for this bruchid, high proportions of eggs hatch (90%) and 80 to 90% of the larvae survive after traversing the seed coat and cotyledons, giving rise to adults (15).

Table 1 - Characteristics of *Phaseolus lunatus* ("Ceará" landrace) and *Vigna unguiculata* (EPACE-10) seeds.

The data reported are the mean ± SD of 20 determinations.

	Seed color	Mass of one seed (g)	Testa thickness (mm)
P. lunatus	White	0.55 ± 0.03	0.14 ± 0.10
V. unguiculata	Brown	0.21 ± 0.007	0.006 ± 0.004

Table 2 - Tannic acid, HCN, protein and vicilin (or phaseolin) contents (as percent fresh weight) of testa and cotyledon tissues of *Phaseolus lunatus* and *Vigna unguiculata* (EPACE-10) seeds.

Data are reported as the mean ± SD of % fresh weight for three independent experiments. *P<0.05 compared to corresponding tissue in *V. unguiculata* (*t*-test).

	Phenols (tannic acid equivalents)	HCN	Protein	Vicilin (or phaseolin)
Phaseolus lunatus				
Cotyledon	0.11 ± 0.00	0.11 ± 0.00*	17.3 ± 0.6*	13.7 ± 2.1
Testa	0.22 ± 0.00*	0.02 ± 0.01	20.7 ± 0.6*	16.7 ± 2.6*
Vigna unguiculata (EPACE-10)				
Cotyledon	0.14 ± 0.03	0.0017 ± 0.0005	15.2 ± 0.3	12.7 ± 0.6
Testa	7.44 ± 0.24	0.0016 ± 0.0004	4.0 ± 0.01	1.1 ± 0.06

Testa components

In order to understand why this high proportion of larvae die in the testa we first measured the concentrations of phenols (tannic acid equivalents) and HCN since the presence of both has been suggested to impair development of *C. maculatus* on nonhost seeds (5,16). The levels of tannic acid in the tissues of *P. lunatus* seeds are equivalent to (cotyledons) or lower (testa) than those found in the host cowpea seeds (Table 2). The cyanide contents in cotyledons and testa of the lima bean seeds utilized by us, although being much higher than those in the same tissues of cowpea seeds, are much lower than those that kill 50% of the C. *maculatus* larvae in artificial seeds containing KCN (0.2% CN^- by weight) (Xavier-Filho J and Pinto MSP, unpublished results). The levels detected by us are also lower than those reported in wild *P. lunatus* seeds, known to be resistant to *C. maculatus* (2-3%) (5). Measurement of the levels of proteins and phaseolin (vicilin) in the testa and cotyledon tissues of both *P. lunatus* and *V. unguiculata* shows that the seed coat of lima beans contains large amounts of these proteins in contrast with cowpea seeds (Table 2).

Detrimental effects of seed fractions on *C. maculatus* development

To measure the effect of protein fractions of lima bean seeds on *C. maculatus* development seeds were carefully dissected and the cotyledon and testa tissues were separately ground to fine meals. These were extracted with buffer for the preparation of albumins, globulins, a low molecular weight fraction and a residue from cotyledons, and a soluble and an insoluble fraction from the testa. The soluble fraction from testa was further fractionated by gel filtration into four fractions according to their molecular masses (F1, 90 kDa; F2, 80 kDa; F3, 25 kDa; F4, <10 kDa). All these fractions plus phaseolin (vicilin)-

rich preparations from both cotyledons and testa of *P. lunatus* and vicilin from *V. unguiculata* seeds were incorporated into artificial seeds and offered to *C. maculatus* females for oviposition. After 20 days artificial seeds were opened and the number and weight of the larvae were determined. Table 3 shows that most of the isolated protein fractions were somewhat detrimental to the bruchid. We believe that all fractions are inherently detrimental to the insect although we did not try to identify individual constituents in all of them. We have chosen to further investigate the F1 fraction from testa tissues because it showed the lowest LD_{50} of all fractions examined (Table 3). Polyacrylamide gel electrophoresis of F1 showed three major proteins of high molecular mass that dissociated to lower molecular mass species after heating (Figure 1). This behavior was identical for both phaseolin isolated from cotyledonary (lanes C) and testa (lanes T) tissues by the method of Sammour et al. (10) as modified by Macedo et al. (11). We noticed the presence of three abundant components after heat treatment, B1 (20.5 kDa), B2 (25 kDa), and B3 (36.5 kDa), in all three fractions (lane C, lane T, and lane F1). These polypeptides correspond to phaseolin components, as also shown by others (17). Staining for the presence of sugars with the periodic acid-Schiff reagent (data not shown) suggested that band B2 protein has a higher level of glycosylation than proteins in bands B1 (2 times less) and B3 (9 times less).

Phaseolin analysis

Confirmation that fraction F1 is composed mostly of phaseolin polypeptides (17) came from N-terminal amino acid sequencing of the three protein bands found after heat treatment as shown in Figure 1. All three bands (B1, B2 and B3) originating from the F1 fraction of testa tissue presented N-terminal sequences that are homologous to the sequence of phaseolin from *P. lunatus* (Figure 2) as deduced from c-DNA data reported by Kami and Gepts (18), who also proposed five putative glycosylation sites for this protein (19). These workers have shown that 7S storage proteins (phaseolins)

Table 3 - Effect of the addition of several protein fractions to the diet of *C. maculatus*.

Effective doses for a 50% response (ED_{50}) are the concentrations of protein fractions (as percent weight of flour) that decrease the mass of the larvae to 50% of the mass of the control. Lethal doses (LD_{50}) are the concentrations of protein fractions (as percent weight of flour) that decrease the number of larvae to 50% of the number found in control seeds. The values were calculated from dose-response curves and are the average of two determinations.

	ED_{50} (%)	LD_{50} (%)
Fractions isolated from cotyledonary tissues		
Albumin	0.9	7.2
Globulin	0.8	6.3
Low molecular weight fraction	7.8	>16.0
Residue	>16.0	>16.0
Phaseolus lunatus phaseolin (vicilin)	1.7	2.0
Vigna unguiculata (EPACE-10) vicilin	6.3	8.0
Fractions isolated from testa tissues		
Insoluble material	0.9	5.1
Soluble material	2.2	12.0
Sephadex G-100 F1 (90 kDa)	0.6	1.4
Sephadex G-100 F2 (80 kDa)	0.7	>2.0
Sephadex G-100 F3 (25 kDa)	0.7	>2.0
Sephadex G-100 F4 (<10 kDa)	0.5	>2.0
Phaseolus lunatus phaseolin (vicilin)	3.5	>2.0

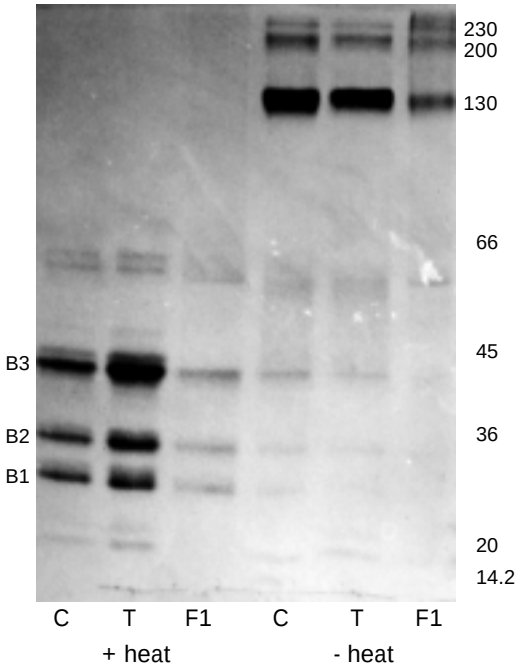

Figure 1 - SDS-PAGE of phaseolin proteins isolated from *P. lunatus* seeds. Samples contained 10-20 µg proteins. *Lane F1*, Fraction F1 isolated from testa tissue by Sephadex G-100 chromatography; *lane T*, 70-90 ammonium sulfate fraction from testa tissue; *lane C*, 70-90 ammonium sulfate fraction from cotyledon tissue. Nonheated samples (- heat); heated samples (+ heat). No reducing agent was used. The numbers on the right refer to molecular mass markers (130, 200 and 230 were extrapolated values). After electrophoresis the gel was stained with Coomassie brilliant blue and a similar gel run alongside the first one was used for transfer of proteins to a PVDF membrane.

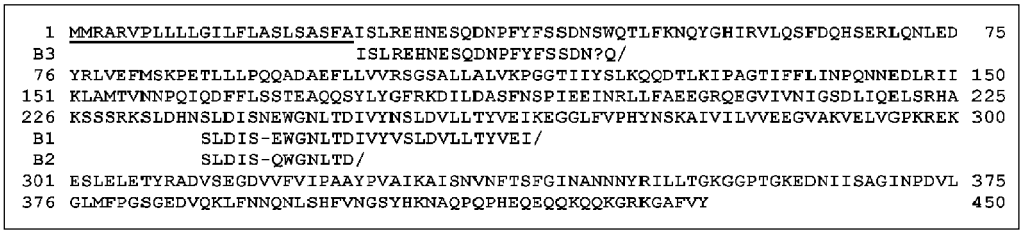

Figure 2 - N-terminal amino acid sequences of phaseolin polypeptides. N-terminal amino acid sequences of the polypeptides B1, B2 and B3 from fraction F1 aligned with the sequence of *Phaseolus lunatus* phaseolin as deduced from c-DNA data reported by Kami and Gepts (18). Underlined residues correspond to the signal sequence of phaseolin.

are generally glycosylated at Asn-X-Thr (Ser) sequences. Alignment of B1 and B2 N-terminal sequences with the sequence of phaseolin (Figure 2) shows that they align at positions 237 to 257 (B1) and 237 to 249 (B2) with only one substitution (for B1) at position 253 where a Val replaces an Asn residue. We suggest that both B1 and B2 polypeptides should be glycosylated at their position 6 (corresponding to position 246 of phaseolin) since no residue was found there during sequencing. On the other hand, B2 is 9 times more glycosylated than B1 as shown by glycoprotein staining after SDS-PAGE (data not shown). The molecular mass of B1 is 20.5 kDa and this corresponds to the mass of the polypeptide chain having 237 to 429 residues, which is the C-terminal amino acid sequence of phaseolin. The difference in molecular mass between B2 and B1 is 4.5 kDa and it can be explained by the higher glycosylation level observed for this polypeptide. The N-terminal sequence of the polypeptide B3 (36.5 kDa) showed total identity with the N-terminal sequence of phaseolin (positions 25-46). The proposed glycosylation site (18,19) was not demonstrable; indeed, a free Asn was found at position 7 (sequence Asn-Glu-Ser).

Discussion

In the present study we have confirmed observations by others that seeds of lima beans do not support larval development of the bruchid *C. maculatus* (4). Although female weevils oviposit on the testa and a high proportion of the eggs laid hatch, most of the larvae formed do not penetrate cotyledonary tissues, dying in the attempt. This behavior contrasts with that of *C. maculatus* in cowpea seeds: almost all eggs laid give rise to larvae that perforate the testa, consume the cotyledonary tissues and give rise to normal adults (15,20). Previous studies have suggested that the reason for the mortality of *C. maculatus* larvae is the presence of cyanogenic glucosides in the testa of lima beans (5).

The levels of tannic acid, which are thought to impair insect development (21) in both cotyledons and testa tissues of *P. lunatus* seeds, do not justify the high mortality of the larvae since *C. maculatus* develops on cowpea seeds with equivalent (cotyledons) or higher levels (testa). On the basis of the testa thickness of both seeds, we calculate that a larva that perforates this tissue will consume equivalent amounts of tannins (Tables 1 and 2). Our data for the cyanide contents of cotyledons and testa are much lower than those for wild *P. lunatus* seeds, which are reportedly known to be resistant to *C. maculatus* (2-3%) (5). It is interesting to note that this bruchid does not breed well in stored domesticated *P. lunatus* seeds, which have low levels of HCN (5). However, it seems that the levels of HCN in commercial lima beans bred for human consumption (2,22) are not completely responsible for the resistance of these seeds to *C. maculatus.*

Incorporation into artificial seeds of isolated fractions from both cotyledons and testa of lima beans shows that albumins and globulins from cotyledonary tissues and several soluble fractions obtained from the testa are detrimental to the cowpea weevil (Table 3). A fraction isolated from the testa and

composed of phaseolin (vicilin) polypeptides (17) was shown to affect development (ED_{50} = 0.6%, w/w) of the cowpea weevil in a manner comparable to the other fractions. Survival (LD_{50} = 1.4%) of larvae in artificial seeds containing this fraction was nevertheless lower than with the other fractions (Table 3). The identification of these polypeptides as phaseolin polypeptides was confirmed by N-terminal amino acid sequencing. This showed that the polypeptides originate from a phaseolin molecule by hydrolysis of internal peptide bonds (Figure 2). That vicilins (phaseolins) are detrimental to this bruchid has been recently demonstrated by us for the case of proteins isolated from *C. maculatus* nonhost seeds (23,24).

Results reported by others have shown that legume storage proteins, especially vicilins, are very resistant to proteolysis in the undenatured state and for this reason they are nutritionally poor without previous cooking (25). Furthermore, it has been reported that *P. vulgaris* vicilins strongly bind to tissues in the small intestine of rats, suggesting that the low digestibility of these proteins may be due to their binding to the surface of the gut (26).

It is noteworthy that phaseolin prepared from cotyledons and testa showed slightly different results in the artificial seed assay as shown in Table 3. We attribute the discrepancies to the probably different degrees of heterogeneity of the several subunits of phaseolin as demonstrated by SDS-PAGE (Figure 1). In the same way, the different contributions of several phaseolin bands in fraction F1 from testa tissues may be related to the greater detrimental effects observed for this fraction (Table 3 and Figure 1).

It is well known that the testa of seeds, which is a maternal tissue, contains proteins, some of them enzymes involved in sugar and amino acid transport (27-30). Nevertheless the finding that storage proteins are expressed in the testa of both monocots (31) and dicots (32) was only recently reported. Work in progress in our laboratory suggests that vicilin-type storage proteins are also present in the seed coat of *P. vulgaris*, *V. unguiculata* and *Canavalia ensiformis*. In the case of the Jack bean (*C. ensiformis*) we have shown that the levels of canavalin peptides present in the seed coat are sufficient to deter *C. maculatus* development (7).

Borisjuk et al. (32) suggest that the synthesis of both legumin and vicilin in developing seeds of *Vicia faba* is related to the hexose to sucrose ratio, which in turn is linked to the developmental gradient. Since it was recently shown that legume 7S globulins (vicilins, phaseolins) associate with glucose, N-acetylglucosamine and chitin (23, 24,33), the presence of these proteins in seed coats could be associated with the transport of sugars in this tissue during development.

The finding that phaseolin is expressed in the seed coats of *P. lunatus* in sufficient amounts to deter *C. maculatus* larval development suggests that 7S storage globulins of legume seeds may have played a strong role in the evolutionary discrimination of these by insects of the family Bruchidae (5-7,23,24).

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