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Molecular Cloning and Sequence Analysis of Four New cDNA Fragments of Cytochrome P450 from *Liposcelis bostrychophila* Badonnel (Psocoptera: Liposcelididae)

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Abstract: P450 enzymes are an important metabolic system involved in the metabolism of a phenomenal number of endogenous and exogenous compounds. In China, Liposcelis bostrychophila is a dominant species in stored products and the resistance to PH_3 has been a severe problem. In order to reveal whether P450 enzymes were involved in the resistance to PH_3 or not and the molecular mechanism of resistance, four different P450 cDNA fragments (243 bp) were cloned from the susceptible strain of L. bostrychophila, according to the RT – PCR strategy with a pair of degenerate primers designed on the basis of the heme-binding region of CYP6 family. The putative conserved domains of P450 were found in fragments 1, 2 and 4, by the Blast search in GenBank database. It was the first time that P450 genes had been cloned from L. bostrychophila, which verified the existence of P450 enzymes in L. bostrychophila. The homologous alignment by ClustalX (Ver. 1.81) indicated that the fragments 1, 2 and 4 shared higher identities of deduced amino acid sequences with certain known members of CYP6 family (42% – 67%, 42% – 56% and 41% – 66%, respectively), while fragment 3 showed lower identities (38% – 56%). And the result was further tested by the phylogeny analysis by MEGA (Ver. 3.1) utilizing the UPGMA (unweighted pair group method with arithmetic mean).

Key words: *L. bostrychophila*, P450, RT – PCR, homologous analysis

1 Introduction

P450 enzymes (mixed function oxidases, cytochrome P450 monooxygenases), one of the most important enzyme systems involved in insecticide detoxification or activation, are a complex family of heme containing enzymes found in most organisms. Various kinds of P450 enzymes have been found in animals, microorganisms and plants, and classified into more than 36 families [1,2]. P450 enzymes bind molecular oxygen and receive electrons from NADPH to introduce an oxygen atom to the substrate. In insects, the diverse functions of P450 enzymes range from the synthesis and degradation of ecdysteroids and juvenile hormones to the metabolism of xenobiotics [3,4]. P450 enzymes play important roles in adaptation of insects to toxic compounds in their host plants and are involved in metabolism of all commonly used insecticides. P450 enzymes metabolize organophosphorus insecticide compounds to more active toxicants by activation of a P = S bond to a P = O bond [4,5,6]. However, in general, P450 enzymes mediate metabolic detoxification of other insecticides.

So far, the nomenclature of P450 gene has been established to designate all gene members of the P450 super-family with a CYPprefix, followed by a numeral for the family, a letter for the subfamily, and a number for the individual gene [4,7]. This system defines that members of a family share > 40% identity in amino acid sequence, and members of a subfamily share > 55% identity [4].

Because of the property of instability, most researchers prefer molecular methods to biochemical methods in the insecticide resistance mechanism of P450 enzymes study. The first insect P450 gene (CYP6A1) was isolated from an insecticide-resistant strain of housefly, *Musca domestica* [8]. After that, more and more P450 genes were cloned. To date, more than 1958 P450 genes have been registered in the GenBank database. In China, the biochemical characteristics of P450 enzymes in insects were given more attention and the molecular cloning just started recently. Three new full-length cDNA were cloned from *Aedes albopictus* [9]. A xanthotoxin-inducible cytochrome P450 cDNA (CYP6 B8) was isolated from *Helicoverpa zea* [10], and the full length of $CYP6BF_1$ was obtained from

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Plutella xylostella through the SMART (Switching Mechanism At 5' end of the RNA Transcript) technique [11]. Apart from these, a few cDNA fragments were also reported. Nine CYP4 fragments from *Culex pipiens* Pallens [12], two CYP6 [13] and ten CYP4 [14] fragments from a susceptible strain of *Helicoverpa armigera* were cloned successively. Besides, two new P450 cDNA fragments were gained from deltamethrin resistant strain of *Musca domestica* using differential display PCR technique [15].

Liposcelis bostrychophila, an important stored-product insect pest, is worldwide and commonly found in various processed and unprocessed dry foods in households, granaries, and warehouses [16]. Outbreaks of *L. bostrychophila* have been reported in humid tropical countries such as Indonesia, Malaysia, Singapore, the Philippines, Thailand, the People's Republic of China and India [17,18]. Routine fumigations of warehouses and storage facilities with methyl bromide have failed to control the pest [19]. In addition, the rapid development of resistance to physical and chemical treatments by the psocids has also been reported [20]. In Australia, detection of high level resistance to phosphine in psocids infesting stored commodities [21] has elevated their pest status enormously and put them alongside the major beetle pests [22]. The metabolic resistance mediated by P450 enzymes may be the most important resistance mechanism[3,6,23,24] while the study about the enzymes was lack in psocids. The objectives of this study were to clone the P450 genes and analyze their functions to supply some basic information for further study on the molecular resistance mechanism of psocids.

2 Materials and Methods

2.1 Insects

The susceptible strain of *L. bostrychophila* was cultured in the Key Laboratory of Entomology and Pest Management, Southwest University, Chongqing. The insects were reared on a diet consisting of whole wheat flour, skimmed milk and yeast powder (10∶1∶1) in an air-conditioned room at 27 ± 1℃, RH 75% - 80% and a scotoperiod of 24 h [25]. This strain was not exposed to blended gas or insecticides for 16 years.

2.2 Isolation of Total RNA

Total RNA was isolated from *L. bostrychophila* adults using TRNzol Reagent (Tiangen). One thousand healthy adults of *L. bostrychophila* were homogenized with at least 1 mL TRNzol Reagent in the glass homogenizer. The particular process of total RNA extraction and purification was carried out following the manufacturer's instructions of the reagent kit. Finally the total RNA (A_{260}/A_{280} = 1.8) dissolved in 40 μL DEPC treated H_2O for future use.

2.3 Synthesis of the First Strand cDNA

The first strand cDNA was synthesized by M - MLV RTase cDNA Synthesis Kit (Takara). The reverse transcriptional system included 2 μL total RNA, 1 μL Oligo(dT_{15}) and 8 μL DEPC treated H_2O at 70℃ for 5 min in water and for 10 min on ice. It was then mixed with 5 μL M - MLV 5 × Reaction Buffer, 1.25 μL dNTPs, 1 μL rRNasin and 4.75 μL DEPC treated H_2O at 42℃ for 5 min. Then 1 μL M - MLV reverse transcriptase was added to the mixture above. At last, the mixture was kept at 42℃ for 1 h, 95℃ for 5 min and hold at 4℃.

2.4 PCR Amplification

PCR cloning was carried out using the Takara PCR Amplification Kit (Takara). The 25 μL reproducing system contained 2 μL cDNA template, 2.5 μL 10 × Reaction Buffer, 4 μL 25 mM $MgCl_2$, 4 μL 2.5 mM dNTPs, 3 μL 10 mM forward or reverse primer, 0.5 μL 5 U/μL Taq DNAse and 6 μL DEPC treated H_2O. The system was kept at 94℃ for 2 min, then 30 cycles of polymerase chain reaction (94℃ for 1 min, 50℃ for 1 min, 72℃ for 1 min), 72℃ for 5 min, and was finally hold at 4℃. The degenerate primers used in PCR cloning were designed based on the homologous region of CYP6 family and their nucleotide sequences were as follows [26].

Forward: 5'-CGGARACNHYNMGNAARTAYCC-3'

Reverse: 5'-CGGGNCCNKCNCCRAANGG-3'

Where, H, K, M, N, R and Y are the IUB (International Union of Biochemistry) standard code for degenerate bases. H = A/C/T, K = G/T, M = A/C, N = A/C/G/T, R = A/G and Y = C/T.

The PCR products were purified and recovered by Gel Extraction Mini Kit (Watson Biotechnologies, inc) and ligated to pMD - 18T vector (Takara) at 16℃ overnight. The ligation solution was transformed to *Escherichia coli* competent cell HB101 (Takara). After that, the transformed competent cells were transferred onto LB medium containing Ampicilin, X-gal

and IPTG at 37℃ for 12 h. In succession, the bacteria colonies screened and the white colonies were selected to culture in the LB medium containing no Ampicilin at 37℃ for 12 h. Finally, PCR amplification was applied for plasmid DNA isolated from the culture to confirm the positive cloning that would be sequenced.

2.5 Sequence Analysis

The obtained sequences were translated into proteins. Searches for similar sequences were carried out by Blast Search of GenBank. The multiple alignments of deduced amino acid sequences with other members of CYP6 and CYP3 family were processed using ClustalX software (Ver. 1.81), meanwhile, the identity comparison was executed by Blastp in GenBank. The phylogenetic relationship was analyzed by MEGA(Ver. 3.1).

3 Results

3.1 The Agarose Gel Electrophoresis of RNA

The integrity of mRNA isolated from *L. bostrychophila* was showed indirectly by the agarose gel electrophoresis of rRNA (Fig. 1).

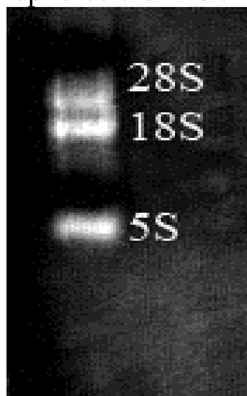

Fig. 1 The agarose gel electrophoresis of total RNA

The three electrophoresis strips marked with 28S, 18S and 5S represented 28S rRNA, 18SrRNA, and 5S rRNA, respectively. The proportion of 28S and 18S seemed to be 1:1 in virtue of strips brightness and width.

3.2 The Agarose Gel Electrophoresis of PCR Products and Their Sequences

The PCR products amplified with a pair of degenerate primers were displayed with the agarose gel electrophoresis (Fig. 2). Four different cDNA fragments, all 243 bp in length, were obtained through the RT – PCR. The corresponding nucleotides sequences and the deduced amino acid sequences (underlined sequences were used for designing degenerate primers) were showed in Fig. 3. According to the Blast search in GenBank database, the putative conserved domains of P450 were found in fragments 1, 2 and 4 while no in fragment 3. Meanwhile, fragments 1, 2, and 4 shared higher identities (42% – 67%, 42% – 56% and 41% – 66%, respectively) than fragment 3 to other members of CYP6 family (38% – 56%), based on the identities comparison of the cloned sequences with corresponding part of other members of P450 gene (Table 1).

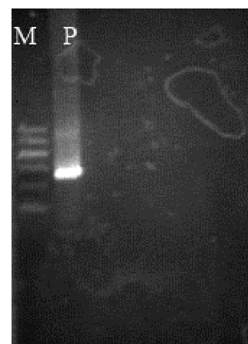

Fig. 2 The agarose gel electrophoresis of the cDNA fragments from PCR cloning. Lane M represents molecular marker, lane P represents PCR product

Table 1. Identities comparison of the cloned sequences with corresponding part of other members of P450 gene

P450 members	Source	Identities of the deduced amino acid sequences (%)			
		Fragment 1	Fragment 2	Fragment 3	Fragment 4
CYP3A1	*Rattus norvegicus*	45	42	46	45
CYP6A2	*Tribolium castaneum*	67	56	46	66
CYP6B1	*Papilio polyxenes*	48	47	41	50
CYP6B2	*Helicoverpa armigera*	46	45	38	45
CYP6D1	*Musca domestica*	46	45	41	47
CYP6D2	*Drosophila melanogaster*	42	45	41	41
CYP6E1	*Culex pipiens quinquefasciatus*	48	48	50	50
CYP6G1	*Apis mellifera*	62	56	43	61

P450 members	Source	Identities of the deduced amino acid sequences (%)			
		Fragment 1	Fragment 2	Fragment 3	Fragment 4
CYP6J1	*Blattella germanica*	53	48	42	50
CYP6K1	*Apis mellifera*	62	56	43	61
CYP6P2	*Anopheles gambiae*	55	56	42	51
CYP6N3v3	*Aedes albopictus*	52	51	45	55
CYP6AY1	*Nilaparvata lugens*	53	50	56	56
CYP6Y1	*Anopheles gambiae*	46	43	43	43

3.3 Homology Analysis of the Sequences

The deduced amino acid sequences, 80 amino acids in length, were aligned by ClustalX (Ver. 1.81) with CYP6 family P450 genes from other insects and CYP3 family P450 genes from rats. By virtue of the alignment, it was concluded that the regions where the degenerate primers were designed were highly conserved, and those were also the conserved regions of CYP6 family (Fig. 4). The dendrogram of the cDNA frgments and certain members in P450 gene family (Fig. 5) was drawn by MEGA 3.1 utilizing the UPGMA. Fragments 1, 2, and 4 were found more closely related to CYP6A2 from *Tribolium castaneum*, while fragment 3 shared higher homology to CYP6AY1 from *Nilaparvata lugens*.

```
Fragment 1
      1 gaaactcttaggaagtacccagtggcaggagtaataattaggcaatgtaataaagactat
      1  E  T  L  R  K  Y  P  V  A  G  V  I  I  R  Q  C  N  K  D  Y
     61 aaaattccggatagcgatatggtgattcccaaaggaacttctacgcatataccgatttat
     21  K  I  P  D  S  D  M  V  I  P  K  G  T  S  T  H  I  P  I  Y
    121 tcccctcattacgattcaaagtatttccgaatcctgagaaatttgatccggatcgtttc
     41  S  P  H  Y  D  S  K  Y  F  P  N  P  E  K  F  D  P  D  R  F
    181 acagaagaagtcaaatctcaacgacctcgttatgcatatttacctttcggcgaaggaccc
     61  T  E  E  V  K  S  Q  R  P  R  Y  A  Y  L  P  F  G  E  G  P
    241 g

Fragment2
      1 gaaaccccaagcaaatatccagtagcgggattattgcttcgagaatgcaacaaagattat
      1  E  T  P  S  K  Y  P  V  A  G  L  L  L  R  E  C  N  K  D  Y
     61 aaagtcccaaatagcgacatggtaataccgaaagggacttcgatacaagtacccatttat
     21  K  V  P  N  S  D  M  V  I  P  K  G  T  S  I  Q  V  P  I  Y
    121 tccctccattatgatccaaaatatttccctgatccacaacgttttgatccggatcgtttc
     41  S  L  H  Y  D  P  K  Y  F  P  D  P  Q  R  F  D  P  D  R  F
    181 aaagaagaagtcaaatcccaacgacatcgttacgcttatttaccgttcggtgaaggcccc
     61  K  E  E  V  K  S  Q  R  H  R  Y  A  Y  L  P  F  G  E  G  P
    241 g

Fragment3
      1 gagacgcctagaaagtaccctcccctacctttgttgttaagaaggtgtacaaaagcgtac
      1  E  T  P  R  K  Y  P  P  L  P  L  L  L  R  R  C  T  K  A  Y
     61 aagattccggattccgatgtaattttagaaccaggaaatctggtttttttcccgacatat
     21  K  I  P  D  S  D  V  I  L  E  P  G  N  L  V  F  F  P  T  Y
    121 tcttatcaacgcgatccagaatattatccggatccggaggaattccgaccagaaagattt
     41  S  Y  Q  R  D  P  E  Y  Y  P  D  P  E  E  F  R  P  E  R  F
    181 tcaccggaaaatcgggaaaaactggtagctcatacgatttttgcccttcggcgacggaccc
     61  S  P  E  N  R  E  K  L  V  A  H  T  I  L  P  F  G  D  G  P
    241 g

Fragment4
      1 gaaacaactagaaaatatccacctgctggtgtagtaagcagagcatgctcaagagactat
      1  E  T  T  R  K  Y  P  P  A  G  V  V  S  R  A  C  S  R  D  Y
     61 caaatccctgattctgatgtgaccattgaaaaaggtatacaagttataatacccgtgttt
     21  Q  I  P  D  S  D  V  T  I  E  K  G  I  Q  V  I  I  P  V  F
    121 ggacttcatcatgatgaaaaatatttccaaatccggaaaagttcgatcctgatcgattt
     41  G  L  H  H  D  E  K  Y  F  P  N  P  E  K  F  D  P  D  R  F
    181 acggaagagggaaaagcctcccggccaaattatacgtacttgccgttcggagctggaccc
     61  T  E  E  G  K  A  S  R  P  N  Y  T  Y  L  P  F  G  A  G  P
    241 g
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Fig. 3 The nucleotides and deduced amino acid sequences of four cDNA fragments (the letters underlined are the nucleotides sequence of the primers)

4 Discussion

Gene cloning by PCR using degenerate primers derived from conserved amino acid sequences from other species has been proved to be a powerful method to obtain related DNA sequences from the target species. Although the P450 super-family has a very divergent sequence and the overall homology may be less than 40% even within the same family, particularly in insects (Wang and Hobbs, 1995), there are still some conserved regions preserved during evolution. And the degenerate primers used in the experiment were designed based on the conserved region of CYP6 family (heme – binding region).

Though the primers applied were degenerate, the agarose gel electrophoresis of the PCR products showed good specificity. Four different cDNA fragments, similar to previous reports in length (Ai et al., 2004a; Li et al., 2005), were obtained after eight.

```
Fragment1     ETLRKYPVAGVIIRQCNKDYKIPDSDMVIPKGTSTHIPIYSPHYDSKYFPNPEKFDPDRF
Fragment2     ETPSKYPVAGLLLRECNKDYKVPNSDMVIPKGTSIQVPIYSLHYDPKYFPDPQRFDPDRF
Fragment4     ETTRKYPPAGVVSRACSRDYQIPDSDVTIEKGIQVIIPVFGLHHDEKYFPNPEKFDPDRF
CYP6A2        ETLRKYPAASIITRTCVKDYKIPDQDIVIEKGTSVIIPVLGIHHDEKFYPNPEKFDPERF
CYP6G1        ETLRKYPPLGFLDRVALHDYKIPNSDVTIDKDTPVIIPMIAFHYDPKYFPNPEKYDPLRF
CYP6K1        ETLRKYPPLGFLDRVALHDYKIPNSDVTIDKDTPVIIPMIAFHYDPKYFPNPEKYDPLRF
CYP6B1        ETLRKYPVADFTQRNAKTDYVFPGTDITIKKGQTIIVSTWGIQNDPKYYPNPEKFDPERF
CYP6B2        ETLRMYSIVEPLQRKAIRDYKLPGTDVVIEKDTVVLISPRGIHYDPKYYDNPKQFNPERF
CYP6E1        ETLRKYPPVANLFREITKNYKVPETDITLEKGYRVVIPVYGIHHDPDIYPNPEVFNPERF
CYP6N3v3      ESLRKYPPASNLTRIVSKDYKLPNCDVVLQQGSTIIVPVYALHHDAEYYPNPEKYDPDRF
Fragment3     ETPRKYPPLPLLLRRCTKAYKIPDSDVILEPGNLVFFPTYSYQRDPEYYPDPEEFRPERF
CYP6AY1       ETMRKYPPVSVLARVCTKPYTIPGTKISIDPGTSVAIPVYSFHHDHKYFPDPETFDPERF
CYP3A1        ETLRLYPIGNRLERVCKKDVEI--NGVFMPKGSVVMIPSYALHRDPQHWPEPEEFRPERF
CYP6Y1        ETLRKHPPVAILERNADKDYRLPDSGLLLRRGQKIMIPIYAMHHDPAHFPEPEQYRPERF
CYP6P2        ETLRKYPPLETVTRAPEHDYTVPGTAHVIPKGTMIQIPIYALHHDAQYYPDPERFDPERF
CYP6D1        ETTRKYPGLPFLNRKCTQDFQVPDTKLTIPKETGIIISLLGIHRDPQYFPQPEDYRPERF
CYP6D2        ETIRKYPGLPFLNRECTEDYPVPGTNHIIAKGTPILISLFGMQRDPVYFPNPNGYDPHRF
CYP6J1        ETLRKYPAIPFLDRRCQEDYPL-TQDLMLPAGTGVYIPVYALHHDSKYFPSPAKFDPERF
              *:   :.       *        .        :         .. . : *   : .*  : * **

Fragment1     TEE-VKSQRPRYAYLPFGEGP
Fragment2     KEE-VKSQRHRYAYLPFGEGP
Fragment4     TEE-GKASRPNYTYLPFGAGP
CYP6A2        TEE-NKAARHHYAHLPFGEGP
CYP6G1        SEE-VKKTRPSYVYMPFGEGP
CYP6K1        SEE-VKKTRPSYVYMPFGEGP
CYP6B1        NPE-NVKDRHPCAYLPFSAGP
CYP6B2        FAE-EVGKRHPCAYLPFGLGP
CYP6E1        IPE-LSTNRHPMAYLPFGEGP
CYP6N3v3      TSE-EVAKRNPYCFLPFGEGP
Fragment3     SPE-NREKLVAHTILPFGDGP
CYP6AY1       SPE-NQEKSINYTYLPFGAGP
CYP3A1        SKE-NKGSIDPYVYLPFGNGP
CYP6Y1        SPD-EVARRDPYCYLPFGEGP
CYP6P2        RPE-VANARPAYVYMPFGEGP
CYP6D1        ADE-SKDYDPA-AYMPFGEGP
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Fig. 4 Homologous analysis of cloned P450 amino acid sequences from *L. bostrychophila* susceptible strain and corresponding sequences of other sources of P450 members

" – " indicates the gaps, " * " indicates the completely conserved residue, " : " indicates one of the "strong" groups (STA, NEQK, NHQK, NDEQ, QHRK, MILV, MILF, HY, FYW) is fully conserved, " . " indicates one of the "weaker" groups (CSA, ATV, SAG, STNK, STPA, SGND, SNDEQK, NDEQHK, NEQHRK, FVLIM, HFY) in amino acids is fully conserved[27].

White plaques (positive cloning) were sequenced, suggesting that more than one gene were cloned. It verified the diversity of P450 genes [6].

The degenerate primers were derived from the conserved region (heme-binding region) of CYP6 family [26]. The putative conserved domains were found in fragments 1, 2, and 4 through the Blast search while no in fragment 3, and it might suggest that fragment 3 was not conserved in the putative conserved domains of CYP6 family or fragment 3 may be a pseudogene evolved from a gene of CYP6 family [29,30].

It is known that the identity comparison is different from homologous analysis. Thus, two methods were used in the process of homologous analysis. That is to say, both sequences identity and phylogeny were analyzed here. And the analysis revealed that the results of the two methods were approximately identical. According to the homologous analysis, the four fragments were probably assumed to be new members of CYP6 family, suggesting P450 genes were cloned from *L. bostrychophila* for the first time. Fragments 1, 2, and 4 shared higher identity to CYP6A2 from *T. castaneum*. The identities amounted to 67%, 52% and 66%, respectively, while an identity of 56% was found between fragment 3 and CYP6AY1 from *N. lugens*. These probably suggested that fragments 1, 2, and 4 belonged to CYP6A subfamily and fragment 3 might be classified to CYP6AY subfamily.

Most of the deduced amino acid sequences were about five hundred residues long, and the four fragments obtained from *L. bostrychophila* just accounted for one eighth of the P450 gene full length. Consequently, the nomenclature and classification of these P450 genes could not be carried out until the gene full lengths were obtained and identified.

Whatever, it was the first time that the P450 genes were cloned from *L. bostrychophila*. Undoubtedly, the fragment cloning was the fist step for the acquisition of gene full length and

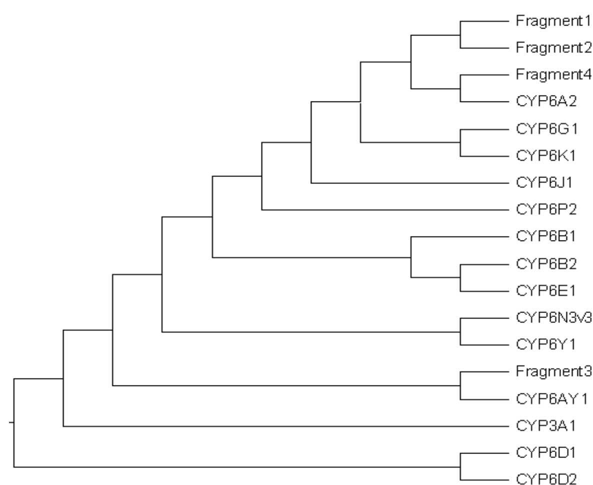

Fig. 5 Dendrogram of certain members in P450 gene family

the results will contribute to the thorough understanding of P450 genes soon and molecular resistance mechanism mediated by P450 enzymes in further study.

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