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Glycine-aspartic_ acid-serine-leucine esterase *Xcc_ est* from *Xanthomonas campestris* pv. *campestris* 8004 and its esterase domain: gene expression in *Escherichia coli*, refolding and characterization

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Abstract: [Objective] To characterize the GDSL (glycine, aspartic acid, serine and leucine motif in protein sequence) esterase *Xcc_ est* from *Xanthomonas campestris* pv. *campestris* (*Xcc*) 8004. [Methods] *Xcc_ est* gene and different domains of *Xcc_ est* gene were PCR amplified and expressed in *Escherichia coli*, the HIS-Tagged fusion proteins were purified by Ni-NTA chromatography. [Results] The optimum pH and temperature of partly purified *Xcc_ est* were 8.0 and 52℃ when *p*NPB (4- nitrophenylbutyrate) was used as substrate. The K_m and V_{max} value of *Xcc_ est* and the passenger domain (*Xcc_ est*N1-334) for pNPB were 47.6 ± 4.6 mol/L, 67.6 ± 7.8 U/mg and 469.4 ± 9.8 mol/L, 2.5 ± 0.9 U/mg respectively. Inclusion bodies of mature domain *Xcc_ est* (*Xcc_ est*N26-606) could be refolded but inclusion bodies of the passenger domain (*Xcc_ est*N26-334) could not be refolded. Refolded mature domain had broad substrate spectrum and showed higher stability than *Xcc_ est* when stored at 25℃. [Conclusions] Refolded *Xcc_ est*N26-606 can be a candidate for biotransformation application.

Keywords: GDSL esterase; *Xanthomonas campestris*; refolding
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Xanthomonas campestris pv. *campestris* (*Xcc*) is a pathogenic bacterium which infects a wide range of plants in the crucifer family by invading and multiplying in plant vascular tissues[1]. *Xcc* 8004 is a strain widely used for phytopathological studies, especially in studies of secretion of extracellular enzymes and exopolysaccharides[2,3]. The extracellular enzymes from pathogenic bacteria often play important roles as virulence factors[4] and also can be used as biocatalysts in industrial applications[5]. GDSL esterase is one of those enzymes which related to quorum sensing in gram negative bacteria[6], cell motility and biofilm formation[7]. There are also evidences indicated GDSL esterases have broad hydrolytic activity toward different substrates[8]. Therefore, GDSL esterases are good candidates for both biotransformation application and physiological study. Most of the GDSL esterases consist of two domains; one domain is a surface-exposed N-terminal passenger domain (or α-domain) which harbors an active site serine in a GDSL motif and other residues of the catalytic site, and the other domain is a C-terminal β-domain located in the outer membrane[9]. The C-terminal domain (β domain) of the GDSL esterase is similar to a newly identified family of autotransporting

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bacterial virulence factors[10]. Generally, GDSL esterases belong to the family II of lipolytic enzymes[11]. According to the distance-based phylogenetic analysis[12], GDSL esterase genes were classified into five distinct clades, most of the bacterial GDSL esterases were represented in clades I and II, while most of the higher green plant GDSL esterases were represented in clades III, IV and V.

In this paper, we reported the expression of GDSL esterase *Xcc_est* gene and its passenger domain *Xcc_est*N1-334 gene in *E. coli*, purification of the HIS-tagged fusion proteins and refolding of the inclusion bodies of the mature domain of *Xcc_est*.

1 MATERIALS AND METHODS

1.1 Bacterial strains, plasmids and culture media

Xcc 8004 was a kindly gift from Prof. Rong xiang Fang (Institute of Microbiology, Chinese Academy of Sciences), *Escherichia coli* DH5α was used for cloning work. *E. coli* BL21 (DE3) was used as host for expression experiments. pET30a (+) was used for standard cloning experiments and expression in *E. coli*. *E. coli* cells were routinely grown in LB medium or on LB agar plates at 37℃. *Xcc* 8004 cells were grown at 30℃ in LB medium or on agar plates. For plasmid selection, 50 μg ml/L of kanamycin was added in medium.

1.2 Extraction and purification of DNA

Isolation of plasmid was performed using the TIANprep Mini Kit of TIANGEN (Beijing, PR China) according to the protocol provided. Isolation of chromosomal DNA of *Xcc* 8004 strains was performed using the TIANamp Bacteria Kit of TIANGEN (Beijing, PR China) according to the protocol provided. DNA was purified using the TIANgel Midi Kit of TIANGEN (Beijing, PR China) according to the protocol provided.

1.3 Gene cloning and expression

Xcc_est gene was PCR amplified directly from the *Xcc* 8004 genomic DNA using Red-*Pfu* DNA Polymerase (BIOCOLORS, Beijing, PR China) with forward *Xcc_est* primer and reverse *Xcc_est* primer (listed below). Primers were designed according to the genome sequence annotation of *Xcc* 8004 (Accession No. CP000050). forward *Xcc_est*: 5'-GGAATTC CATATGGCTTCAACCCTTCGCCCGATCCG-3'; reverse *Xcc_est*: 5'-CGAGCTCGAAGTTGCCGCT GAAGTTC-3'.

The PCR product was double digested with *Nde*I and *Xho*I and cloned into pET30a (+). The plasmid harboring the PCR product insert was named as pET*Xcc_est*. The plasmid was transformed into *E. coli* BL21 (DE3) cells and induction of esterase expression was performed at an optical density at 580 nm (OD 580) of 0.8, by adding isopropylthiogalactoside (IPTG) to a final concentration of 1.0 mmol/L. After expression the cells were harvested by centrifugation (4000 g for 10 min).

Gene amplifications of the mature domain (*Xcc_est*N26-606 gene, numbers indicated the amino-acid sequence number from N-terminal), the passenger domain (*Xcc_est*N1-334 gene) and the mature passenger domain (*Xcc_est*N26-334 gene) were conducted with the according primers listed below respectively using pET*Xcc_est* as the template.

*Xcc_est*N26-606 gene, forward *Xcc_est*N26-606: 5'-GGAATTCCATATG GACTCGGCCTTCGA TCAAA-3' and reverse *Xcc_est*; *Xcc_est*N1-334 gene, forward *Xcc_est* and reverse *Xcc_est*N1-334: 5'-CCTCGAGGGGCTTGCCGTCGAGATGCCAC GCC-3'; *Xcc_est*N26-334 gene, forward *Xcc_est*N26-606 and reverse *Xcc_est*N1-334.

Restriction enzymes digestion and ligation were the same strategies as for *Xcc_est* gene. The pET30a (+) harboring the PCR fragment were named as pET*Xcc_est*N26-606, pET*Xcc_est*N1-334 and pET*Xcc_est*N26-334 respectively. Expressions of the three genes were also the same strategies as for *Xcc_est* gene.

1.4 Single step purification of *Xcc_est* and *Xcc_est*N1-334 on Ni-chelating column

Purification on Ni-chelating column was conducted using the protocol and buffer supplied by Novagen. Cells of the 250 mL expression culture were suspended in 20 mL binding buffer and then sonicated. The

supernatant was applied into a 1 mL Novagen His Band gravity flow column which was equilibrated with 20 mL Ni-NTA binding buffer, then washed with 20 mL wash buffer. His-tagged proteins were eluted with 10 mL elution buffer. The eluate was collected and dialyzed against phosphate buffer (pH 7.2) for 24 h and concentrated by lyophilization.

Protease inhibitor cocktail (500 μmol/L AEBSF, 150 nmol/L Aprotinin, 1 μmol/L E-64 proteinase inhibitor, 0.5 mmol/L EDTA, 1 μmol/L Leupeptin; Merck, Darmstadt, Germany) was added into the elution when needed.

1.5 Purification and refolding of inclusion bodies

Purification and refolding of inclusion bodies was conducted according to the method applied for refolding of *E. coli* outer-membrane phospholipase A[13].

Refolding solution was applied into a DEAE sepharose column equilibrated with buffer A (30 mmol/L Tris, 5 mmol/L EDTA, pH 8.0). Proteins were eluted with a linear NaCl gradient (0 – 2 mol/L) with ten column volumes of buffer A. Fractions with enzyme activity were pooled and freeze dried.

1.6 Esterase activity assay (pNPB method)

Esterase activity was determined spectrophotometrically at 405 nm using 1 mmol/L *para*-nitrophenyl butyrate (*p*NPB, Sigma) as previously described[8]. One unit of enzyme activity is defined as the amount of enzyme forming 1 mol of substrate per min.

1.7 Esterase activity staining

Native gels were incubated in a solution of 0.1 mol/L sodium phosphate (pH 7.0), then activity staining was carried out as described previously[8].

1.8 Lipase activity assay

Lipase activity was analyzed using an olive emulsion method[14]. One lipase unit was defined as the enzyme required to release 1 μmol of fatty acid per minute at 50℃, pH 7.0.

1.9 Protein methods and SDS-PAGE

Protein concentration was determined using the BCA Protein Assay Kit of Pierce (Rockford, USA) with bovine serum albumin (BSA) as a standard.

SDS-PAGE was performed with a 6% stacking gel and a 12% separating gel.

1.10 Software and online service

The program BLAST X[15] was used for protein homology searching, SignalP Server was used for prediction of protein signal peptide[16].

2 RESULTS

2.1 Clone and expression of *Xcc_est* gene, mature domain of *Xcc_est* gene (*Xcc_est*N26-606), passenger domain of *Xcc_est* gene (*Xcc_est*N1-334) and mature passenger domain of *Xcc_est* gene (*Xcc_est*N26-334)

The GDSL esterase gene was amplified by PCR from the genome DNA of *Xcc* 8004. Sequencing of the 1.8 kb insert (designated as *Xcc_est* gene) revealed an ORF of 1,818 bp, encoding a polypeptide of 606 amino acid residues (MW 62kDa). The average G + C content of the esterase-encoding sequence was 67%.

Because purification of *Xcc_est* was unsuccessful, other two purification strategies were applied. The outline of the first strategy is, because a potential signal peptide of 25 amino acids of *Xcc_est* was predicted[16], based on the result *Xcc_est*N26-606 gene was supposed to express in the form of inclusion body. The inclusion bodies could be purified, and then the purified inclusion bodies were refolded and further separated. Another strategy was based on the structure analysis information in Pfam online database (http://www.sanger.ac.uk/Software/Pfam) which indicated the passenger esterase domain is from Met-1 to Pro-334, therefore *Xcc_est*N1-334 gene (passenger domain) and *Xcc_est*N26-334 gene (mature passenger domain) were cloned.

The four genes could be expressed in *E. coli* (Fig. 1). As expected, no esterase activity could be detected in the expression lysates of *Xcc_est*N26-606 and *Xcc_est*N26-334 gene, and over-expressed protein only existed in the pellet fraction on the SDS-PAGE (Fig. 1, lane 2 and lane 4).

2.2 Purification and kinetic parameters of *Xcc_est* and *Xcc_est*N1-334

*Xcc_est*N1-334 could be purified to 92% homogeneity by single-step purification on Ni-chelating

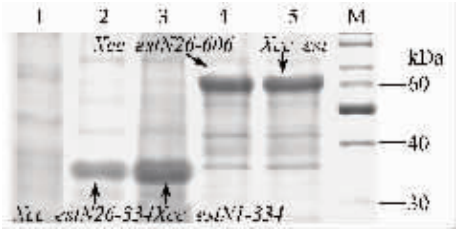

Fig.1 SDS PAGE of expressions of *Xcc_est*, *Xcc_est*N26-606 *Xcc_est*N1-334 and *Xcc_est*N26-334 genes. Lane 1: induction of pET30a empty plasmid; lane 2: *Xcc_est*N26-334; lane 3: *Xcc_est*N1-334; lane 4: *Xcc_est* N26-606; lane 5: *Xcc_est*; M: protein marker.

column (Fig. 2, lane 2). However, *Xcc_est* could only be purified to about 65% homogenecity (Fig. 2, lane 1); furthermore, when the protein concentration of *Xcc_est* exceeded 2 mg/mL in phosphate buffer (pH7.2), the protein aggregated into pellets and severe degradation occurred even though multiple protease inhibitors (protease inhibitors cocktail) were added. Though *Xcc_est* was not well purified, kinetic parameters of the two enzymes were determined (Table 1). It was obvious that *Xcc_est* showed higher affinity to *p*NPB substrate than *Xcc_est*N1-334.

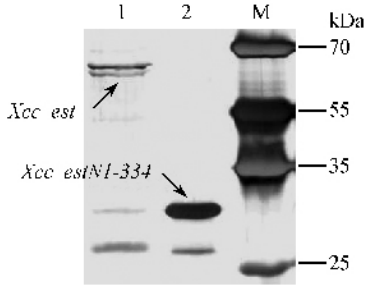

Fig.2 SDS PAGE of *Xcc_est* and *Xcc_est*N1-334 purified by single step purification on Ni-chelating column. lane1: *Xcc_est*; lane2: *Xcc_est*N1-334; M: protein marker.

Table 1 Apparent kinetic parameters of *Xcc_est* and *Xcc_est*N1-334

Purified enzyme	K_m/(μmol/L)	V_{max}/(U/mg min)
Xcc_est	47.6±4.6	67.6±7.8
*Xcc_est*N1-334	469.4±9.8	2.5±0.9

Lineweaver-Burk analysis was used to calculate the V_{max} and K_m. 0.1 mg of enzyme was added to the substrate solution, different concentration of *p*NPB (0.1 – 1 mmol/L) was dissolved in substrate solution and tested. Reactions were carried out in standard condition. Numbers are averages of three replicates.

2.3 Refolding of inclusion bodies of *Xcc_est*N26-606 and *Xcc_est*N26-334 and purification of refolded *Xcc_est*N26-606

Inclusion bodies of *Xcc_est*N26-606 were well purified (Fig. 3, lane 3). The refolding process was carried out with two types of detergents (charged and neutral). Refolding efficiency strongly depended on the type of detergent in the refolding solution. Refolding occurred in the presence of the neutral detergent like Triton X-100 or Brij 35. After optimization of the refolding process (0.1 mg/mL inclusion bodies, 10 mmol/L Triton X-100, 20 mmol/L tris HCl, pH 8.0 5 mmol/L EDTA, 16 h), about 5 % of the inclusion bodies could be refolded (Fig. 3, lane 2) according to the band scanning result. Motility difference could be achieved on SDS-PAGE gel between unfolded protein and refolded protein as shown in Fig. 3 which was called 'heat modifiability'[17] (All the samples were not boiled before loaded). However, attempts to purify the refolded *Xcc_est*N26-606 failed, severe degradation occurred during the purification process.

The inclusion bodies of *Xcc_est*N26-334 (mature passenger domain of *Xcc_est*) could not be refolded after much condition optimizations.

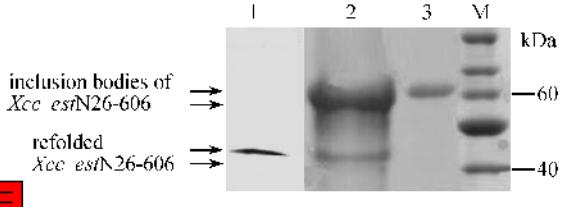

Fig.3 SDS PAGE of inclusion bodies, refolding mixture of *Xcc_est*N26-606 and activity staining of *Xcc_est*N26-606 refolding mixture after SDS PAGE (All the samples were not boiled before loaded). lane1: activity staining of *Xcc_est*N26-606 refolding solution after SDS PAGE; lane2: refolding solution of *Xcc_est*N26-606; lane3: inclusion bodies of *Xcc_est*N26-606; M: protein marker Same samples were applied in lane 1 and lane2 respectively.

2.4 General properties of the purified *Xcc_est*

Maximum activity was found at pH 8.0 in 100mmol/L potassium phosphate buffer for pNPB hydrolysis. The activity was measured at various temperatures, and the hydrolysis activity increased with increasing temperature, reaching a maximum at 52℃. *Xcc_est* also had weak specific activity (8.6 U/mg) when oliver oil was used as substrate. The thermostability of the enzyme was also tested, and it remained 90% activity after incubation for 30 min at 50℃.

2.5 Stability of refolded *Xcc_est*N26-334 at 25℃

The refolded *Xcc_est*N26-606 in refolding solution stored at 25 C was applied for stability test. As shown in Fig. 4, refolded *Xcc_est*N26-606 had much higher stability than purified *Xcc_est*. After a 60-day storage, for *Xcc_est* 95% of activity was lost while only 7% of activity was lost for refolded *Xcc_est*N26-606.

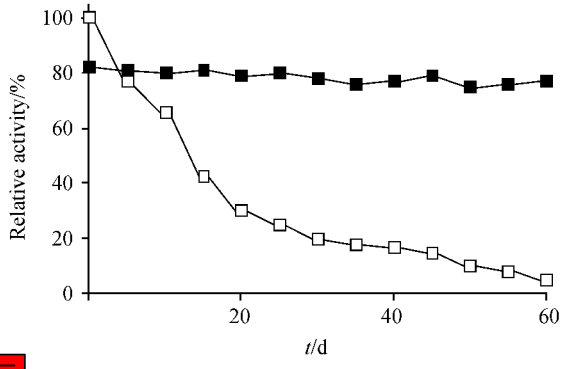

Fig.4 Stability of purified *Xcc_est* (□) and refolded *Xcc_est*N26-606 (■) stored at 25 °C. Initial specific activity: *Xcc_est*, 32.5U/mg (100% relative activity); refolded *Xcc_est*N26-606, 26.6 U/mg (82% relative activity), Reactions were carried out in standard condition using *p*NPB as substrate.

3 DISCUSSION

The BLAST result of *Xcc_est* revealed 99% identity to a GDSL esterase *Xv_*EstE from *Xanthomonas vesicatoria* DSM 50861, which was another pathogenic variant of specie *Xanthomonas campestris*[8]. *Xcc_est* was a typical GDSL esterase which had five highly conserved blocks (Fig. 5), a GDSL-like lipase/acylhydrolase domain (from Thr-32 to Ala-298) and an autotransporter β-domain (from Glu-335 to Thr-596). According to the block alignment and comparison to the site-directed mutagenesis results of *A. hydrophilia* actyltransferase[18], it could be predicted that catalytic triad of the *Xcc_est* was formed by Ser-38, Asp-282 and His-285 (Fig. 5, marked with asterisk). Strikingly, both *Xcc_est* and *Xv_*EstE had a fifth threonine in the GDSLT motif (Fig. 5, underlined), which was a characteristic of the higher green plant GDSL esterases/lipases[12].

The β-domain is the translocator domain of the GDSL esterase[19]. Two distinct models about the structure of the translocator and translocation process of the passenger protein were proposed.[19,20]. The β domain is very important to passenger proteins in both models; it is probably related to the maturation of the passenger protein by triggering the folding and releasing the passenger proteins to the cell surface.

Comparison of the refolding results between *Xcc_est*N26-606 and *Xcc_est*N26-334 implied the β domain

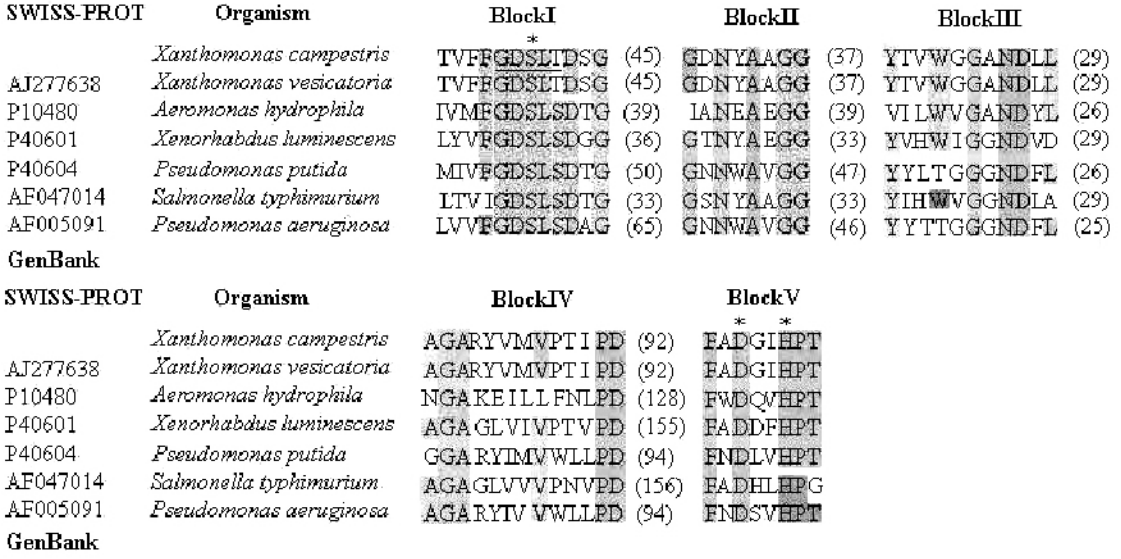

Fig.5 Sequence comparison between *Xanthomonas campestris* esterase (*Xcc_est*) and members of GDSL lipolytic enzymes. Numbers in parentheses refer to the number of amino acid residues between the conserved blocks; Identical amino acids are shaded in grey; The putative catalytic triad residues are marked with asterisk (*) and the G-D-S-L-T consensus motif is underlined.

was probably a key role in the refolding process of *Xcc_est*. Comparison of K_m and V_{max} values between *Xcc_est*-act and *Xcc_est* indicated the β domain was also important for the catalyzing performance of *Xcc_est*. To elucidate the essential role of β domain of *Xcc_est*, it is better to express *Xcc_est* gene and its composition domain genes respectively in *Xanthomonas campestris* mutant which has no background esterase activity. Those works are underway in our lab.

Refolded *Xcc_est*N26-606 showed a same broad acceptance of substrates (Table 2) as *Xv_*EstE from *Xanthomonas vesicatoria* DSM 50861[8]. Unlike *Xv_*EstE, *Xcc_est* also had a lipase activity according to our work. Considering the outstanding room temperature stability of refolded *Xcc_est*N26-606, the enzyme is a good candidate for biotransformation application.

Table 2 Specific activity for hydrolysis of various substrates

Substrate	Specific activity /(U/mg)
p-NP acetate	24.3
p-NP butyrate	26.6
p-NP caproate	50.3
p-NP laurate	8.2
p-NP tetradecanoic acid	2.1
p-NP oleic acid	0
p-NP stearic acid	0
Oliver oil	8.6

p-NP: para-nitrophenyl. One unit of esterase activity is defined as the amount of enzyme forming 1 mol of substrate per min. One lipase unit was defined as the enzyme required to release 1 μmol of fatty acid per minute under 37℃, pH 7.0. The numbers are averages of three replicates, standard deviation < 5%.

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野油菜黄单胞菌 8004 甘天丝亮特征序列酯酶及其酯酶结构域在大肠杆菌中的表达，包涵体复性及性质

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摘要：【目的】了解野油菜黄单胞菌(*Xanthomonas campestris* pv. *campestris*)8004 GDSL(蛋白序列中甘氨酸、天冬氨酸、丝氨酸和亮氨酸特征序列)酯酶的性质。【方法】利用 PCR 方法扩增 *Xcc_est* 及其不同结构域的基因，这些基因以组氨酸标签融合蛋白的形式在大肠杆菌中获得表达。融合蛋白通过镍亲和色谱纯化。【结果】部分纯化的 *Xcc_est* 在催化对硝基苯丁酸酯时，最适 pH 值为 8.0，最适温度为 52℃。*Xcc_est* 对于对硝基苯丁酸酯的 K_m 值和 V_{max} 值分别是 47.6 ± 4.6 μmol/L，和 67.6 ± 7.8 U/mg，*Xcc_est* 的酯酶结构域(*Xcc_est*N1-334)对于同一底物的 K_m 值和 V_{max} 值分别是 469.4 ± 9.8 μmol/L 和 2.5 ± 0.9 U/mg。*Xcc_est* 的成熟结构域(*Xcc_est*N26-606)可以获得成功复性，但是成熟酯酶结构域(*Xcc_est*N26-334)不能获得复性。复性后的 *Xcc_est*N26-606 底物谱较广，在室温下具有较高稳定性。【结论】复性的成熟结构域蛋白(*Xcc_est*N26-606)具有一定的生物转化应用前景。

关键词：GDSL 酯酶；野油菜黄单胞菌(*Xanthomonas campestris*)；复性

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