

## Baculo-expression and enzymatic characterization of CES7 esterase

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**The male reproductive tracts in different species are characterized by similar patterns of male-dependent overexpression of carboxylesterases. This phenomenon indicates male sex-associated functions of these enzymes for spermatogenesis, sperm maturation, and sperm use. Recently, a novel epididymis-specific gene named *Ces7* was cloned and characterized, which belongs to the carboxylesterase family. To study the functions of CES7 in sperm maturation and storage, CES7 recombinant protein was expressed in baculovirus system. The recombinant protein had carboxylesterase activity hydrolyzing cholesterol ester and choline ester. CES7 as carboxylesterase might be involved in ester hydrolysis, sperm maturation, and storage in male reproductive tract.**

**Keywords** esterase; carboxylesterase; baculo-expression

Received: April 17, 2009 Accepted: May 18, 2009

### Introduction

Carboxylesterases (CEs) are ubiquitous proteins identified in species ranging from bacteria to men, which represent a multigene family of serine-dependent enzymes (carboxyl-ester hydrolase; EC 3.1.1.1). They can hydrolyze carboxylester, amide, and thioester bonds in a variety of exogenous and endogenous compounds [1]. Male-dependent carboxylesterases are overexpressed in the male reproductive tracts of different animal species (bivalve mollusks, fruit-flies, and mammals). This phenomenon indicates male sex-associated functions of these enzymes. It was proposed that male genital tract carboxylesterases might protect the male reproductive

system against xenobiotic influences, and provide advantageous metabolic capabilities to sperm protection, maturation, and sperm use [2,3].

During their passage through the epididymis, the spermatozoa undergo a process of maturation leading to acquire their fertilizing ability. The epididymis displays regional variations in the morphology and metabolic properties of its epithelium which are relevant for the progressive development of mature sperm characteristics [4]. In our recent report, a novel gene named *Ces7*, a member of carboxylesterase family, was identified in the rat epididymal cDNA library, and was mainly expressed in the corpus and cauda epididymis. In the present study, we studied the enzyme kinetics, substrates, and characterizations of CES7 in the eukaryotic baculo-express system.

### Materials and Methods

#### Total RNA preparation and cDNA synthesis

Healthy male Sprague-Dawley rats (SLACCAS, Shanghai, China) at 12 weeks of age were used. The rats were sacrificed and the whole epididymides were removed and stored at  $-80^{\circ}\text{C}$  until used. The RNA isolation was performed as previously described [5]. Small pieces of the frozen epididymis were homogenized in TRIzol reagent (Invitrogen, Foster City, USA), and total RNA was extracted with chloroform. After being purified by isopropyl alcohol, the extract was treated with DNase I (Takara, Tokyo, Japan). cDNA was synthesized from the total RNA using ReverTra Ace (ToYoBo, Tokyo, Japan) with an oligo(dT) primer as previously described [6].

#### cDNA cloning and baculovirus construction of *Ces7*

The PCR amplification was carried out to obtain the full length of rat epididymal *Ces7* cDNA using rat

epididymal cDNA described above as a template. The following primers were used: 5'-GTTGAATTCATGAGTGGGGACTG-3' (sense) and 5'-GAAGTCGACCTAAGAAGCAATGGA-3' (anti-sense). The PCR reactions with 30 cycles were performed at denaturing, annealing, and extension temperatures of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s, respectively, with Ex-Taq (Takara, Dalian, China). The amplified DNA fragment was sub-cloned into the pGEM-T easy vector (Promega, Madison, USA).

### Expression of CES7 recombinant protein

Recombinant baculovirus expressing the full-length CES7 protein were generated using Bac-to-Bac system (Invitrogen, Carlsbad, USA). Expression of CES7 protein using baculovirus was carried out according to the instructions of the manufacturer (Invitrogen). The cDNA in the cloning vector was sub-cloned into the pFAST BAC1 vector. The pFAST BAC1 vector containing *Ces7* cDNA was transformed into DH10Bac cells followed by transposition of the inserts into bacmid DNA. The recombinant bacmid DNAs were separately transfected into Sf9 cells with CELL FECTIN reagent (Invitrogen) and the virus was harvested 72 h later. The supernatant containing the virus was stored at 4°C in the dark with 5% fetal bovine serum. The viruses were used to infect High Five cells (Invitrogen) to produce the secreted CES7 protein in Express Five STM media (Invitrogen). The media were routinely harvested 48 h after infection, and stored -20°C until used. The concentrated (Microsep centrifugal super-filter, PALL, Ann Arbor, USA) and dialyzed media were analyzed for hydrolase activities. Protein concentration was determined using Bradford assay with bovine serum albumin as standard.

### Enzyme activity assay

The carboxylesterase activity was determined using spectrophotometric assay. The hydrolysis of *p*-nitrophenylacetate (PNPA) (Sigma, St. Louis, USA) was determined in PBS buffer by measuring the amount of released *p*-nitrophenol with the spectrophotometer (Model GQ1300, GE, Buntinghamshire, UK) according to the method previously described [7] at concentrations ranging from 100 to 800 μM.

The hydrolysis of cholesterol ester was assayed by an enzymatic determination using the Cholesterol/Cholesterol Ester Quantitation Kit (BioVision, Mountain View, USA) according to the manufacturer's recommendations. Cholesterol acetate (Sigma) of the final

concentration 200 μM was used as a substrate in PBS buffer (pH 7.4, 37°C). The hydrolysis of cholesterol acetate was determined by measuring the amount of released free cholesterol.

According to the instructions provided in the Acetylcholinesterase Quantitation Kit (Genmed, Shanghai, China), the acetylcholinesterase activity was determined using spectrophotometric assay (GE GQ1300) with acetylthiocholine iodide (Genmed) of the final concentration 300 μM as a catalytic substrate and 5,5'-dithiobis-2-nitrobenzoic acid (DTNB, Ellman reagent; Genmed) as a chromogenic substrate.

Specific activities of CES7 toward all substrates used were expressed in terms of the amount of substrate hydrolyzed in 1 min under specified conditions.

### Other methods

The cDNA fragment encoding the selected peptide (68 amino acids) of *Ces7* was cloned into the pQE expression system (Qiagen, Amtsgericht Düsseldorf, Germany). The cDNA fragment was amplified by PCR using the following primer pairs, forward primer 5'-GCGGGATCCATGAGGGAAGCGCCT-3' with *Bam*HI site, and reverse primer 5'-GCGGGTACCTGTAACCAGCCCAGG-3' with *Kpn*I site. After enzyme digestion, the fragment was inserted into the pQE30 vector (Qiagen). The expression vector was constructed according to the standard protocol in pQE expression manual. The purification of the recombinant protein from the inclusion bodies and the preparation of the rabbit polyclonal anti-sera against CES7 recombinant peptide were performed as previously described [8,9].

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting were performed as described previously [10]. Total protein extract for each sample was electrophoresed on 8% polyacrylamide gels (SDS-PAGE), electroblotted onto Hybond-P membranes (GE), immunoblotted, and visualized by the ECL Plus system (GE). Anti-CES7 anti-serum (dilution 1:10,000) was used as primary antibody. The secondary antibody (dilution 1:10,000) was the goat horseradish peroxidase-conjugated anti-rabbit immunoglobulin G (Calbiochem, Darmstadt, Germany).

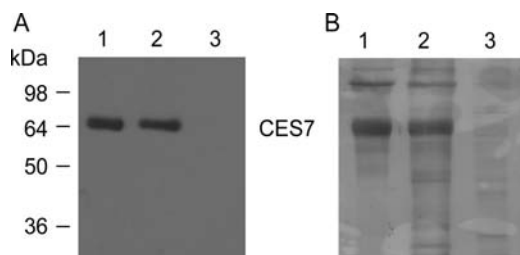
## Results

### Baculovirus construction and expression of CES7 recombinant protein

The cDNA encoding CES7 protein (GenBank accession No. AF479659) was obtained from cDNA of rat

epididymis by PCR amplification. The specific primers were designed on the nucleotide sequence of *Ces7*. The obtained cDNA, referred to as *Ces7*, contained an in-frame initiation codon (ATG) and a termination codon (TAG). The cDNA possessed an open reading frame of 1728-bp encoding a polypeptide of 575 amino acid residues, which was identical to that encoded by *Ces7* (L. Zhang, submitted paper to ABBS).

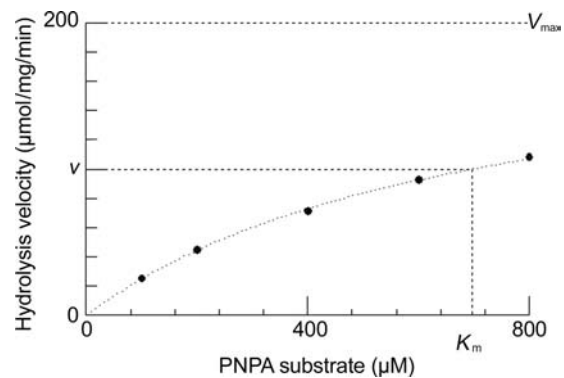
The *Ces7* was inserted into baculovirus and successively transfected into Sf9 cells and High Five cells. Western blot analysis using anti-CES7 serum identified a major immuno-reactive protein produced by the recombinant virus in the media (Fig. 1). The estimated molecular mass of this protein expressed in the insect cells was 64 kDa which was lower than 70 kDa, the apparent mass of epididymal CES7 (L. Zhang, submitted paper to ABBS), perhaps because of their different glycosylations. On the other hand, no protein in non-infected media was detected by western blotting (lane 3).



**Figure 1 Baculo-expressed CES7 protein identity** Western blot analysis (A) and silver staining (B) of CES7 protein recovered from the media with anti-CES7 sera. Lane 1, media recovered by filtration concentration; lane 2, concentrated media recovered by dialysis; lane 3, media of mock infected recovered by concentration. Each lane of samples loaded was concentrated from 1 ml media.

### Enzymatic characterization of CES7 recombinant protein

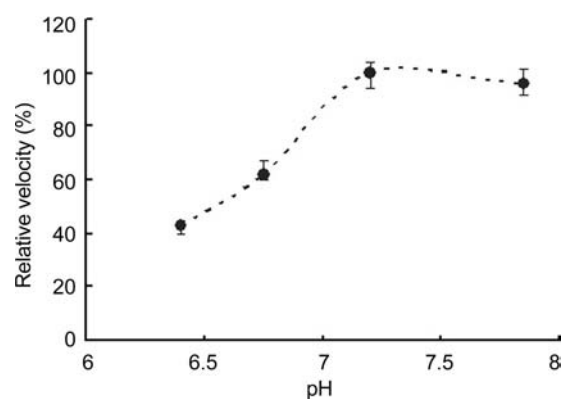
The enriched recombinant CES7 preparation from the media after concentration and dialysis was analyzed for carboxylesterase activity with PNPA as substrate. The media of the High Five cells infected with the recombinant virus showed a high level of PNPA hydrolase activity. Except the spontaneous hydrolysis of the substrate, the reaction obeyed Michaelis–Menten kinetics (Fig. 2), with  $K_m$  value of 670  $\mu\text{M}$  and  $V_{max}$  value of 200  $\mu\text{mol}/\text{mg}/\text{min}$ .  $K_m$  and  $V_{max}$  were determined from Lineweaver–Burk plots. As a control, the media of non-infected High Five cells were also subjected to the same process. No hydrolase activity could be detected in this case (data not shown).



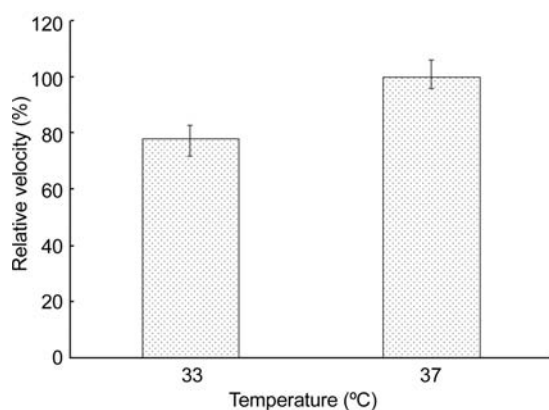
**Figure 2 Kinetic characterization of CES7 carboxylesterase activity** Substrate concentration dependency of the CES7 reaction velocity was shown. Carboxylesterase activity was determined in PNPA hydrolysis. The data are the mean of three independent measurements.

The enzymatic kinetic characterizations are affected by the pH and temperature of the reaction buffer. The pH or temperature of male reproductive tract is different from that of other tissues. For further kinetic characterization, the relative reaction velocity ( $v$ ) observed for saturating PNPA concentrations at pH 7.2 or 37°C was referred to the highest carboxylesterase activity (100%). The pH response curves shown in Fig. 3 revealed distinct pH optima for the hydrolysis reaction. There was a broad pH-optimum between pH 7 and pH 8. Furthermore, the temperature responses to the reaction velocity were shown in Fig. 4. The reaction velocity at 33°C was about 78% of the one at 37°C.

The recombinant CES7 protein showed hydrolytic activity toward PNPA as a usual substrate of carboxylesterase. It also showed catalytic activity toward



**Figure 3 pH dependency of the CES7 reaction velocity** Carboxylesterase activity of CES7 was determined at saturating PNPA substrate concentrations. The data are the mean of three independent measurements.



**Figure 4 Temperature dependency of the CES7 reaction velocity** Carboxylesterase activity of CES7 was determined at saturating PNPA substrate concentrations. The data were the mean of three two independent measurements.

cholesterol acetate and acetylthiocholine iodide, which are also typical esterase substrates. The kinetic parameters of CES7 for the hydrolysis of cholesterol ester and choline ester are listed in **Table 1**. Average values from three independent measurements were presented.

**Table 1 Cholesterol esterase and cholinesterase activities of CES7 protein**

Substrate	Substrate concentration (mM)	Reaction velocity (nmol/mg/min)
Cholesterol acetate	0.2	$6.0 \pm 0.5$
Acetylthiocholine iodide	0.3	$2.6 \pm 0.3$

## Discussion

To identify the CES7 esterase in rat epididymis, we expressed and characterized the protein in baculovirus expression system. The results showed that recombinant CES7 protein exhibited the high levels of carboxylesterase activities and of cholesterol or choline esterase activity.

Overexpressed male-dependent carboxylesterases are abundant in the male reproductive tissues and fluids [11]. Up to 70 esterases isoforms from mouse testis, epididymis and vas deferens have been identified by 2D gel electrophoresis [2]. Carboxylesterase recruitment by male reproductive system may be adaptive for spermatogenesis, sperm maturation, and sperm use, although the

natural *in vivo* substrates of most carboxylesterases are unknown. The presence of Est-6 (the major carboxylesterase in *Drosophila melanogaster*) has effects on the duration of copulation, remating of female, sperm use, and progeny production [12–14]. Furthermore, carboxylesterases such as CES7 in mammalian animals might also hydrolyze glycerides to yield glycerol backbones of glycerol-phospho-choline, which are found in abundance in the mammalian epididymal fluids, and are believed to serve as organic osmolytes [15,16].

Cholesterol, cholesterol derivative sterols are common lipids in male reproductive tracts and sperm [17,18]. Cholesterol distribution is highly organized in spermatid membranes, whose level determines sperm membrane fluidity [19]. In the testes, cholesterol/esterified cholesterol ratio is argued to manipulate normal spermiogenesis process [20]. Furthermore, the free cholesterol in the epididymal lumen fluids and the sperm membrane could be involved in the spermatozoa maturation [21]. The cholesterol/phospholipids ratio of the epididymal sperm membrane is the important factor on sperm capacitation and acrosome reaction [22]. The epididymal esterases might be critical for cholesterol ester hydrolysis and free cholesterol supply. CES7 as a cholesterol esterase was highly specifically synthesized in distal epididymis, and it might be involved in this process.

Cholinergic neurotransmitter system molecules are found to play a role during fertilization in invertebrate and vertebrate organisms [23]. Recent studies showed acetylcholine could stimulate human spermatozoa motility, and affect the initiation of acrosome reaction [24,25]. CES7 as a cholinesterase in the epididymis could hydrolyze cholinergic agents, and might contribute to prevent mature sperm from being activated.

In the present study, cDNA cloning was performed to identify the *Ces7* gene. The deduced amino acid sequence of CES7 contained several motifs characteristics of carboxylesterase isozymes and other serine hydrolases [26]. For example, CES7 contained several Cys residues which are highly conserved in the serine hydrolase family and may function as disulfide bonds. Its sequence also possessed common structures of the carboxylesterase and serine hydrolase superfamily, Ser<sup>226</sup>, Gln<sup>251</sup>, and His<sup>454</sup>, which comprise a catalytic triad, and Gly<sup>214</sup>–Gly<sup>215</sup>, which may be a part of the oxy-anion hole for substrates. All of the residue motifs are highly conserved in the typical serine hydrolase family. NXSX motifs, putative N-glycosylation sites found in most mammalian CES genes, are very interesting because most carboxylesterases are high-mannose-

type glycosylated proteins [27]. There were four N-glycosylation sites (amino acid sequences and sites: NVSD at 281–284, NEST at 363–366, NESY at 515–518, and NIST at 524–527) in CES7 protein. Moreover, the glycosylation is essential for maximal catalytic activity of mammalian carboxylesterases. Carboxylesterases had conservative catalytic domains and high level of glycosylations. Carboxylesterase proteins with catalytic activities should only be synthesized in eukaryotic expression systems because the glycosylations of carboxylesterase in ER processing are crucial to their hydrolase activity, which perhaps contribute to maintain 3D structure of enzymatic catalytic motifs [28]. For the efficient esterase production and purification, baculoviral-mediated expression of CES7 should be the best choice. In the expression system using baculovirus, recombinant CES7 mostly existed in the media not in the cytosols. The reason why recombinant CES7 was secreted to the media with the fact that it did not contain a C-terminal endoplasmic reticulum retention signal, HXEL motif, is currently unknown [1].

In our study, the esterase activities of CES7 were temperature and pH sensitive. In some reports, the pH of caput lumen fluids and cauda lumen is  $\sim 6.5$  and  $\sim 6.9$ – $7.3$ , respectively [29,30]. The physiological pH of corpus and cauda fluids was matched to the optimal activity of CES7 esterase. It was reasonable that CES7 proteins were mainly expressed in the corpus and cauda of epididymis, and contributed to the esterase activities of epididymal lumen fluids in these regions.

In conclusion, we cloned the cDNA encoding an epididymis-specific *Ces7* esterase, and expressed it in insect cells and characterized it. The results of the present study suggested that CES7 was an esterase that could hydrolyze cholesterol and choline esters, and it belonged to the member of the CES7 family. This CES7 might play an important role in sperm maturation and storage through its ester hydrolase activity in male reproductive tract.

## Funding

This work was supported by grants from the National Basic Science Research and Development Project of China (2006CB504002 & 2006CB944002), the Chinese Academy of Sciences Knowledge Innovation Program (KSCX1-YW-R-54), the National Natural Science Foundation of China (30770473), the State 863 High Technology R&D Project of China (2004AA221120).

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