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Cloning and molecular modeling of a thermostable carboxylesterase from the chicken uropygial glands

Ahmed Fendri*, Fakher Frikha, Hanen Louati, Madiha Bou Ali, Hela Gargouri, Youssef Gargouri, Nabil Miled

Laboratoire de Biochimie et de Génie Enzymatique des Lipases, Ecole Nationale d'Ingénieurs de Sfax (ENIS), route de Soukra, BPW 3038 Sfax, Tunisia

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ABSTRACT

Starting from total uropygial glands mRNAs, chicken uropygial carboxylesterase (cuCES) cDNA was synthesized by RT-PCR and cloned into the PGEM-T vector. Amino acid sequence of the cuCES is compared to that of human liver carboxylesterase 1 (hCES1). Given the high amino acid sequence homology between the two enzymes, a 3-D structure model of the chicken carboxylesterase was built using the structure of hCES1 as template. By following this model and utilizing molecular dynamics (MD) simulations, the resistance of the chicken carboxylesterase at high temperatures could be explained. The docking of substrate analogs into the cuCES active site was used to explain the fact that the chicken carboxylesterase cannot hydrolyze efficiently large substrate molecules.

1. Introduction

Carboxylesterase (EC 3.1.1.1) is a class of enzymes that catalyze hydrolysis of the carboxyl ester bond. They prefer monoesters with short to medium acyl chains as a substrate. They are also able to hydrolyze short-chain triglycerides. Like lipases, carboxylesterases have broad substrate tolerance, high regio- and stereoselectivity, and stability in organic solvent, rendering them useful as biocatalysts for the synthesis of important bio-molecules [1].

Due to the relative abundance of these proteins and their involvement in chemical and pharmaceutical synthesis, synthesis of food ingredients and other various fields, many carboxylesterases (CES) have been purified to apparent homogeneity, and corresponding cDNAs were also isolated [2–4]. In addition, structures for human CES genes have been reported, including liver and intestinal enzymes (CES1 and CES2) genes [5–7].

Carboxylesterases belong to a superfamily of hydrolytic enzymes sharing a common structural framework namely the α/β hydrolyse fold [8]. The active site of these enzymes contains the same catalytic triad (serine–histidine–aspartic/glutamic acid) similar to that found in serine proteases. As in all serine hydrolases, the nucleophilic serine is part of a consensus peptide sequence Gly-X-Ser-X-Gly and catalysis proceeds via a two-step mechanism involving an acyl-enzyme intermediate.

Three dimensional structures for human CES1 have been determined at high resolution (2.8 Å) [9–11] and three ligand binding sites have been described and designated as the active site, the side door, and the Z-site. The side door apparently assists with the release of the hydrolyzed product (e.g. fatty acids) following catalysis, whereas the Z-site is supposed to play a role in regulating catalysis following ligand binding, and opening up the active site to substrate and subsequent catalysis [12]. Other key sites have also been identified in human CES1, based on the 3-D studies [9,10]: two disulfide bonds: Cys95/Cys123 and Cys280/Cys291 and the 'gate' site which may facilitate product release following catalysis [9,10,12].

Recently, a carboxylesterase was purified from the chicken uropygial glands and some biochemical properties were determined [13]. The purified esterase displayed its maximal activity (200 U/mg) on short-chain triacylglycerols (tributyrin) at a temperature of 50 °C and had no significant lipolytic activity on medium chain (trioctanoin) or long chain (olive oil) triacylglycerols.

In this work, we report the cloning of the chicken uropygial carboxylesterase (cuCES) cDNA and the comparison of the corresponding amino acid sequence with that of human liver carboxylesterase 1 (hCES1). Molecular modeling of cuCES was used

Abbreviations: cuCES, chicken uropygial carboxylesterase; hCES1, human carboxylesterase 1; MD, molecular dynamics; RMSF, root-mean-square fluctuation.

* Corresponding author. Laboratoire de Biochimie et de Génie Enzymatique des Lipases, ENIS, route de Soukra, 3038 Sfax, Tunisia. Tel.: +216 74675055; fax: +216 74675055.
E-mail address: Ahmed_Fendri@yahoo.fr (A. Fendri).

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to explain the thermostability and the substrate specificity of this enzyme.

2. Materials and methods

2.1. Chemicals

All enzymes used in DNA manipulations were from Promega and Invitrogen; oligonuclotides were synthesized by Invitrogen.

2.2. Bacterial strains, plasmids and media

Escherichia coli strain DH5α was used as a cloning host for the gene part encoding the mature lipase. *E. coli* strain was grown in Luria-Bertani medium, supplemented with 100 μg/ml ampicillin whenever plasmid maintenance was required. The plasmid PGEM-T Easy (Promega) was used as a cloning vector. PCR products were purified using Wizard PCR Preps DNA purification System (Promega).

2.3. cDNA synthesis and amplification

The coding sequence of cuCES was determined by reverse transcriptase-polymerase chain reaction (RT-PCR) and amplification of mRNA from chicken uropygial glands. Total mRNAs were isolated from chicken uropygial glands using the single step guanidine isothiocyanate-phenol-chloroform isolation method developed by Chomczynski and Sacchi [14]. cuCES cDNA was obtained from total mRNAs by the reverse transcription procedure (Promega).

First strand cDNAs were prepared using heat-denaturated (5 min at 70 °C) total mRNAs (10 μg) as template, 200 U MMLV reverse transcriptase (Invitrogen), 20 pmol of each deoxynucleoside triphosphate, and 20 pmol of each primer, (forward primer, 5′-AAAGCAGAGCAACCAGAA-3′; reverse primer, 5′-TCACAAATCTGTGCGTTC-3′). Primers were predicted, respectively, from the N-terminal sequence of the purified cuCES and the C-terminal of an enzyme similar to a fatty acyl-CoA hydrolase precursor of *Gallus gallus* (GenBank accession no. XM_001232057), chosen according to the high homology between the N-terminal sequence deduced from this hydrolase and that of cuCES.

Reverse transcription was carried out in a total reaction volume of 20 μl for 5 min at room temperature and then for 60 min at 42 °C. The cDNA/RNA heteroduplex was then denaturated at 70 °C for 15 min and cooled on ice.

2.4. Cloning of the mature esterase gene region

Amplification of the specific cuCES cDNA was carried out by PCR using the single strand cDNAs as template with primers, 5′-AAAGCAGAGCAACCAGAA-3′, and 5′-TCACAAATCTGTGCGTTC-3′. PCR was performed in a 0.2 ml eppendorf tube with a Gene Amp® PCR System 2700. The PCR mixture contained 20 pmol of both primers, 20 pmol of each deoxynucleoside triphosphate, polymerization buffer, and 5 U Taq polymerase (Amersham Pharmacia Biotech) in 100 μl. The single strand cDNAs were directly used as template. The thermal profile involved 35 denaturing cycles at 94 °C for 1 min, primer annealing at 55 °C for 1 min, and extension at 72 °C for 3 min.

The PCR product (1.7 kb) was isolated and ligated into the *EcoRI* linearized and dephosphorylated PGEM-T Easy vector, using the PGEM-T Easy blunt ended cloning kit, according to the manufacturer's protocol (Promega). Protoplasts of *E. coli* DH5α were transformed with the ligation mixture. The resulting recombinant plasmid was named pCES. The presence of the appropriate insert was determinated by restriction analysis. DNA products were analyzed on a standard 1% agarose gel containing ethidium bromide (1 μg/ml). DNA sequences were elucidated by the dideoxynucleotide chain termination method according to a cycle sequencing protocol using thermosequenase (Amersham Pharmacia Biotech). The DNA sequencing reactions were carried out at the Biotechnological Center of Sfax (Tunisia) with the DNA sequencer ABI PRISM 3100/3100-Avant Genetic Analyser (California, USA). The sequencing was performed three times, using the recombinant vector (pCES) as template with T7 promotor primer and the M13 reverse primer (Invitrogen).

2.5. Nucleotide sequence access number

The nucleotide sequence of mature CES, determined in this study, was deposited in the GenBank database under Accession No. JQ714283

2.6. Software for infrastructure

The sequence alignment was performed with BioEdit Version 7.2.5 software [15]. The Molecular Operating Environment 2009.10 (MOE) software [16] was used for homology modeling, molecular dynamics and structures visualization. The models were stereochemically evaluated by the program PROCHECK [17]. The Visualization and figures generation were performed with PyMol program version 0.99beta06 [18].

2.7. Homology modeling

The 3-D coordinates of the Human liver carboxylesterase 1 in complex with tocrine (hCES1) (PDB code: 1MX1) was extracted from the Protein Data Bank (http://www.rcsb.org). The hCES1 structure was used as template to build the model of the cuCES structure by using the structure-modeling program Molecular Operating Environment 2009.10. The model of the cuCES was then subjected to molecular mechanics optimization using CHARMM27 force field [19]. Energy minimization (geometry optimization) was performed until the gradient of 0.05 kcal/(Å mol) was reached. The RMS deviations involving α-carbons between the initial and the optimized models were 1.36 Å.

2.8. Docking

The tributyrin (TC_4, short chain triacylglycerols) and trioctanoïn (TC_8, medium chain triacylglycerols) were modeled by the ChemBioDraw Ultra software v12.0 from CambridgeSoft. The Molegro Virtual Docker v.6.0.0 software [20] was used for docking substrates, TC_4 and TC_8 to the cuCES model.

The potential binding sites (also referred to as cavities or active sites) were being identified using the built-in cavity detection algorithm, molecular surface with 0.3 Å grid resolutions and 1.2 Å Probe size.

After preparation of the protein and the ligand, the docking was performed using MolDock Score function and MolDock SE search algorithm [20]. Ten runs of energy minimization were performed to optimize H-Bonds after docking. 2500 iterations and 500 steps Simplex Evolution were applied to generate the best five poses scores which were visually analyzed.

Only one of these poses corresponding to the highest score was used. The protein–substrate complex was then subjected to molecular mechanics optimization using CHARMM27 force field as described previously. The Score of the final protein–substrate complex was evaluated using the total interaction energy between the active site and the substrate.