Carboxylic ester hydrolases: Classification and database derived from their primary, secondary, and tertiary structures

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Abstract: We classified the carboxylic ester hydrolases (CEHs) into families and clans by use of multiple sequence alignments, secondary structure analysis, and tertiary structure superpositions. Our work for the first time has fully established their systematic structural classification. Family members have similar primary, secondary, and tertiary structures, and their active sites and reaction mechanisms are conserved. Families may be gathered into clans by their having similar secondary and tertiary structures, even though primary structures of members of different families are not similar. CEHs were gathered from public databases by use of Basic Local Alignment Search Tool (BLAST) and divided into 91 families, with 36 families being grouped into five clans. Members of one clan have standard α/β-hydrolase folds, while those of other two clans have similar folds but with different sequences of their β-strands. The other two clans have members with six-bladed β-propeller and three-α-helix bundle tertiary structures. Those families not in clans have a large variety of structures or have no members with known structures. At the time of writing, the 91 families contained 321,830 primary structures and 1378 tertiary structures. From these data, we constructed an accessible database: CASTLE (CArboxylic eSTer hydroLasEs, http://www.castle.cbe.iastate.edu).

Keywords: carboxylesterases; cholinesterases; cocaine esterases; cutinases; lysopholipases; phospholipases; triacylglycerol lipases

Introduction

Carboxylic ester hydrolases (CEHs) catalyze the hydrolysis of ester bonds into alcohols and carboxylic acids, and they are ubiquitous throughout life. Members of this enzyme group that attack different substrates, form different products, and have different names are listed as EC 3.1.1.1 to EC 3.1.1.98, with seven deleted entries.[1] Carboxylesterases (EC 3.1.1.1), triacylglycerol lipases (EC 3.1.1.3), phospholipase A2s (EC 3.1.1.4), lysophospholipases (EC 3.1.1.5), acetylcholinesterases (EC 3.1.1.7), butyrylcholinesterases (EC 3.1.1.8), aminoacyl-tRNA hydrolases (EC 3.1.1.29), and cocaine esterases (EC 3.1.1.84) are the most extensively researched CEHs.

According to the CATH database,[2] many CEHs have standard α/β hydrolase folds, which are composed of three α/β/α layers, with the second β-strand

Additional Supporting Information may be found in the online version of this article.

*Correspondence to: Peter J. Reilly, Department of Chemical and Biological Engineering, 2114 Sweeney Hall, Iowa State University, Ames, IA 50011-2230. E-mail: reilly@iastate.edu being antiparallel to generally seven others in the β-sheet.[3,4] Other CEHs with other types of α/β hydrolase folds have different arrangements of their α-helices and β-strands. Some CEHs have six-propeller folds, which consist of a six-bladed β-sheet with a central axis. Others have four-layer β-sandwich folds, where several antiparallel β-strands are arranged in two β-sheets. Three-solenoid folds are also found in CEH structures; they consist of many parallel β-strands arranged into three β-sheets. The outer-membrane CEHs are commonly found in β-barrel folds.

To this point, there is no systematic structural classification of the CEHs. Such a classification is likely to yield very different results than found with Enzyme Commission (EC) numbering, because enzymes with different EC numbers and different names may have very similar amino acid sequences (primary structures) and three-dimensional (tertiary) structures. This has been demonstrated in the Carbohydrate-Active EnzYme (ThYme) databases and Thioester-Active EnzYme databases,[5,6] as well as elsewhere.

Earlier research has partially covered this topic, but with many fewer primary structures than amassed in this project. The ESTerases and α/β-Hydrolase Enzymes and Relatives (ESTHERs) database[7,8] covers some CEHs, focusing on α/β hydrolase structures. It is not limited to CEHs, but includes other enzymes such as peptidases and thioesterases that have this fold. ESTHER classifies sequences into three levels: blocks, rank 1 families, and rank 2 families, where each block is based on conserved and characteristic parts of sequences. At the time of writing, it had over 40,000 primary structures classified into four blocks, 94 rank 1 families, and 93 rank 2 families further divided from 11 rank 1 families.

The CAZy database has classified 15 homologous families of carbohydrate esterases, which catalyze the de-O- or de-N-acylation of substituted saccharides, by their primary structures. Twelve of these families contain CEHs, mainly acetyl xylan esterases, and mainly with α/β hydrolase folds. At the time of writing, about 32,000 primary structures of carbohydrate esterases were included, of which almost 21,000 were CEHs.

The lipase engineering database (LED)[9] classified three classes and 38 superfamilies with α/β hydrolase folds, of which 16 included lipases and 10 covered other CEHs, by their functions, sequences, and crystal structures. Its founders employed much smaller *E*-values in their use of Basic Local Alignment Search Tool (BLAST)[10] to gather primary structures than used in this work, implying that the family members in LED were more similar to each other than here, perhaps leading to more families. It encompassed 112 homologous families and almost 25,000 primary and over 1100 tertiary structures. However, it has not been maintained since 2009.

The MELDB database sorted microbial carboxylesterases and triacylglycerol lipases by their primary structure similarities.[11] It corresponded to parts of the LED database, but it appears to be no longer available.

The research reported in this article systematically classifies the CEHs by their primary, secondary, and tertiary structure similarities, as opposed to classifying them by their EC numbers. This will cast light on the various ways that CEHs with different structures catalyze the hydrolysis of ester bonds to yield carboxylic acids and alcohols.

Potential Family Identification

CEH families were generally identified by the techniques used for classifying fatty acid synthesis enzymes.[6,12,13] All the primary structures of CEHs, chosen by their EC numbers, with evidence at protein level in the UniProt database[14] were collected. These totaled 752 sequences. The criterion of evidence at protein level is to ensure that wet laboratory experiments had been conducted on these proteins to verify their functions as CEHs. This criterion ruled out most available CEH sequences, mainly those obtained from whole-genome projects, whose functions are putative because their sequences have been compared only with those of known CEHs rather than being verified experimentally.

The collected primary structures were checked on the Pfam database[15] to obtain their catalytic domains only. BLAST was used consecutively to find primary structures similar to these catalytic domains (query sequences) from the National Center for Biotechnology Information's up-to-date nr database,[16] which gathers nonredundant protein sequences from various sources such as the GenBank,[17] Protein Data Bank (PDB),[18] Protein Information Resource,[19] Protein Research Foundation,[20] RefSeq,[16] and Swiss-Prot[14] databases. The threshold *E*-value in BLAST was set to 0.001. Protein sequences with *E*-values lower than 0.001 were regarded as similar enough to the query sequence to be included in one potential family.[10] In-house Python and shell scripts were implemented to automate the process of obtaining catalytic domains of query sequences in Pfam, to conduct BLAST consecutively, and to further analyze the results of structure comparison. All the scripts were run on the Google cloud platform with Linux Cent OS7 installed.

Family Verification

Multiple sequence alignment (MSA), secondary structure comparison, and tertiary structure superposition are the three main techniques to verify membership in the potential families, possibly merging or splitting them. It is assumed that all members of a family have the same protein ancestor.

A random sample of sequences in each potential family was used to perform MSA with ClustalX 2.1.[21] The alignment is to ensure that these sequences are similar enough, with several positions of amino acid residues conserved along the entire sample. Different potential families gathered by BLAST were subjected to joint MSA to ascertain whether they could be merged into one family. Conversely, if no amino acid residue is conserved and if clear differences are observed in the MSA result, then the potential family was split into two or more families. Occasionally no residue is conserved in what clearly is a family because of a sequence error in one or a few of its members.

Up to 50 tertiary structures from each potential family, if available in the PDB, were superimposed by MultiProt.[22] The monomer of each tertiary structure was extracted and compared. The root mean square deviation (RMSD) of the α-carbon atoms of the different tertiary structures was calculated, together with the P_{avg}, a measure of the percentage of these atoms that are close enough (<4.0 Å) to be compared.[12,13]

A further criterion to verify family membership is that active sites of potential members should remain in similar positions within each family. Also, secondary structure elements, based on the DSSP database[23,24] embedded in the PDB, were compared and analyzed to ensure that potential members of each family have almost the same elements.

Finally, memberships of potential families were manually inspected to confirm that they held a significant number of entries with names and EC numbers specific to CEHs.

Clan Identification and Verification

Clans are composed of two or more different families, where their active sites, reaction mechanisms, and secondary and tertiary structures are conserved from family to family, although their primary structures may not be significantly similar from one family to the next. It is assumed that family members of different clans are more distantly derived from the same protein ancestor than are members of the same family.

We used CATH-defined folds to first divide available tertiary structures in different families into separate groups. We then used two separate procedures to determine membership of families in clans. In the first, tertiary structure representatives from different families with similar tertiary structures were superimposed by MultiProt. Varying from previous methods to calculate RMSD and P_{avg} values of members of all potential families in a clan, pairwise RMSD and P_{avg} values after overlapping representative tertiary structures from pairs of families were calculated. This variation was caused by the large number of families with similar folds, making it difficult to visually distinguish them by PyMOL.[25] The MultiProt superposition, along with RMSD and P_{avg} calculations, were implemented by Python scripts, and RMSD and P_{avg} values were recorded in matrices. To cluster similar structures into potential clans, the pairwise RMSD matrices were imported into MEGA 6.06,[26] and neighbor-joining trees were produced as curved and circular trees. Although MEGA was intended to produce phylogenetic trees for the study of molecular evolution, the pairwise distance matrix used in MEGA is similar enough to be used for RMSD matrices. Potential clans were proposed according to the trees. Then the structures of potential clan members were superimposed and inspected in PyMOL.

In the second procedure, similar secondary and tertiary structures from different families were grouped roughly into potential clans, and then their structures were superimposed by MultiProt and visually inspected by PyMOL. If the superposition were satisfactory, RMSD and P_{avg} values for single representatives of all the potential family members of a potential clan were calculated. The proposed classification was tuned until the structures superimposed in PyMOL were in good alignment and RMSD and P_{avg} values were minimized. Active sites were checked, if available, to see whether the catalytic residues are in similar positions to act on the substrates, and whether they share the same mechanism in each clan.

Interestingly, the initial pairwise alignment procedure did not perform as well as the initial visual inspection procedure. Therefore, the latter technique was chosen to assign families to clans.

Results

BLAST searches using the 752 query sequences yielded 480,148 primary structures of CEHs and other enzymes. In addition, 2101 tertiary structures were gathered from the PDB. The primary structures were classified into 130 potential families.

The membership of each potential family was verified by three methods: MSA of primary structures, secondary structure analysis, and tertiary structure superpositions. The potential 130 families obtained by BLAST became 91 families after MSA using ClustalX, secondary structure analysis, and tertiary structure superposition by MultiProt and PyMOL, and after noting that some potential families had no or very few CEH members (Table I). After these operations, 321,830 primary structures, 1490 sequences with evidence at protein level, and 1378 tertiary structures remained.

The ClustalX sequence alignment files, using 50 representative sequences of each family (or all that were available, if <50), are in Supporting Information

Table I. *Clans and Families of Carboxylic Ester Hydrolases*

Family	Number of sequences	Number of sequences with evidence at protein level	Number of known tertiary structures (representative PDB structures)	Producing organisms[a]	Dominant enzyme names	Dominant EC numbers
Clan A (α/β-hydrolase, three-layer α/β/α sandwich, Rossmann fold, second β-strand antiparallel with sequence 1, 2, 4, 3, 5, 6, 7, and 8)						
1	1216	5	1 (3QIT)	**B**, E[a]	α/β-Hydrolase, esterase, thioesterase	3.1.1.–
2	31,202	41	131 (5ALM)	A, **B**, E	α/β-Hydrolase, 3-oxoadipate enol-lactonase	3.1.1.24
3	26,277	69	53 (3L1J)	A, **B**, E	α/β-Hydrolase, acetylesterase, esterase/lipase	3.1.1.–
4	497	5	10 (4CG1)	**B**	Lipase, triacylglycerol lipase	3.1.1.3
5	2191	5	14 (3FYU)	**B**	Acetyl xylan esterase	3.1.1.72
6	1181	10	2 (3C5V)	B, **E**	Protein phosphatase methylesterase, peroxidase	3.1.1.89, 1.11.1.–
7	1437	8	6 (3D59)	B, **E**	Carboxylic ester hydrolase, platelet-activating factor acetylhydrolase	3.1.1.–, 3.1.1.47
8	730	2	4 (4G4G)	**B**, E	Acetyl xylan esterase	3.1.1.72
9	2432	13	2 (1K8Q)	**E**	Lysomal acid lipase, lipase member M	3.1.1.–
10	3896	3	2 (3HXK)	**B**, E	Xylanase, pectin acetylesterase, esterase	3.2.1.8, 3.1.1.–
11	1359	4	14 (4UYU)	B, **E**	Pectin acetylesterase, protein notum homolog	3.1.1.–
12	24,560	169	280 (2JGJ)	A, B, **E**	Carboxylesterase, carboxylic ester hydrolase, acetylcholinesterase, cholinesterase, cocaine esterase	3.1.1.8, 3.1.1.84
13	4538	6	19 (3ZI7)	**B**, E	Esterase	3.1.1.–
Clan B (α/β-hydrolase, three-layer α/β/α sandwich, Rossmann fold, all β-strands parallel with sequence 2, 1, 3, 4, and 5)						
14	410	5	2 (1YQE)	**A**, E	D-Aminoacyl-tRNA deacylase	3.1.1.96
15	5264	4	7 (1U8U)	**B**	GDSL-like lipase, arylesterase, acyl-CoA thioesterase	3.1.1.–, 3.1.1.2, 3.1.2.2
16	1869	2	13 (1R50)	**B**, E	Lipase	3.1.1.–
17	2960	12	60 (1XZG)	**B**, E	Cutinase, acetyl xylan esterase	3.1.1.74, 3.1.1.72
18	1463	14	10 (1BWQ)	**B**, E	GDSL-like lipase, acetylhydrolase	3.1.1.–
19	2262	6	6 (1DEO)	A, **B**, E	Rhamnogalacturonan acetylesterase, GDSL family lipase, carbohydrate esterase family 12 protein	3.1.1.86, 3.1.1.–
20	1717	15	43 (1EB8)	B, **E**	α/β-Hydrolase, esterase	3.1.1.–
21	1985	5	4 (1ESD)	**B**, E	GDSL family lipase, triacylglycerol lipase	3.1.1.–, 3.1.1.3
22	2374	9	18 (1CVL)	**B**, E	Lipase, lactonizing lipase	3.1.1.3
23	498	8	7 (4X92)	B, **E**	Phospholipase A2, lecithin-cholesterol acyltransferase	3.1.1.4, 2.3.1.43
24	1537	17	15 (4X71)	**B**, E	Lipase, triacylglycerol lipase	3.1.1.–, 3.1.1.3
Clan C (α/β-hydrolase, three-layer α/β/α sandwich, Rossmann fold, first β-strand antiparallel with sequence 1, 3, 2, 4, 5, 6, and 7)						
25	6115	6	19 (1ZJ5)	A, **B**, E	Carboxymethylenebutenolidase, dienelactone hydrolase	3.1.1.45

Table I. *Continued*

Family	Number of sequences	Number of sequences with evidence at protein level	Number of known tertiary structures (representative PDB structures)	Producing organisms[a]	Dominant enzyme names	Dominant EC numbers
26	8649	9	14 (4ETW)	A, **B**, E	α/β-Hydrolase, hydrolase (biotin biosynthesis), carboxylesterase	3.1.1.1
27	4643	15	5 (4UUQ)	A, **B**, E	α/β-Hydrolase, lysophospholipase	3.1.1.5
28	5655	30	9 (3CN9)	**B**, E	Carboxylesterase, phospholipase	3.1.1.1, 3.1.1.–
29	4262	3	18 (1TQH)	A, **B**	Esterase, carboxylesterase	3.1.1.–, 3.1.1.1
30	1818	4	2 (3BF8)	A, **B**, E	α/β-Hydrolase, 3-oxoadipate enol lactonase, 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase	3.1.1.24, 4.2.99.20
31	314	1	14 (1LBS)	B, **E**	Lipase	3.1.1.–
Clan D (six-bladed β-propeller)						
32	8864	16	42 (4GN9)	A, **B**, E	Gluconolactonase, SMP-30/gluconolactone/LRE-like region	3.1.1.17
33	672	18	7 (3SRE)	B, **E**	Serum paraoxonase/ arylesterase 2	3.1.1.2, 3.1.1.81
Clan E (three α-helix bundle)						
34	2791	325	288 (1TG1)	**E**	Phospholipase A2	3.1.1.4
35	738	14	1 (1POC)	B, **E**	Phospholipase A2	3.1.1.4
36	269	5	3 (2WG7)	B, **E**	Phospholipase A2	3.1.1.4
Not part of a clan						
(*α/β-Hydrolase, three-layer α/β/α sandwich, Rossmann fold, various β-strand arrangements*)						
37	1715	15	8 (3ERJ)	A, B, **E**	Peptidyl-tRNA hydrolase	3.1.1.29
38	7996	29	15 (3EB9)	**B**, E	6-Phosphogluconolactonase	3.1.1.31
39	4994	56	12 (1LPB)	B, **E**	Pancreatic glycerol lipase, phospholipase A1	3.1.1.3, 3.1.1.32
40	2593	32	35 (4GBG)	B, **E**	Lipase	3.1.1.–
41	1493	11	1 (2YIJ)	B, **E**	Phospholipase A1, lipase	3.1.1.32
42	1059	11	1 (1CJY)	**E**	Lysophospholipase	3.1.1.5
43	688	2	1 (3KVN)	**B**	Esterase	3.1.1.–
44	869	6	4 (1UWC)	**E**	Diacylglycerol lipase	3.1.1.–
45	11,783	17	43 (4HOY)	**B**, E	Peptidyl-tRNA hydrolase	3.1.1.29
46	43	1	1 (1TIA)	**E**	Lipase	3.1.1.–
47	12,511	8	4 (1CHD)	A, **B**	Chemotaxis-specific regulator protein, protein-glutamate methylesterase	3.1.1.61
Patatin-like fold						
48	11,787	40	4 (4PKB)	B, **E**	Patatin, patatin-like phospholipase family	3.1.1.–
α/β TIM barrel						
49	8906	10	15 (3CL6)	A, **B**, E	Polysaccharide deacetylase	3.1.1.58
50	3765	7	7 (4DI9)	**B**, E	2-Pyrone-4,6-dicarboxylate hydrolase, amidohydrolase	3.1.1.57, 3.5.1.–, 3.5.2.–
Seven-bladed β-propeller						
51	8410	8	5 (3FGB)	A, **B**, E	6-Phosphogluconolactonase, 3-carboxy-muconate cyclase	3.1.1.31, 5.5.1.5

Table I. *Continued*

Family	Number of sequences	Number of sequences with evidence at protein level	Number of known tertiary structures (representative PDB structures)	Producing organisms[a]	Dominant enzyme names	Dominant EC numbers
Three-solenoid fold						
52	8166	22	13 (2NSP)	A, B, **E**	Pectinesterase, pectin methylesterase	3.1.1.11
Four-layer α/β/β/α fold						
53	1325	2	3 (2WYM)	A, **B**	L-Ascorbate 6-phosphate lactonase, β-lactamase	3.1.1.–, 3.5.2.6
Three-layer β/β/α fold						
54	8877	14	19 (3KO9)	**B**, E	D-Tyrosyl-tRNA(Tyr) deacylase, D-aminoacyl-tRNA deacylase	3.1.–.–
β-barrel						
55	1024	2	1 (2ERV)	**B**	Outer membrane enzyme, deacylase, lipid A 3-*O*-deacylase	3.1.1.77
56	3296	2	7 (1ILZ)	**B**, E	Phospholipase A1	3.1.1.32
Two-layer sandwich fold						
57	3136	94	4 (1J26)	**B**, E	Peptidyl-tRNA hydrolase, peptide chain release factor I	3.1.1.29
β-sandwich						
58	2129	16	3 (1BCI)	B, **E**	Cytosolic phospholipase A2	3.1.1.4
Not described						
59	5037	4	5 (2RTX)	**B**, E	Peptide chain release factor 1, peptidyl-tRNA hydrolase	3.1.1.29
60	395	10	9 (4C7W)	**V**	Hemagglutinin-esterase, E3 glycoprotein	3.1.1.53
61	521	3	9 (2JZ7)	**B**	Lipase, polyurethanase, hemolysin E	3.1.1.–
62	386	4	1 (4NFU)	**E**	Senescence-associated carboxylesterase	3.1.1.1
63	3312	5	1 (3WMT)	**B**, E	Feruloyl esterase, tannase	3.1.1.20, 3.1.1.73
64	944	14	5 (3FBX)	B, **E**	Phospholipase B-like 2	3.1.1.–
No known tertiary structure						
65	13	1	0	**E**	Phospholipase A2	3.1.1.4
66	438	7	0	**E**	Putative peptidyl-tRNA hydrolase	3.1.1.29
67	11	11	0	**E**	Phospholipase A1, phospholipase A2	3.1.1.4, 3.1.1.32
68	728	8	0	**E**	Groups XIIA and XIIB secretory phospholipase A2	3.1.1.4
69	1234	4	0	**B**, E	Poly(3-hydroxybutyrate) depolymerase, feruloyl esterase	3.1.1.75, 3.1.1.73
70	1334	1	0	**B**, E	Carboxymethylenebutenolidase, dienelactone hydrolase	3.1.1.45
71	34	1	0	**B**	Poly(3-hydroxyoctanoate) depolymerase	3.1.1.76
72	2686	10	0	A, **B**, E	Polyhydroxybutyrate depolymerase family, acetylxylan esterase, feruloyl esterase, carbohydrate esterase family 1	3.1.1.75, 3.1.1.72, 3.1.1.73
73	335	1	0	**B**	Phospholipase A1	3.1.1.32
74	3091	1	0	**B**, E	Lysophospholipase L2	3.1.1.5
75	7331	14	0	B, **E**	GDSL family esterase/lipase	3.1.1.–

Table I. *Continued*

Family	Number of sequences	Number of sequences with evidence at protein level	Number of known tertiary structures (representative PDB structures)	Producing organisms[a]	Dominant enzyme names	Dominant EC numbers
76	261	4	0	B, **E**	Chlorophyllase	3.1.1.14
77	284	1	0	**E**	Triacylglycerol lipase	3.1.1.3
78	688	2	0	B, **E**	Acetylhydrolase, esterase, lipase, α/β-hydrolase	3.1.1.–
79	514	3	0	**E**	Triglyceride lipase, cholesterol esterase, lysosomal acid lipase	3.1.1.–, 3.1.1.13
80	1828	10	0	B, **E**	Patatin-like phospholipase domain	3.1.1.–
81	212	1	0	**E**	ATG15 protein, triacylglycerol lipase	3.1.1.3
82	192	2	0	**E**	Steroyl esterase, YEH1p, YEH2p	3.1.1.13
83	2953	3	0	**B**, E	Sialate *O*-acetylesterase, 9-*O*-acetylesterase	3.1.1.53
84	332	3	0	**E**	Acyloxyacyl hydrolase	3.1.1.77
85	55	3	0	**B**	EstP (carboxylesterase)	3.1.1.1
86	799	1	0	**B**, E	Lipase	3.1.1.–
87	439	2	0	**B**	Lipase	3.1.1.–
88	2378	34	0	**E**	Phospholipase DDHD	3.1.1.–
89	465	3	0	**B**	D-(−)−3-hydroxybutyrate oligomer hydrolase, cytochrome C1	3.1.1.22
90	1666	17	0	B, **E**	Patatin-like phospholipase domain protein, triacylglycerol lipase	3.1.1.–, 3.1.1.3
91	862	14	0	B, **E**	Phospholipase B1	3.1.1.–

Most prevalent producers are bolded.

[a] A, archaea; B, bacteria; E, eukaryota; V, virus.

Figure S1. The conserved amino acid counts in each family are summarized in Supporting Information Table SI. In the great majority of cases, at least one and usually substantially more amino acid residues are totally conserved over all primary structures. A roughly equal number of residues of chemically similar but not identical amino acid residues are also conserved. The secondary structures of a representative of each family with a known tertiary structure are shown in Supporting Information Figure S2. Each family member has similar secondary structures in its core, but some members have either extra or missing α-helices or β-strands. RMSD and P_{avg} values obtained by tertiary structure superposition also appear in Supporting Information Table SI. In a large fraction of all cases where multiple tertiary structures in a single family are available, RMSD < 1.5 Å and $P_{\text{avg}} > 90\%$.

A large majority of families have >1000 primary structures, and most of the rest have 100–1000 sequences (Table I). As expected, all families have at least one sequence with evidence at protein level. Among them, 64 of the 91 families have known tertiary structures, with 27 families having none.

Many families have members produced by organisms in all three life kingdoms (Table I). Members of other families are produced by organisms in two kingdoms, usually bacteria and eukaryota, or in a single kingdom (Table I).

A total of 36 families were grouped into five clans by having secondary and tertiary structures that could be closely superimposed (Table I), even though their primary structures may not be significantly similar. Supporting Information Table SII shows RMSD and P_{avg} values and protein folds of each clan. In all cases, RMSD < 2.5 Å. However, P_{avg} values cover a wide range. Tertiary structures of families within clans are more variable than those of family members, as the former do not share similar primary structures while the latter do.

Each clan has characteristic tertiary folds (Fig. 1). Clan A CEHs all have standard α/β hydrolase folds, in which the second β-strand is antiparallel to the others in the β-sheet.[3,4] The β-sheet generally has eight β-strands, which are found in the order 1, 2, 4, 3, 5, 6, 7, and 8, based on their amino acid residue numbers. Clan B members have similar tertiary structures as those in clan A; however, all their β-strands are parallel to each other, proceeding in the same direction, and they are arranged in the order 2, 1, 3, 4, and 5. A sixth β-strand, if present, may be found

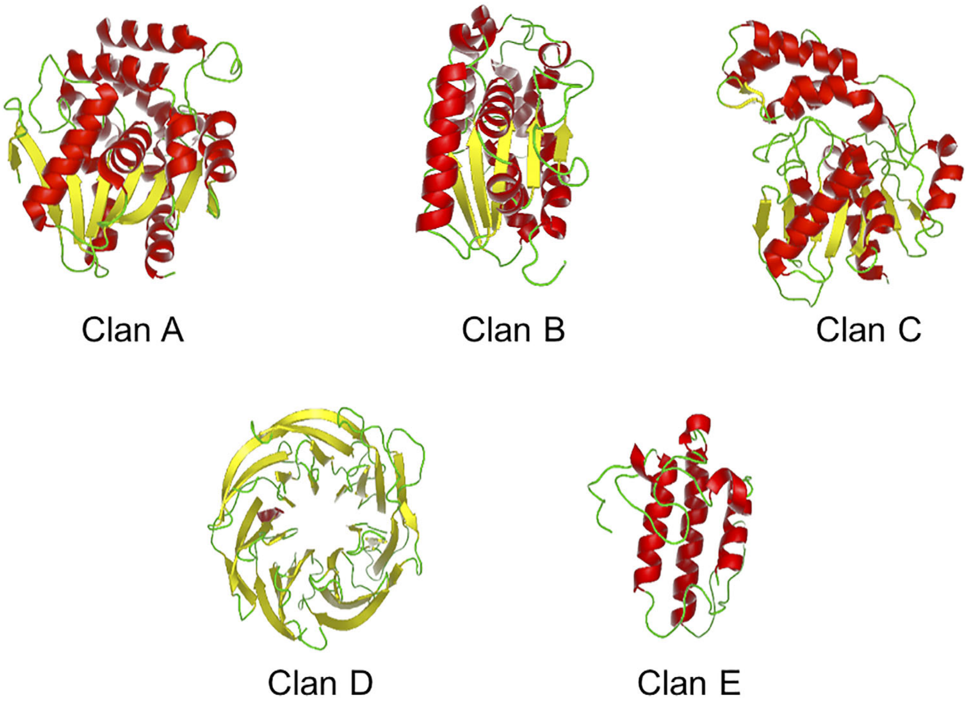


Figure 1. Tertiary structures of representative families from the five CEH clans. Clan A: family 2, *Burkholderia xenovorans* 3-oxoadipate enol-lactonase, PDB 2XUA. Clan B: family 17, *Trichoderma reesei* cutinase, PDB 4PSD. Clan C: family 30, *Escherichia coli* esterase, PDB 3BF8. Clan D: family 32, mouse SMP30/GNL hydrolase, PDB 4GN9. Clan E: family 34, *Daboia russelii* phospholipase A_2, PDB 1TG1.

before or after the fifth one. Clan C enzymes have α/β hydrolase-like tertiary structures as well, but their first rather than their second β-strands are antiparallel to the others on the same β-sheet. The β-strand order is 1, 3, 2, 4, 5, 6, and 7. The tertiary structures of clan D members are six-bladed β-propeller folds, where each blade, consisting of four β-sheets, shares a central axis. Clan E enzymes have three-α-helix up-down bundle tertiary structures.

At present, over 60% of the CEH families are not part of clans, either because they have no known tertiary structures or because their members cannot be superimposed well on the tertiary structures of the members of existing clans or with each other, even when they share much the same fold. A total of 22 of these families have known tertiary structures designated by CATH or the Structural Classification of Proteins (SCOP) database,[27] such as three-layer α/β/α sandwiches (Rossmann folds), α/β-hydrolase structures, patatin-like folds, α/β-TIM barrels, seven-bladed β-propellers, three-solenoid folds, three-layer β/β/α sandwiches, β-barrels, two-layer sandwich folds, and β-sandwiches (Table I). A further six families have tertiary structures that are not classified by either CATH or SCOP.

Of the most studied CEHs, large numbers of named carboxylesterases or EC 3.1.1.1 designations are found in families 12 (clan A), 26, 28, 29 (clan C), 62, and 85, of those not in any clan (Table I). Families 4 (clan A), 21, 24 (clan B), 39, 77, 79, 81, and 90 (no clan) have triacylglycerol lipases (EC 3.1.1.3). Phospholipase A2s (EC 3.1.1.4) exist in families 23 (clan B), 34–36 (clan E), 58, 65, 67, and 68 (no clan). Lysophospholipases (EC 3.1.1.5) are in families 27 (clan C), 42, and 74 (no clan). Acetylcholinesterases (EC 3.1.1.7) and butyrylcholinesterases (often simply called cholinesterases) (EC 3.1.1.8) are found only in family 12 (clan A), as are cocaine esterases (EC 3.1.1.84). Cutinases (EC 3.1.1.74) are in family 17 (clan B). Enzyme name and EC number assignments tend to be somewhat elastic and arbitrary, causing some spurious variation, and some families now not assigned to clans will eventually find their way into them once they have known tertiary structures. Therefore, these lists are subject to future revision. However, it is evident by the number of sequences in each family that carboxylesterases are mainly in families 26, 28, and 29 (clan C), triacylglycerol lipases exist largely in families 21, 24 (clan B), and 39 (no clan), and phospholipase A2s are found predominantly throughout clan E and in family 58 (no clan).

CEH Mechanisms

Table II presents catalytic residues from representative families from each of the 64 families that have known tertiary structures, gathered from the articles corresponding to the PDB structures listed there. Clans A, B, and C all have catalytic residues characteristic of serine protease mechanisms, with

Table II. *Catalytic Residues of Carboxylic Ester Hydrolase Families with Known Tertiary Structures*

Clan	Family	PDB designation	Producing species and enzyme	Catalytic residues
A	1	1QIT	*Moorea producens* (*Lyngbya majuscula*) decarboxylating thioesterase	S100/E124/H266
A	2	4CCY	*Bacillus subtilus* carboxylesterase CesB	S130/E245/H274
A	3	4KRX	*Escherichia coli* acetyl esterase Aes	S165/D262/H292
A	4	4CG1	*Thermobifida fusca* polyethylene terephthalate degrading hydrolase	S130/D176/H208
A	5	2XLB	*Bacillus pumilus* (*Bacillus mesentericus*) acetyl xylan esterase	S181/D269/H298
A	6	3C5V	Human PP2A-specific methylesterase	S156/D181/H349
A	7	3D59	Human plasma platelet-activating factor acetylhydrolase	S273/D296/H351
A	8	4G4G	*Sporotrichum thermophile* (*Myceliophthora thermophila*) glucuronyl esterase	S213/E236/H346
A	9	1K8Q	Dog gastric lipase	S153/D324/H353
A	10	–	–	–
A	11	4UYU	Human WNT deacylase notum	S232/D340/H389
A	12	2JGJ	Mouse acetylcholinesterase	S203/E334/H447
A	13	1JJF	*Clostridium thermocellum* feruloyl esterase	S172/D230/H260
B	14	–	–	–
B	15	1U8U	*Escherichia coli* thioesterase/protease/lysophospholipase L_1	S10/D154/H157
B	16	1I6W	*Bacillus subtilis* lipase	S77/D133/H156
B	17	4PSD	*Trichoderma reesei* cutinase	S164/D216/H229
B	18	1VYH	Mouse PAF-AH holoenzyme	S48/D/193/H196
B	19	1DEO	*Aspergillus aculeatus* rhamnogalacturonan acetylesterase	S9/D192/H195
B	20	1EB8	*Manihot esculenta* hydroxynitrile lyase	S80/D208/H236
B	21	4HYQ	*Streptomyces albidoflavus* phospholipase A1	S11/H218
B	22	1CVL	*Chromobacterium viscosum* lipase	S87/D263/H285
B	23	4X92	Human lysosomal phospholipase A2	S165/D327/H359
B	24	4FDM	*Bacillus* L2 lipase	S113/D317/H358
C	25	1DIN	*Pseudomonas knackmussi* dienelactone hydrolase	C123/D171/H202
C	26	4ETW	*Shigella flexneri* enzyme/ACP substrate gatekeeper	S82/D207/H235
C	27	3HJU	Human monoglyceride lipase	S121/D239/H269
C	28	3CN9	*Pseudomonas aeruginosa* carboxylesterase	S113/D166/H197
C	29	1TQH	*Geobacillus stearothermophilus* carboxylesterase Est30	S94/D193/H223
C	30	3BF7	*Escherichia coli* esterase	S89/D113/S206/H234
C	31	1LBS	*Moesziomyces antarticus* triacylglycerol hydrolase	S105/D187/H224
D	32	2DG0	*Staphylococcus aureus* lactonase	D138/D236
D	33	1V04	Human/rabbit/mouse/rat serum paraoxonase	H115/H134
E	34	1PPA	*Agkistrodon piscivorus* lysine 49 phospholipase A2	H48/Y73/D99
E	35	1POC	*Apis mellifera* venom phospholipase A2	H34/D35
E	36	2WG7	Rice class X1b phospholipase A2	H61/D62
–	37	1RZW	*Archaeglobus fulgidis* peptidyl-tRNA hydrolase	H20/D93/H113
–	38	3EB9	*Trypanosoma brucei* 6-phosphogluconolactonase	D163/H165
–	39	1RP1	Dog pancreatic lipase related protein	S152/D176/H263
–	40	1TGL	*Rhizomucor michei* triacylglycerol lipase	S144/D203/H257
–	41	–	–	–
–	42	1CJY	Human cytosolic phospholipase A2	S228/D549
–	43	3KVN	*Pseudomonas aeruginosa* autotransporter EstA	S14/D286/H289
–	44	1UWC	*Aspergillus niger* feruloyl esterase	S133/D194/H247

Table II. *Continued*

Clan	Family	PDB designation	Producing species and enzyme	Catalytic residues
–	45	4HOY	*Acinetobacter baumanni* peptidyl-tRNA hydrolase	H22/N70/D95/N116
–	46	1TIA	*Penicillium camemberti* lipase	S145/D199/H259
–	47	1CHD	*Salmonella enterica* chemotaxis receptor methylesterase	S164/H190/D286
–	48	4PKB	*Solanum cardiophyllum* patatin-17	S77/D215
–	49	3CL6	*Pseudomonas fluorescens* allantoinase	E36/H259
–	50	4DI9	*Sphingomonas paucimobilisi* 2-pyrone-4,6-dicarboxylic acid carboxylase	R124/D248
–	51	–	–	–
–	52	2NSP	*Dickeya dadantii* (*Erwinia chrysanthami*) pectin methylesterase	Q177/D178/D199
–	53	2WYM	*Escherichia coli* L-ascorbate-6-phospholactonase	D121/H122/D226/H281
–	54	3KO9	*Plasmodium falciparum* D-amino acid deacylase	T90
–	55	2ERV	*Pseudomonas aeruginosa* 3-*O*-deacylase	H126/S128/E140
–	56	1ILZ	*Escherichia coli* phospholipase A	H142/S144/N156
–	57	–	–	–
–	58	1CJY	Human cytosolic phospholipase A2	S228/D549
–	59	2JY9	*Thermus thermophilus* YaeJ bound to ribosome	Gln28
–	60	1FLC	Influenza C virus hemagglutinin-esterase fusion glycoprotein	S57/D352/H355
–	61	2Z8X	*Pseudomonas sp*. family I.3 lipase	S207/D255/H313
–	62	4NFU	*Arabidopsis thaliana* signaling node	S123/D187/H317
–	63	3WMT	*Aspergillus oryzae* feruloyl esterase B	D17/S203/H457
–	64	–	–	–

catalytic serine residues followed by catalytic aspartate or glutamate residues and then catalytic histidine residues. In only four families in these three clans is this pattern not followed. There is not enough information to identify characteristic catalytic residues in families 10 and 14, a full set of three catalytic residues is not available for family 21, and a catalytic cysteine residue replaces a serine residue in family 25.

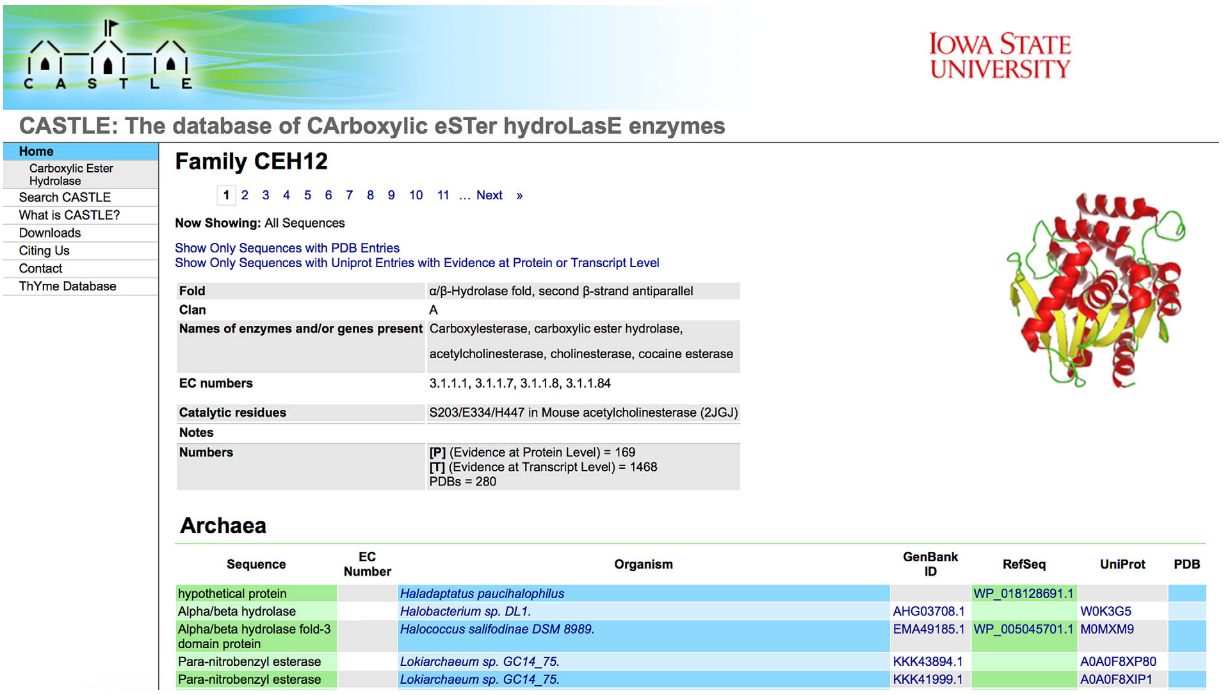
Clans D and E present different catalytic residue patterns than Clans A, B, and C. Clan D, with six-bladed β-propeller folds, has two characteristic patterns, two aspartate residues in family 32 and two histidine residues in family 33. Clan E enzymes, with three-α-helix up-down bundle tertiary structures, have histidine and aspartate catalytic residues.

Identities and locations of catalytic residues are more variable in families 37–64. Families 37–47, less families 41, 45, and 47, which have various α/β-hydrolase structures, have all or some of the catalytic residues characteristic of serine proteases. Families 60–62, which at present do not have settled tertiary structures, have Ser/Asp/His catalytic residue patterns, suggesting that they may be composed of serine proteases when more is known about them.

Database Construction

The information covered earlier has been gathered into a freely accessible database labeled CASTLE (CArboxylic eSTer hydroLasEs, http://www.castle.cbe.iastate.edu). This database at present is divided into the 91 CEH families defined earlier, each of them containing a listing of enzyme names or other identifiers, EC numbers, producing organism genus and species names, and GenBank, RefSeq, UniProt, and PDB accession codes in columns (Fig. 2). Most entries are of proteins gathered from genomic studies that have not undergone experimental verification, and therefore, only a minority have EC numbers. Producing organisms are separated into archaea, bacteria, and eukaryota. Some families have entries in all three kingdoms, while others have entries in one or two. Entries in each kingdom are alphabetically ordered by the genus of the producing organism. In general there are 500 entries on each page.

The top of each page (Fig. 2) shows an addressable list of pages, prompts to see only sequences with PDB entries or only sequences with UniProt entries having evidence at protein level (labeled by [P]) or transcript level (labeled by [T]). Other information is the characteristic fold of the family (if known), dominant names of enzymes and/or genes present, dominant EC numbers, catalytic residues (if known), and numbers of sequences with evidence at protein and transcript levels and with PDB entries. All enzyme names and EC numbers are taken from either the UniProt database or the NCBI protein



CASTLE: The database of CArboxylic eSTer hydroLasE enzymes

Family CEH12

Now Showing: All Sequences

Show Only Sequences with PDB Entries
Show Only Sequences with Uniprot Entries with Evidence at Protein or Transcript Level

Fold	α/β-Hydrolase fold, second β-strand antiparallel
Clan	A
Names of enzymes and/or genes present	Carboxylesterase, carboxylic ester hydrolase, acetylcholinesterase, cholinesterase, cocaine esterase
EC numbers	3.1.1.1, 3.1.1.7, 3.1.1.8, 3.1.1.84
Catalytic residues	S203/E334/H447 in Mouse acetylcholinesterase (2JGJ)
Notes	
Numbers	**[P]** (Evidence at Protein Level) = 169 **[T]** (Evidence at Transcript Level) = 1468 PDBs = 280

Archaea

Sequence	EC Number	Organism	GenBank ID	RefSeq	UniProt	PDB
hypothetical protein		*Haladaptatus paucihalophilus*		WP_018128691.1		
Alpha/beta hydrolase		*Halobacterium sp. DL1.*	AHG03708.1		W0K3G5	
Alpha/beta hydrolase fold-3 domain protein		*Halococcus salifodinae DSM 8989.*	EMA49185.1	WP_005045701.1	M0MXM9	
Para-nitrobenzyl esterase		*Lokiarchaeum sp. GC14_75.*	KKK43894.1		A0A0F8XP80	
Para-nitrobenzyl esterase		*Lokiarchaeum sp. GC14_75.*	KKK41999.1		A0A0F8XIP1	

Figure 2. Screen shot of the family 12 (clan A) introductory page.

database; we do not assign enzyme names or EC numbers.

Entries in all columns except that of enzyme names or identifiers are linked to other electronic resources: those of EC numbers to the ExPASy SwissProt enzyme nomenclature database (http://enzyme.expasy.org/EC/*x*), those of producing organisms to the NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/Taxonomy/Browser/wwwtax.cgi?id = *y*), those of GenBank and RefSeq identifiers to the NCBI protein database (http://www.ncbi.nlm.nih.gov/protein/*y*), those of UniProt identifiers to the UniProt database (http://www.uniprot.org/uniprot/*y*/), and those of PDB identifiers to the PDB database (http://www.rcsb.org/pdb/explore/explore.do?structureId=*y*), where *x* is the EC number and *y* is the NCBI taxonomy, GenBank, RefSeq, UniProt, or PDB accession codes.

Two features ease the task of navigating and retrieving information in CASTLE. A search tool allows keywords, EC numbers and GenBank, RefSeq, UniProt, or PDB accession codes to be searched. Furthermore, each family can be downloaded into a comma-separated value file, which can be viewed in a spreadsheet. These files can be obtained by contacting the authors.

Conclusions

Classifying CEH enzymes into families and clans provides valuable insights about them. Several observations may be made about their structural classifications:

1. Often CEHs with the same enzyme function appear in multiple families and clans. For instance, phospholipase A2s are found in eight families. Four of these families are in two clans, with a fifth family having a known tertiary structure but not being in a clan. Three further families have no known tertiary structures. Carboxylesterases occur in six families, four of them in two clans. A fifth has a known but not firmly described structure, while a sixth has no known tertiary structure.
2. On the other hand, some major enzyme groups are found in single families. Acetyl- and butylrylcholinesterases and cocaine esterases all occupy family 12 in clan A.
3. All clans except clan E include diverse enzymes with various functions, as do families not in clans. The clan with the largest number of families (clan A) includes enzymes with eight different EC numbers, plus the generic EC 3.1.1.–, and many more than eight enzyme functions. Clan B contains enzymes with seven EC numbers plus EC 3.1.1.–.
4. It is common for some families to contain enzymes with not only EC numbers denoting CEHs, but also with other EC numbers.
5. Some families show little experimental work on their enzymes. Twenty-seven families (those from 65 to 91) at present have no known tertiary structures. Eight of them, plus two others, have only one sequence with evidence at protein level for each family. These families need more attention from researchers, because they may have novel substrate specificities that will be useful or important to industrial or medical applications.
6. To emphasize the sharp difference between classifying enzymes by function and classifying them

by structure, only enzymes from 33 EC numbers out of the total 91 EC numbers starting with EC 3.1.1 (1–5, 7, 8, 11, 13, 14, 17, 20, 22, 24, 29, 31, 32, 45, 47, 53, 57, 58, 61, 72–77, 81, 84, 89, and 96) are found in Table I. This indicates that the other 68 supposed CEHs either do not actually exist, have been combined with other CEHs, or have been researched very rarely.

7. To extend the utility of this work, it has been gathered into a freely accessible database, entitled CASTLE (CArboxylic eSTer hydroLasEs, http://www.castle.cbe.iastate.edu).

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