DEPARTMENT OF BIOTECHNOLOGY

Decoding Enzymes
Structure, Mechanism, Production and Utility

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Introduction:

This project uncovers these two interesting enzymes, reversing their Enzyme Commission (EC) numbers, structures, and biochemical functions, while also delving into how they are manufactured, purified, and utilized in real-life situations.

sEH (EC 3.3.2.10) is a central enzyme in the hydrolysis of epoxides, especially lipid signalling molecules, and thus an attractive target in cardiovascular and inflammatory disease therapy. Its two-domain architecture, comprising an N-terminal lipid-binding domain and a C-terminal hydrolase domain, positions it ideally for its function.

Conversely, CGTase (EC 2.4.1.19) is dedicated to transforming starch to cyclodextrins—ring-shaped sugar molecules that find extensive application in the field of pharmaceuticals, food processing, and even environmental clean-up. Its catalytic domain with a conserved α-amylase fold enables it to perform this intricate conversion with ease.

Learning about these enzymes is not merely a matter of their chemistry—there's also the way we make and purify them for industrial application. The upstream process is where we optimize microbial fermentation systems to obtain the maximum enzyme yield, whereas the downstream process is all about purification via methods such as chromatography, filtration, and precipitation. All these steps are essential in making the enzymes not just functional but commercially acceptable as well.

Outside the laboratory, the contribution of these enzymes is considerable. sEH inhibitors are in development to treat hypertension, neurodegenerative conditions, and metabolic disorders, whereas cyclodextrins produced by CGTase are utilized to increase drug solubility, extend food shelf life, and even assist in pollution management. Through the investigation of these enzymes from their molecular structure to their real-world applications, this project seeks to underscore their potential to fuel scientific and industrial advancement.

Soluble Epoxide Hydrolase (sEH)

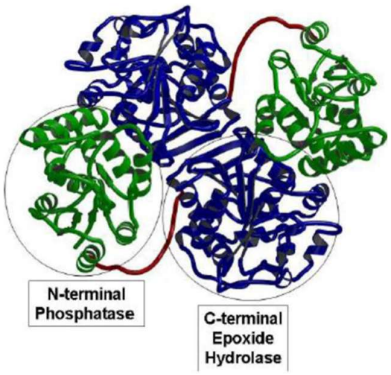
EC Number: 3.3.2.10

The soluble epoxide hydrolase (sEH) comes under the EC 3.3.2.10 category of the hydrolase enzymes. The EC number is a classification of enzymes according to the reactions they catalyse:

EC 3	Hydrolysing (breaking bonds with the use of water).
EC 3.3	Acting on ether bonds.
EC 3.3.2	Epoxide hydrolases (hydrolases acting on epoxide bonds).
EC 3.3.2.10	Refers specifically to soluble epoxide hydrolase (sEH).

Structure and Functions:

Soluble epoxide hydrolase is a two-activity enzyme present in the entire body, from the brain to the liver, kidneys, heart, lungs, intestines, and blood vessels. sEH plays an important role in lipid metabolism, regulation of inflammation, and cardiovascular function. sEH is a 62 kDa enzyme consisting of two different domains that are joined by a short proline-rich linker and that act as a homodimer in mammalian cells. Each domain is involved in a separate catalytic activity:



• The C-terminal region has hydrolase activity, which cleaves epoxides to yield their corresponding diols. Such activity has been linked with cardiovascular protection, anti-inflammatory, and pain modulation through the bioactivation of lipophilic-lipid mediator lipids such as epoxyeicosatrienoic acids (EETs).

• The N-terminal region is active as a phosphatase that hydrolyses lipid phosphates, although the latter's function in biology, especially in relation to the central nervous system, is less explored

Industrial Production (Upstream):

To start sEH production we need a strain to work with

Strain Isolation

As the goal is to study human related sEH, I'm selecting Human sEH gene (EPHX2) as a strain to go for genetic engineering. For bacterial studies we have Pseudomonas aeruginosa or Bacillus megaterium as a potential strain.

Selecting a Vector(plasmid)

An appropriate vector is selected according to the needs of production:
pET-28a(+) Has a His-tag for simpler purification.
pGEX-4T-1 Assists in protein solubility by incorporating a GST fusion tag.

Cloning the sEH Gene

Isolate mRNA with the sEH gene and transcribe it into cDNA with RT-PCR.
Amplify the gene with PCR (Polymerase Chain Reaction) with particular DNA primers.
Verify that the gene was replicated accurately with gel electrophoresis.

Placing the Gene into the Plasmid

Digest both the sEH gene and the plasmid with restriction enzymes (e.g., NdeI, XhoI).
Combine them together with T4 DNA ligase to create the final recombinant plasmid.
Transform this plasmid into E. coli DH5α bacteria so that it can replicate for future use.

Plasmid Transfer to the Production Bacteria

Transfer the plasmid to E. coli BL21(DE3) (a specific bacteria for protein expression) via heat shock transformation.

Culture the bacteria on LB agar plates with kanamycin (an antibiotic)
to only get those bacteria that were successfully transfected with the plasmid.

Protein Expression

Once the bacteria is containing the sEH gene, the next step is to make them to produce the enzyme:

1. Grow the transformed E. coli BL21(DE3) in liquid culture (LB broth with kanamycin).
2. When the bacteria are at the right growth stage, IPTG (Isopropyl β-D-1-thiogalactopyranoside) will be added to activate the T7 promoter, triggering sEH production.
3. Allow the bacteria to produce sEH for a few hours before moving to purification.

Purification (Downstream):

Once sEH has been produced, it needs to be extracted from the bacterial cells:

Cell Harvesting & Lysis

• Centrifuge the culture at 8,000 rpm for 10 min at 4°C.
• Resuspend cell pellet in lysis buffer:

- 50 mM Tris-HCl (pH 8.0)
- 300 mM NaCl
- 10 mM imidazole
- 1 mM PMSF (Protease Inhibitor)

• Lyse cells using sonication (10 sec on, 30 sec off, for 10 min) or high-pressure homogenization.
• Centrifuge at 12,000 rpm for 20 min at 4°C to separate soluble proteins.

Affinity Purification (His-Tag Ni-NTA Chromatography)

1. Load supernatant onto a Ni-NTA column pre-equilibrated with buffer:

   50 mM Tris-HCl (pH 8.0), 300 mM NaCl, 10 mM imidazole.
2. Wash column with 40 mM imidazole to remove non-specific proteins.
3. Elute sEH using 250 mM imidazole in the same buffer.

Dialysis and Final Purification

- Dialyze protein against 50 mM Tris-HCl (pH 7.4), 150 mM NaCl to remove imidazole.

- Further purify using Size Exclusion Chromatography (SEC).
- Filter (0.22 µm) and store at -80°C.

Quality Control

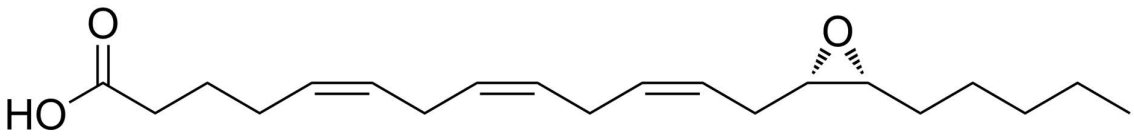
- Confirm molecular weight (~62 kDa) using SDS-PAGE.
- Verify identity with Western blot using anti-sEH antibodies.

Once the process is optimized at the lab scale, it can be scaled up using 100L–1000L bioreactors for commercial production.

Applications

sEH is practically a very useful and an important enzyme for industrial as well as medicinal applications. sEH being a double activity enzyme it can be deployed in various medicinal fields such as cardiovascular problems, pain relief, oncology, etc. The studies for sEH and its potential use cases in various other industries is being conducted.

EETs and some context for applications



CHEMICAL STRUCTURE OF 14, 15- EPOXYEICOSATRIENOIC ACID

EETs or otherwise called Epoxyeicosatrienoic acids are very important biomolecules which act as a lipid signalling molecule, these EETs are derived from arachidonic acids by action of cytochrome P450 enzymes.

These molecules play an important role in cardiovascular, inflammatory and neurological functions by taking the role of anti-inflammatory agents and cellular messengers.

EETs are produced in endothelial cells by a three- step enzymatic pathways and these are released into our bloodstream by binding to GPCRs (G-Protein-Coupled-Receptors)

These EETs are regulated and rapidly metabolised by sEH to maintain the correct concentration and the correct cellular functions which may be inhibited by EETs

As EETs have many therapeutic effects such as anti-inflammatory, neurological functions, cardiovascular, etc. sEHs by inhibiting EETs causing some problems which may be solved by blocking sEH which prevents EET breakdown and this is exactly what many researchers are exploring

Many research fields on EET retention are our potential medical applications for sEH inhibition They include:

Cardiovascular Health

epoxyeicosatrienoic acids (EETs) are key biomolecules which are involved in anti-inflammatory and vasodilatory activity of our body. These EETs are regulated by sEH enzyme. sEH often inhibit their activity and leading to loss of their therapeutic effect, which leads to hypertension and other heart related problems. sEH inhibition is a potential guard against cardiac arrest and hypertension.

Pain relief and anti-inflammatory treatments

Chronic pain and inflammation are most frequently linked to the over-degradation of lipid mediators by sEH. she inhibition can be very helpful way to prevent these lipid mediatory therapeutic agents from degradation and retain their effect. By this way we can prevent inflammation and chronic pain. Experiments show that sEH inhibitors can be used instead of opioids to treat diseases like arthritis, neuropathic pain, and fibromyalgia.

Progressive neural decline Disorders

Recent studies identified that sEH is a part of brain cholesterol regulation and has a link with Alzheimer's and Parkinson's disease. Cholesterol control disruption leads to neurodegeneration, and so researchers are investigating the way controlling sEH activity would delay the progression of the disease and normalize brain function. This has a big potential for drug treatment of cognitive decline and neuroinflammation in the future.

Oncology Studies

Growing evidence supports the suggestion that sEH plays a role in cancerogenesis, particularly prostate and breast cancer. Overexpression of sEH activity is in certain studies hypothosized to drive tumour expansion, whereas activity inhibition here would limit metastasis of the cancer. The question arose then whether sEH inhibitors could constitute an innovative future anticancer approach.

Industrial and Environmental Applications

Chemical and Pharmacy Manufacturing

sEH can be employed to catalyze epoxides into diols, a significant chemical reaction in drug, polymer, and other industrial chemical production. Researchers are seeking to leverage the use of engineered variants of sEH in manufacturing to enhance their efficiency and to minimize toxic by-products.

Agriculture and Pest Control

As sEH is implicated in insect metabolism, it is also a target for environmentally friendly pesticide development. By controlling the activity of sEH in certain insects, researchers hope to create pest control agents that are more targeted and less toxic to non-target organisms.

Environmental Detoxification

sEH is involved in the breakdown of toxic chemicals with epoxide moieties, characteristic of toxic contaminants and industrial residues. The use of sEH-based bioremediation could potentially aid in environmental polluter cleaning, for example, oil spill cleaning and pesticide residue elimination.

Conclusion

sEH is not only a biological enzyme, its uses cross over into healthcare, industry, and environmental science. In medicine, drugs for the treatment of heart disease, pain, and neurodegenerative disease are being designed in the form of sEH inhibitors. In industry, sEH enzymes are being designed and investigated for use in drug synthesis as well as for green chemistry. The potential for using the enzyme for pest control and environmental decontamination continues to show the versatility of the enzyme.

With ongoing research, sEH-based treatments and technologies will become a part of standard medical interventions and industrial procedures.

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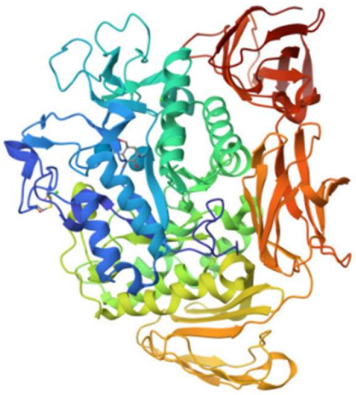
Cyclodextrin Glucanotransferase (CGTase)

EC Number: 2.4.1.19

EC 2	It's a transferase, meaning it moves chemical groups from one molecule to another.
EC 2.4	Specifically, it transfers sugar molecules.
EC 2.4.1	More precisely, it works with hexose sugars (like glucose).
EC 2.4.1.19	This is the unique identifier for CGTase, which specializes in rearranging starch into circular molecules called cyclodextrins.

Structure & Functions



CGTase is a member of glycoside hydrolase family of enzymes that include enzymes like amylase. It is a multi-domain structure that allows it to bind to starch, hydrolyze it and then rebuild the resulting pieces into cyclodextrins. Its structure has 5 domains

For CGTase to break down starch and convert them to cyclodextrins a mechanism known as stacking should take place in which CGTase holds onto starch molecules properly

Domain A (Catalytic Core)

Barrel shaped part which is major functional part of this enzyme, where actual chemical reactions happen. It contains the active site, where starch binds and it then gets transformed to cyclodextrins

Domain B (Connecting Region)

This domain(part) of enzyme helps in positioning the starch molecules correctly inside the enzyme.

Domain C (Stabilisation)

This domain provides structural support and maintains the shape of the enzyme.

Domain D (Cyclodextrin binding)

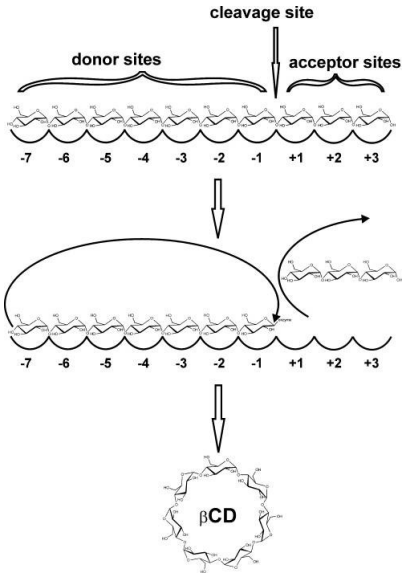
This helps in recognizing and binding to cyclodextrin molecules and ensuring proper product is formed.

Domain E (Starch binding)

This helps the enzyme grip onto large starch molecules and helps in improving efficiency.

Imagine CGTase as a tiny, specialized machine designed to work with starch. It grabs onto the long starch chain, carefully cuts out a small piece, and then folds it into a neat little ring to form cyclodextrins. To make sure this process happens smoothly, CGTase has special components like Tyrosine and Phenylalanine, which help stabilize the reaction. Think of them as hands that gently guide the starch into the right position. It also has flexible loops that can shift and adjust, allowing it to work with different starch molecules and fine-tune the process as needed.

CGTase needs to hold starch molecules properly to break them down and reintegrate to turn them into cyclodextrins, this process is made sure by a mechanism called **stacking**



The Role of "Stacking"

Think of CGTase like a machine gripping a sheet of paper (starch). It uses aromatic amino acids (like tyrosine and phenylalanine) to "stack" onto sugar rings, keeping them steady so the reaction can happen

What Happens If We Change These Key Parts?

Scientists experimented by swapping out some of these amino acids to see what would happen:

Tyrosine 100 to Leucine: This made the enzyme much lesser effective and it lost the grip to starch molecules

Phenylalanine 183&259 to Leucine: This affected how the starch fit into the enzyme by shifting positions in which it gets attached

Industrial Production (Upstream)

Strain Isolation

CGTase is produced by Gram-positive bacteria, Bacillus circulans, Bacillus macerans and Bacillus Sp. are some of the potential strains for production of CGTase.

I'm selecting **Bacillus cirulans** as it is one of the most widely used strains in industrial production of CGTase.

Gene Cloning and Expression

Same vectors used in cloning of sEH genes will be used for cloning and carrying CGTase genes as well

pET-28a (+) for easier purification as it has a histidine tag

pGEX-4T-1 for more solubility and higher efficiency

The CGTase gene is first isolated and then inserted into the plasmid using restriction enzymes and DNA ligase. The recombinant plasmid is introduced into E. coli or Bacillus subtilis. The bacteria that successfully absorb the plasmid are selected using antibiotic resistance markers.

Fermentation

Once the genetically engineered bacteria is ready, they are grown in fermenters/bioreactors under controlled environment to efficiently produce CGTase.

Bacteria are grown in nutrient rich media containing starch, nitrogen sources(ammonia) and minerals. The temperature, pH and oxygen transfer are closely monitored and regulated as per requirement.

Bacteria naturally produce CGTase but for higher efficient production an inducer like IPTG is added to enhance expression system. Most of the bacteria secrete CGTase directly into medium, making it easier to extract.

Downstream processing (purification & recovery)

As CGTase is secreted extracellularly by most species including bacillus circulans, the downstream processing is relatively easier compared to other intracellular methods. It also ensures better concentration and purity of enzymes as there are less contaminants to worry if the enzyme is secreted into the broth itself.

Once CGTase is secreted into the broth in fermentation, it needs to be purified, concentrated and quantified for it to be available for industrial or commercial uses.

Separation of cells & Impurities

As CGTase is secreted extracellularly, the first step is to separate bacterial cells from the liquid fermentation broth. This can be accomplished by centrifugation or membrane filtration methods

This step removes unwanted solid residues and gives us a broth which is rich in CGTase

Concentration

After completion of the cell separation, we do have a CGTase rich broth but it is still mixed with other proteins and unwanted components. To concentrate it, we use precipitation techniques such as:

Ammonium sulphate precipitation

This method helps us separate CGTase out of the solution while keeping the structural aspects intact.

Ultrafiltration

This technique is a membrane-based technique that removes smaller contaminants while retaining the enzyme.

Going with both the techniques will ensure more concentration of CGTase, which we require.

Purification

After successful concentration step, we proceed further with purification via chromatography techniques. This step ensures that our enzyme is separated from other proteins and biomolecules based on size, charge or binding affinity.

As we have included a Histidine tag in the gene cloning process using pET-28a(+) vector, we can proceed with affinity chromatography using the advantage of the tag by through metal ions

Purification via Immobilized Metal Affinity Chromatography (IMAC)

The Histidine tag on CGTase has a strong affinity to bind with metal ions such as Ni+2 or Co+2. The fermentation broth containing extracellular CGTase which has been concentrated earlier will be passed through an IMAC column loaded with Ni+2 (preferably).

CGTase binds specifically to the metal ions (Ni+2) as Histidine group is sterically hindered around oppositely charged Ni+2, while impurities and unwanted proteins flow through the channel.

Elution of purified CGTase can be achieved by using a concentrated imidazole buffer which will replace the position of CGTase's histidine and makes it elute. Imidazole competes with Histidine of CGTase for binding to Ni+2, making the enzyme elute with high purity. Using imidazole makes sure the enzyme is gently disrupted without making any damage to the enzyme itself.

Now, the collection of eluted CGTase is still not completely pure as it might contain imidazole contain
ning buffer and it may reduce the enzymatic activity, therefore we should now go with dialysis of buffer to exchange the buffer with a new one to make sure the CGTase is completely pure. This can be achieved with ultrafiltration techniques.

Final Formulation & Stabilization

Based on the use cases we can proceed with either Freeze Drying or Liquid based formulation of CGTase. Freeze drying will be preferred as it makes sure the enzyme activity and stability are retained and also helps in portability of the enzyme and makes it economically more feasible to transport and large-scale industrial use.

Applications

Food and Pharmaceutical Industries

By now we know that CGTases produce Cyclodextrins (CDs) from starch, CDs are cyclic oligosaccharides which plays a role of natural molecular encapsulators. They enhance solubility, stability and controlled release of bioactive compounds, making them valuable in food, pharmaceuticals and cosmetics.

CGTase converts starch into cyclodextrins of different kinds (α-CDs, β-CDs, γ-CDs). Different types of CDs have a different role in industrial usage:

α-CDs are primarily used in dietary supplements and food emulsification whereas β-CDs are commonly used in drug delivery for hydrophobic drugs and finally γ-CDs are used in flavours, fragrances and cosmetics with controlled release.

Removal of Toxic Compounds

Usually, Industries produce many hydrophobic pollutants such as heavy metals, pesticides and oils into the environment this may cause trouble when not treated properly and at the same time as these pollutants are poorly soluble, they are very difficult to degrade and treat. As this is exactly what CGTase does, employing it in discharge treatment might be very useful.

Cyclodextrins produced by CGTase acts as molecular cages, by capturing hydrophobic toxins they improve the water-soluble property of discharge pollutants. This makes the microorganism present in the water to degrade the pollutants easier. Best example for this us of CGTase is β-CD is used to remove pesticide residues from water

Biofuel & Renewable Energy Applications

These days as the entire world is looking at renewable energy, enzymes like CGTase are being explored for biofuel production. Starch based biofuels like bioethanol require very specific and efficient enzymatic breakdown of starch which can be accomplished by CGTase very effectively.

CGTase can modify starch in such a way that it turns into a soluble sugar precursor, improving bioethanol yield. It also helps in converting agricultural waste into biofuels in a very affordable and efficient manner helping sustainability and economic feasibility. This can be explained using live example of CGTase enhancing starch hydrolysis efficiency, reducing pre-treatment costs of it.

Conclusion

CGTase plays an important role in many industrial applications such as food, pharmaceuticals, environmental protection and renewable energy. It’s ability to convert starch into valuable cyclodextrins is unmatchable

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