Glycosyl Hydrolases: Biochemistry and Applications

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Abstract

Glycoside hydrolases, cleave the glycosidic linkage of glycosides, forming a sugar hemiacetal or hemiketal and a free aglycon. It also catalyzes the hydrolysis of carbohydrates with O-, N- and S-linkages. Since these glycosidases hydrolyze specific linkages they are used for elucidating the structure and function of polysaccharides.

Glycoside hydrolases can be classified in several ways based on their substrate specificity, their molecular mechanism and amino acid or nucleotide sequence similarity which reflects their evolutionary relationship. Lysozyme, (glycoside hydrolase, muramidase), is a small, monomeric protein and was the first protein which underwent extensive structural studies. Antibacterial property of lysozyme against Gram positive organism is being exploited by different industries like food processing, food preservation and pharmaceutical industry making it an commercially important enzyme.

Keywords

Glycoside hydrolase · Muramidase · Lysozyme · Antibacterial · Retaining hydrolases · Inverting hydrolases

Introduction

Carbohydrates are the most abundant biomolecule on Earth. They play a diverse roles in living organisms from being structural elements (cellulose, chitin) and energy molecules (starch, glycogen) to being involved in cell recognition processes.

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S. Shrivastava (ed.), *Industrial Applications of Glycoside Hydrolases*,
https://doi.org/10.1007/978-981-15-4767-6_2

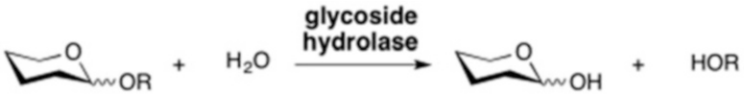

Fig. 1 Reaction catalysed by glycosidases

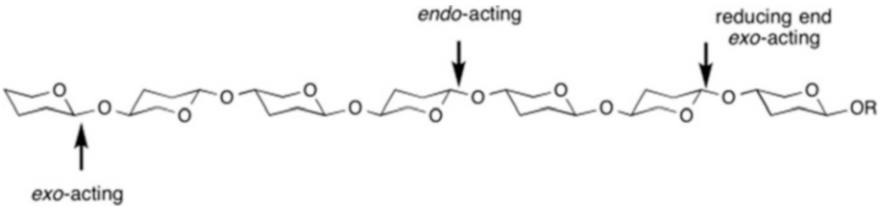

Fig. 2 Position of hydrolysis of endo- and exo-glycosidases

A covalent bond that joins a carbohydrate (sugar) molecule to another group, which may or may not be another carbohydrate, leading to formation of long polymers or polysaccharides is termed a glycosidic bond or glycosidic linkage.

Glycoside hydrolases (EC 3.2.1. . .) are a broad group enzymes that hydrolyze the glycosidic linkage of glycosides, forming a sugar hemiacetal or hemiketal and the free aglycon (Fig. 1). Glycoside hydrolases alternately are also referred to as glycosidases, or glycosyl hydrolases. The glycosidic bond can link two or more sugars, or link a sugar and a non-sugar moiety. Glycoside hydrolases can also hydrolyse linkages of carbohydrates with O-, N- and S-linkages. Since glycosidases hydrolyze definite linkages in polysaccharides they are used for elucidating their structures and functions.

Classification

Glycoside hydrolases are classified in several ways based on their substrate specificity, their molecular mechanism and amino acid or nucleotide sequence similarity which reflects their evolutionary relationship.

One of the easiest ways of classification is as endo- and exo-glycosidases based on the capability of the enzyme to hydrolyse the reducing end of the substrate (mostly, but not always) or in the middle of the chain (Fig. 2). Most cellulases are *endo*-acting, whereas Lac Z β-galactosidase from *E. coli* is *exo*-acting (Davies and Henrissat 1995).

The glycosyl hydrolases can be classified based on sequence or evolutionary similarity. This has divided the glycosyl hydrolases into 128 families and 14 clans. Glycoside hydrolase family 1 (GH1) comprises some common enzymes like beta-glucosidase (EC: 3.2.1.21); beta-galactosidase (EC: 3.2.1.23); 6-phospho-beta-galactosidase (EC: 3.2.1.85); 6-phospho-beta-glucosidase (EC: 3.2.1.86);

lactase-phlorizin hydrolase (EC: 3.2.1.62), (EC: 3.2.1.108); beta-mannosidase (EC: 3.2.1.25); myrosinase (EC: 3.2.1.147) etc.

Nomenclature can also be based on substrate that they act upon. Thus glucosidases act on glucosides and xylanases catalyze the cleavage of the homopolymer of xylose called xylan. Other common examples are lactase, amylase, chitinase, sucrase, maltase, neuraminidase, invertase, hyaluronidase and lysozyme.

Depending on the reaction mechanism, glycoside hydrolases can be classified based on the stereochemistry of the product formed. They can be classified as either *retaining* or *inverting* enzymes. These two most common reaction mechanisms that was first outlined by Koshland. Enzymatic hydrolysis of the glycosidic bond takes place via a general acid base catalysis that requires two vital residues: a proton donor and a nucleophile/base. Some variations of these mechanisms have been found and one catalyzed by an NADH cofactor, has been identified. The reaction takes place by two key mechanisms giving rise to either retention, or an inversion, of the anomeric configuration at the end of the reaction (Gebler et al. 1992).

Retaining Glycoside Hydrolases

Retaining glycosidases catalyze a two-step reaction, each step resulting in inversion, for a net preservation of stereochemistry (Fig. 3). In retaining enzymes, the nucleophilic catalytic base is in close vicinity of the sugar anomeric carbon. This base is more distant in inverting enzymes because a water molecule is accommodated between the base and the sugar. This difference results in an average distance between the two catalytic residues of −5.5 Å in retaining enzymes as against −10 Å in inverting enzymes (Gloster et al. 2008).

Inverting Glycoside Hydrolases

The catalysis of the glycosidic bond by inverting glycoside hydrolases results in a net inversion of the anomeric configuration. This is a single step, single-displacement mechanism involving oxo-carbenium ion-like transition states (Fig. 4). The reaction normally occurs with a general acid–base catalysis with glutamic or aspartic acids usually being two amino acids involved in the catalysis process (McCarter and Withers 1994).

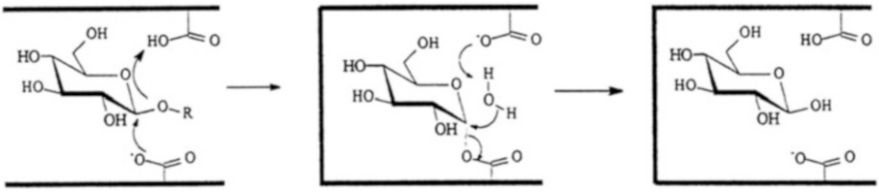

Fig. 3 Mode of action of retaining glycoside hydrolases

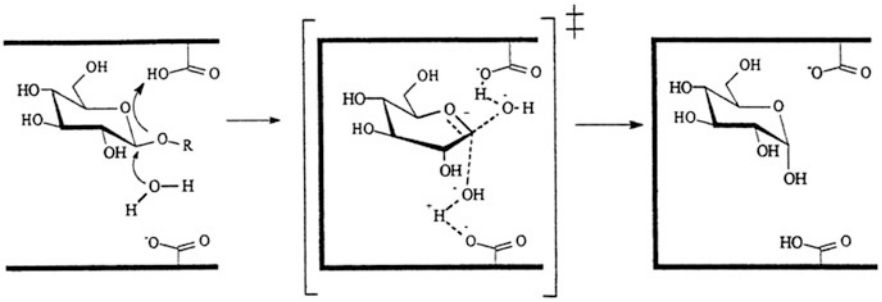

Fig. 4 Mode of action of inverting glycoside hydrolases

Lysozyme: A Representative Glycosyl Hydrolase

Introduction

Lysozyme, also known as muramidase or N-acetylmuramic hydrolase, is a monomeric protein of 129 amino acids, stabilized and cross-linked by four disulfide bridges among the eight cysteine residues of its polypeptide chain. Alexander Fleming accidentally discovered that a drop of his nasal mucus could cause the lysis of bacteria, which enabled him to detect a 'remarkable bacteriolytic element' that he later named lysozyme (Fleming 1922).

Lysozyme, N acetyl-hexosaminidase is ubiquitous in nature. Lysozyme is a naturally occurring enzyme found in bodily secretions such as tears, saliva, serum, and milk. It is also present in cytoplasmic granules of the macrophages and the polymorphonuclear neutrophils (PMNs).

Chicken egg white is the richest source of lysozyme with a concentration ranging between 3400 and 5840 mg/L (Sauter and Montoure 1972; Wilcox and Cole 1957). Similar enzymes are also found in organs and secretions of various vertebrates, invertebrates, bacteria, and even plants (e.g., Papaya latex), thus making lysozymes the most widely distributed substance (Ogawa et al. 1971). Based on different characteristics like structure, catalysis and immunization, lysozymes are classified into three families: chicken-type (c-type), goose-type (g-type) and invertebrate-type (i-type). Several other types of lysozymes, including phagetype, bacterial-type and plant-type lysozyme, have also been documented (Callewaert and Michiels 2010; Cao et al. 2015).

The bacteriolytic properties of hen egg white lysozyme (HEWL) were first described by the Russian scientist Laschtchenko in 1909. It was given the name, lysozyme in 1921 by Sir Alexander Fleming, since it causes bacterial lysis. Lysozyme catalyzes the breakdown of certain carbohydrates found in the cell walls of certain bacteria (e.g., cocci) and functions, in the case of lacrimal fluid, to protect the cornea of the eye from infection. Lysozyme has many firsts to its credit too. Lysozyme being first enzyme having all the 20 usual amino acids to be sequenced

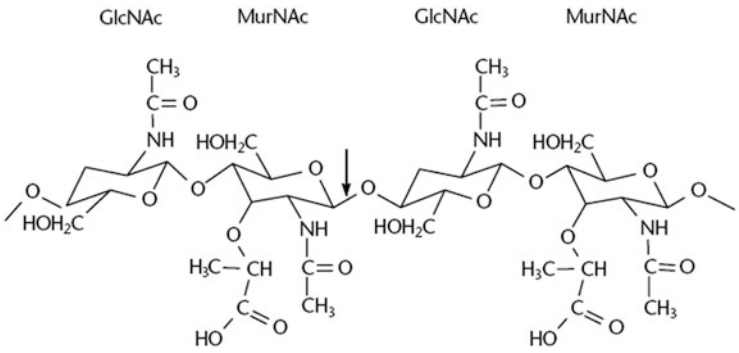

Fig. 5 Substrate for lysozyme is a copolymer of N-acetylmuramic acid (MurNAc, NAM) and N-acetyl glucosamine (GlcNAc, NAG), constituent of bacterial cell walls. The bond hydrolysed by lysozyme is indicated by the arrow (Imoto 2001)

(1963), and the first enzyme whose reaction mechanism and precise X-ray crystallographic study was accomplished (1961–1966). Thus, lysozyme is one of the most thoroughly characterized enzymes and serves as a model enzyme in biochemistry and biology.

Lysozyme being a small, stable enzyme makes an ideal molecule for research into protein structure and function and the recent development of structural biology owes much to it. Brian Matthews performed hundreds of mutations on the bacteriophage lysozyme by replacing one or more amino acids in the protein chain, removed large residues inside the protein, leaving a hole, or crammed a large amino acid inside, where it would not normally fit to study the consequence of such changes in the function of the enzyme. He has also attempted to create new active sites by creating new molecule-shaped pockets. Structures of hundreds of these mutant lysozymes are available at the PDB (Kuroki et al. 1993; Pjura et al. 1990).

Lysozyme catalyzes the breakdown of 1, 4-beta-linkages between C-1 of N-acetylmuramic acid (NAM) and C-4 of N-acetyl-D-glucosamine (NAG) residues in a peptidoglycan, a major component of gram-positive bacterial cell wall. This hydrolysis in turn compromises the integrity of bacterial cell walls causing lysis of the bacteria. It exerts lytic action mainly on Gram-positive bacteria and thus is effective against bacterial and viral infections (Fig. 5).

Hen Egg White Lysozyme (HEWL)

Hen egg white has abundant quantities if lysozyme and hence has been the most researched one. It is a small protein consisting of 129 amino acids, with a molecular weight of 14,307 Da. The 10 carboxyl, 7 amino, 11 guanidyl groups, six tryptophyl residues, and four disulfide bridges in locations between Cys 6-Cys127, Cys30-Cys115, Cys 64-Cys 80 and Cys 76-Cys 94 helps its folding into a compact globular structure and lends stability and unusual compaction. Lysozymes were the first

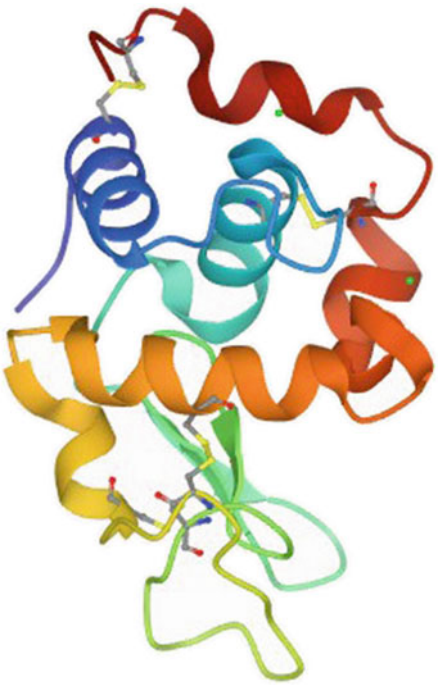

Fig. 6 Ribbon diagram of hen egg white lysozyme

enzyme to have their three-dimensional (3D) structures resolved by X-ray crystallographic method in 1965 by David Philips (Deborah et al. 1984; Fleming 1922). X-ray studies of the lysozyme-inhibitor complex helped identify the structure and location of the active site which is located in a deep cleft of the protein structure. It has both alpha and beta folds, consisting of five to seven alpha helices and a three-stranded antiparallel beta sheet. The enzyme is roughly ellipsoidal in shape, comprising of two domains joined by a long alpha helix forming a cleft. The upper lip of the active site cleft confers potent antimicrobial activity. The N-terminal domain (residues 40–88) has some helices and beta parallel sheets and the second domain (1–39 and 89–129) has mostly alpha helical structure (Fig 6).

Catalytic Mechanism

The two catalytic amino acids residues were identified as aspartate-52 and glutamate-35. At pH 5 (optimum pH for lysozyme), Asp-52 is deprotonated, while Glu-35 is in its undissociated form, the enzymatic reaction is possible due to this successful co-existence. Glu-35 acts as general acid in the reaction donating the proton to oxygen atom of glycosidic bond to accelerate the reaction. Asp-52 stabilizes the oxy-carbonium ion generated at the site of the reaction.

In most of the glycosyl hydrolases studied aspartate and/or glutamate residues have been found to be involved in catalysis.

Glycosyl hydrolases have innumerable industrial and biotechnological applications from biofuel production to drug design. Cellulases are involved in several industries like biofuel, food, feed, beverages, paper, textile, pharmaceutical, agricultural etc. Enzymic hydrolysis of cellulosic biomass results in the generation of sugars that are the starting materials for production of various value added products of commercial interest, such as bioethanol, organic acids, sugars and animal feeds (Kuhad et al. 2011).

Genetically inherited or, in rare cases acquired deficiency or malfunction of glycosyl hydrolases, such as lactase deficiency or break down of cell–cell communication can lead to serious health problems, from allergies and autoimmune diseases to severe recessive lysosomal storage diseases like Tay-Sachs, Hunter, Fabry, Gaucher, and Krabbs diseases, caused by lack of hexosaminidase A, iduronate-2-sulfatase, α-galactosidase A, and glucocerebrosidase, respectively. Glycosyl hydrolases, glycosyl hydrolases inhibitors, and chaperones are used to treat these diseases, but chances of a complete cure are still a long way off.

Applications of Lysozyme

Antibacterial characteristic of lysozyme against Gram-positive organisms is employed by a myriad of industries like food processing, pharmaceutical, and medicine. The food processing industry benefits primarily by its application as a natural preservative. Lysozyme is widely used as a preservative for meat, fish, milk, dairy products, as well as for fruits and vegetables. The pharmaceutical industry utilizes lysozyme for creating adjuvant drugs for antibiotics and analgesics to be used in microbial infections. It is also used in the treatment of leukemia and neoplastic diseases. Furthermore, lysozyme can act as an indicator of the progression of pathological variations in humans and animals.

Lysozyme has the characters of a ferment. There is an increase in activity up to 60 °C, but above 65 °C, it is destroyed more rapidly. Neutral medium is best to act optimally. Lysozyme resists peptic or tryptic digestion thus increasing the superfluity of applications. Lysozyme can be preserved for a long duration, when kept dry. Commercial dried egg albumen is considered as very rich source of lysozyme.

The protein engineering of lysozyme has substantially extended its practical applications. The engineered enzyme, being a dimeric protein exhibits a new specific activity in relation to Gram-negative bacteria and Gram-positive bacteria. In the monomeric form it is more active against Gram-positive bacteria, as reported by Ibrahim et al. (1991, 1996), Lesnierowski et al. (2004) and Kijowski et al. (2006). A drug produced on the basis of lysozyme dimer exhibited immune stimulating and immune corrective activity and was more potent in the treatment of bacterial and viral diseases.

Antimicrobial Activity

Lysozyme being a muramidase, hydrolyses the β-l, 4-glycosidic linkage of peptidoglycans, and leads to breakdown of the murein layer which decreases the mechanical strength of the bacterial cell wall. Hence, nucleic acids do not bind to the cell wall resulting in the disintegration of bacterial genetic material, finally leading to the death of the bacteria (Wang et al. 2005).

Non-lytic activity of lysozyme was recognized by the fact that partially or completely denatured lysozymes deficient in enzymatic activity could still work against both classes of bacteria. This property has been attributed to the cationic and hydrophobic properties associated with the conformational changes (Ibrahim et al. 2002; Masschalck et al. 2000).

Lysozyme modified with polysaccharides by the Maillard reaction, exhibits many enhanced characters such as improved solubility, stability, emulsifying characteristics and antimicrobial activity. These improved characteristics make lysozyme an ideal molecule in food matrices, especially in the fish, meat, dairy, fruit, vegetable and wine processing industries.

Lysozyme from egg white is widely used in food industries. It is effective mainly against Gram-positive bacteria, but through denaturation, chemical modifications, or by combining it with other preservatives its spectrum can be broadened for Gram-negative bacteria also. The stability and safety of lysozyme makes it an ideal preservative for other food applications such as food packaging too (Min and Krochta 2005). It is also used as a preservative for food like sausages, broiler, raw marine products, as well as an antibutyric acid blowing agent in semi-hard cheese production. It increases shelf life of nominally processed and non-sterile foods like meats.

It can be added to infant formula milk and significance of lysozyme is in the improvement of digestibility of the milk by the normalization of the intestinal flora, and also it contributes in the improvement of natural immunity through the augmentation of properdin, r-globulin and agglutinins.

Saliva shows strong antimicrobial effect on the oral pathogens due to the imminent presence of antibodies as well as proteins. One such protein is lysozyme which is one of the most powerful natural antibacterial and antiviral activity compound. According to erstwhile studies, it has been reported that lysozyme may bind and aggregate Gram-positive bacteria and Gram-negative periodontopathic bacteria such as *Capnocytophaga gingivalis* (Jesse Joel et al. 2016). Lysozyme plays a vital role in improvement of digestive function in ruminants (Dobson et al. 1984), leaf-eating monkeys, and birds (Kornegay et al. 1994). In many invertebrates, bacteria represent a substantial part of their diet. Worms and flies often feed on decomposing organic matter including the large biomass of microorganisms, digestion of which is aided by midgut lysozyme (Lemos and Terra 1991).

Lysozyme, the enzyme produced by polymorphonuclear and mononuclear inflammatory cells, is a strong antibacterial molecule that inhibits the growth of pathogens. Lysozyme and β defensin-2 can act synergistically against some microbial strains which is in consistent with the concept that secreted antimicrobial

peptides and other components of innate immunity constitute the first line of defense consequently protecting the host mucosal surfaces (Lee et al. 2004). It is used prophylactically for dental caries, lozenges for sore throat, in contact lens decontamination solutions, and eye drops, topical creams for dystrophic and inflammatory conditions of skin and tissues. It is also used as a prognostic marker for male prostate cancer.

Industrial Applications

Lysozyme, from egg white is extensively used in soluble form to control lactic acid bacteria in different foods. It can be used to control malolactic fermentation (MLF) during winemaking. MLF is only considered necessary in red and in some white wine but requires to be controlled in all other types of wine (Liburdi et al. 2014).

Lysozyme can effectively control the growth of beer spoilage bacteria, chiefly lactic acid bacteria (Commission Regulation 2012). Lysozyme added to beer exerts a strong inhibitory action on any lactic acid bacteria that may be present and is very stable throughout the shelf life without any unfavorable impact on beer savor or foam stability (Silvetti et al. 2010). It is a good alternative to sulphites and prevents malo-lactic fermentation. But it has no effect against other spoilage agents like yeasts.

Lysozyme (as the hydrochloride) is used in cheese production to prevent "late blowing" (cheese spoilage). This phenomenon is caused by the growth of *C. tyrobutyricum* which carries out butyric acid fermentation. Lysozyme exhibits high specificity by preventing microbial contamination without inhibiting starter and secondary cultures required for the ripening process (Mine 2008). *C. tyrobutyricum* metabolize lactate and produce carbon dioxide, hydrogen, butyric acid and acetic acid. In curing process the accumulation of these gases builds up pressure and causes splits or cracks in the cheese. Lysozyme influences the acceleration of cheese ripening by the lysis of starter bacteria leading to the release of cytoplasmic proteolytic enzymes, which plays a key role in the cheese ripening process (Abdou et al. 2013).

Thus lysozyme obtained from comestible sources that is commonly used as food, can be considered acceptable for use in food processing when used in accordance with good production practice.

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