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Oligomeric Structure of β-Glucosidases

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INTRODUCTION

β-Glucosidases (E.C. 3.2.1.21) catalyze the hydrolysis of the β-glycosidic bonds of aryl- and alkyl-β-glucoside, and cellobiose. They are widely distributed in all living organisms, as glycoconjugates are one of the most diverse groups of organic molecules in the biosphere. The aglycone of the glucosides confers substrate specificity of the enzyme [1] and the aglycones released from the glucosides play important physiological roles in the fundamental biological processes and in the industrial applications [2-9].

In plants, β-glucosidases have been implicated in regulating various aspects of principal metabolic events and growth-related responses, e.g. phytohormone activation [2-3], cell wall degradation in the endosperm during germination [4], lignification [5], and pathogen defense reaction [6-7]. An oat (*Avena sativa*) plastidal β-glucosidase is involved in the defense mechanism against fungal infection [8]. Cellulose, our most abundant renewable resource on this planet, breaks down cellulose to cellobiose, and b-glucosidase hydrolyzes the cellobiose to two glucose molecules. In humans, a β-glucosidase hydrolyzes glucosylceramide in the lysosome, and its lack leads to Gauchers disease, with the symptoms of anemia and thinning of the bones [9].

Glycosyl hydrolase (EC 3.2.1.x) have been classified into approximately 60 families on the basis of their amino acid sequence similarities rather than substrate specificity (10-12). These families of glycosyl hydrolase are grouped into 5 larger superfamilies called 'clan GH A-E' by significant similarities in tertiary structure along with conservation of the catalytic residues and catalytic mechanism. Family 1 is included in clan GH-A and characterized by an $(\beta/\alpha)_8$ barrel structure, retaining in the anomeric configuration of the glycone and two catalytic glutamic acid residues in the β strands number 4 and 7. β-Glucosidases appear in 1, 3 and 4 of these families. In particular, a large number of these enzymes are grouped in family 1. In active site of the family 1 β-glucosidases, the two catalytic glutamic acids in the I/VTENG and TNEP motifs play critical roles in catalysis as a nucleophile or an acid/base catalyst, respectively [13]. However, a few of experimental data on molecular determination of aglycone specificity of β-glucosidases are now available [14-15].

In particular, glycosyl hydrolase family 1 (β-glucosidase) has similar tertiary structure and a strong tendency to form homo- and hetero-oligomers [10-12]. A main difference in their tertiary structure of the monomers lies in the surface loops near active site [16]. The quaternary protein structures of family I β-glucosidases are of great **interest** because they exist as various forms of oligimers such as dimer, tetramer, octamer or larger aggregates made of different numbers of subunits [16-25]. Nevertheless, the functional implications of such diverse oligomerization schemes remain unclear. Structural studies of the unique oligomeric β-glucosidases are an important requirement toward uncovering their precise role(s) in biological processes.

OLIGOMERIZATION CHARACTERISTICS OF β-GLUCOSIDASES

The large number of glycoside hydrolase families reflects this diversity of substrates and the need for selective hydrolysis of the glycosidic bond. The three-dimentional crystal structure of several family 1 β-glucosidases (1CBG, white clover (*Trifolium repens*) linamarase; 1MYR, white mustard (*Sinapis alba*) myrosinase; 1BGA, *Bacillus polymyxa* β-glucosidase; 1PBG, *Lactococcus lactis* phospho-β-galactosidase; 1GOW, *Sulfolobus solfataricus* β-glucosidase; 1QVB, *Thermosphaera aggregans* β-glucosidase, 1QOX, *Bacillus circulans* sp. *alkalophilus* β-glucosidase; 1ELE, maize β-glucosidase) has recently been elucidated [16-23].

Glycosyl hydrolase family 1 to which the BGA family belongs has similar tertiary structure, catalytic mechanism and a tendency to form homo- and hetero-oligomers [10-12]. Although multimeric enzyme proteins are generally composed of a fixed number or molar ratio of subunits, some BGA family β-glucosidases have a tendency to form discrete homooligomers having different number of subunits. For example, in *Hevea* β-glucosidase and casava linamarase exist homooligomers of different MW that are composed of different number of single monomers [24-25]. These enzymes

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display several discrete protein bands on native gel electrophoresis of their extracts as does the oat β-glucosidase. Oat β-glucosidase is a linear assembly of a distinct structure consisted of monomers [26-27], whereas the other enzymes are an aggregate of different numbers of subunits (monomers). The function of such oligomerization has not yet been elucidated, although it is assumed to be required for catalytic activity or structural stability [16-23].

TECHNIQUES FOR STRUCTURAL STUDY OF OIGOMERIC PROTEINS

Conventional light microscopy and confocal fluorescence scanning light microscopy can not be used for structural study of individual proteins, because of the low resolution of these techniques. Although atomic force microscopy (AFM) has a source of high-resolution information [28], it allows the visualization only of the surfaces and therefore provides limited insight into the internal three-dimensional (3-D) structure of macromolecules.

Three different techniques have proven successful for reaching beyond the wavelength limits of visible light and into high resolution range in which individual molecules and their internal structure can be visualized: nuclear magnetic resonance spectroscopy (NMR), X-ray crystallography, and high-resolution electron microscopy (EM).

Nuclear magnetic resonance spectroscopy (NMR) [29]

NMR is particularly well suited for small molecules and for individual domains of proteins. It allows the study of the macromolecule in solution, and avoids the time-consuming search for optimal crystallization conditions. However, this technique requires large amounts of protein (several milligrams) and a high solubility in water. NMR can not be successfully applied for the structural studies of typical membrane proteins and filamentous proteins due to a size limit for the proteins to be studied.

X-ray crystallography [30]

X-ray analysis is the first method to solve the structure of large individual protein and currently the excellent source of structural study. It allows the investigation of various biological specimens, ranging from peptides to viruses by irradiating a 3-D crystal, recording the spatial distribution and the intensities of the diffracted X-ray beams and calculating from diffraction patterns. X-ray crystallography is a powerful technique to reveal the internal three-dimensional (3-D) structure of macromolecules. But this method has several limitations to elucidate large multi-component complexes. The critical procedure for an X-ray analysis is to grow large and well-ordered 3-D crystals. Finding the suitable conditions for crystallization is usually a labor-intensive process as the conditions for crystal growth cannot be predicted. In particular, proteins of larger molecular weight often resist attempts at crystallization and crystal may grow after flexible structural regions have been removed. In addition, it is more difficult to crystallize integral membrane proteins and filamentous proteins, such as actin and tubulin, as they often form aggregates rather than well-ordered 3-D crystals.

Transmission electron microscopy (TEM)

NMR and X-ray crystallography are used for determining the structure of proteins at an atomic resolution. However, these methods are limited to the study of small proteins and the protein must be crystallized first. Electron microscopy cannot give high enough resolution to reveal a protein at atomic resolution, but can be especially useful to view large protein complexes that cannot be crystallized. EM, combined with 3-D image reconstruction techniques of cryo-EM, electron tomography and single-particle analysis, offers some distinct advantages over the X-ray crystallography and NMR methods: First, large protein assemblies and complexes, with dimensions of 30-200 nm, are especially suitable for structural study by cryo-EM reconstruction. Second, nonhomogeneous samples can be effectively studied because a homologous group can be manually selected from the cryo-EM micrographs. Third, time-resolved studies can be carried out more easily because biochemical reactions can be terminated at a preferred time point by flash freezing. Forth, phase information for the structure retained as the diffracted electron beam is refocused to form a real-space image on an image plane. Finally, reconstruction at medium resolution (~20 Å) can be easily obtained in a week.

On the other hand, several difficulties must be overcome to obtain the high-resolution information with EM [31-32]. First, because the object of interest must be investigated in a vacuum, special care must be taken to avoid sample drying. Second, since the object is heavily bombarded with high-energy electrons and biological objects are radiation sensitive, radiation damage must be minimized for recording intelligible information. Finally, a 3-D structure must be reconstructed from 2-D projections of the molecule in various orientations, because electron micrographs represent only two dimensional (2-D) projections of a 3-D object.

A brief description for the 3-D image reconstruction techniques of cryo-EM, electron tomography and single-particle analysis are as follows:

Cryo-electron microscopy

The pioneering work of Taylor and Glaeser [33] and of Dubochet and colleagues [34] paves the way for cryo-electron microscopy. Cryo-electron microscopy can be used to visualise the intermediate structures in the reaction pathways of large protein complexes. It has the advantage that the vapor pressure of water becomes negligible at temperatures below -100C. Sample drying can thus be avoided by keeping the protein at liquid nitrogen temperature. Vitrification of the sample using the plunge-freezing method works particularly

well for individual proteins and multiprotein complexes. In addition, there is no distortion of the sample because the sample is always in solution. Also, the electron beam causes less damage to the sample though the low dose methods which are normally used in cryo-EM. However, biological macromolecules are normally made up of carbon, hydrogen, oxygen, and nitrogen and the electron absorption of such molecules is very low. As a result, image contrast is also very low and it is hard to detect features when dealing with just a few images. The important procedure for cryo-EM is to form thin vitreous ice. To prepare the sample for cryo-EM, the specimen grid is rapidly plunged into liquid ethane and then the water turns into vitreous ice. The grid is transferred to liquid nitrogen and mounted in the cryoholder of the electron microscope.

Electron tomography [35]

Electron tomography is particularly well suited for very large particles such as cell organelles and large multiprotein complexes. Images of the same specimen are recorded at tilt angle increments of about 1. Since the tolerable electron dose must be divided over some 120-140 images, and since averaging techniques cannot be applied, the resolution of such tomograms is fairly low (20-70 Å). However, in comparison with large dimensions of such objects, the information content of tomograms can be enormous. The specimen can be either large individual particles or tissue sections. Using vitrification of the sample using the plunge-freezing method in combination with electron tomography allows one to investigate very large particles in three dimensions and in fine detail.

Single-particle analysis

Single-particle analysis is suited for large proteins or protein complexes (usually exhibiting a molecular mass higher than 500 kDa) that can be obtained in sufficient quantities and homogeneity. Upon classification and alignment of the particles, a 3-D structure can be calculated. Averaging of about 73,000 particles yielded a resolution of 11.5 Å for the 70S ribosome [36].

QUATERNARY STRUCTURE OF β-GLUCOSIDASES

The members of family 1 β-glycosidase share high similarity in their tertiary structure from plants to archaeal bacteria that have 17% amino acid sequence homology in the case of maize and *Sulfolobus solfataricus* β-glucosidases [23]. The enzyme is globular in shape, with eight a/b barrel motifs [$(\alpha/\beta)_8$] and two catalytic glutamic acid residues in the b strands number 4 and 7 [13]. The $(\alpha/\beta)_8$ fold is stable structural fold and one of the largest and most regular of all domain structure [37]. It has been found in many different kinds of proteins, most of which are enzymes, with completely different amino acid sequences and functions. A comparison of the structure of these proteins shows that the β strands and a helices are connected with loop regions that have quite different lengths and conformations in the different proteins. This implies that the β strands and a helices have important roles in the structural framework of the enzyme, whereas the loops contain the amino acids responsible for its catalytic chemistry and substrate binding. In some cases the loops are very long and form independent domains in the overall subunit structure. The major structural difference among the family 1 enzymes lies in the surface loops surrounding the active site [16].

Although it is common for the family I β-glycosidases to form oligomers, the regions involved in the formation of the oligomers vary widely from one enzyme to the other. A comparison of critical regions contributing to the dimer formation, among the plant β-glycosidases; maize (*Zea mays*) β-glucosidase Glu1, white clover (*Trifolium repens*) linamarase, and white mustard (*Sinapis alba*) myrosinase shows no similarity [17-18, 23]. For example, the important regions of dimer formation in white clover (*Trifolium repens*) linamarase are located at the C-terminal region, whereas all of the dimeric interface residues in white mustard (*Sinapis alba*) myrosinase are located at the N-terminal region. Also, the dimer interface in the maize (*Zea mays*) β-glucosidase Glu1 is located in the mid-section of the polypeptide chain. None of the above-mentioned interface residues is conserved universally among family 1 glycoside hydrolases, suggesting that the specific monomer surface sites and domains interacting to form a quaternary structure are not critical as long as they do not block the active site.

Maize β-glucosidase

In maize (*Zea mays*), b-glucosidase (2-*O*-b-d-glucopyranosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one hydrolase) occurs in two different forms, ZMGlu1 and ZMGlu2 [23]. The deduced protein products of two isoenzymes cDNAs share 90% sequence identity. The catalytically active form of maize β-glucosidase is a 120 kDa homodimer. As with other β-glucosidases, the ZMGlu1 monomer folds as a $(\beta/\alpha)_8$ barrel fold (Fig. 1A). The connections between β-strands and a-helices within each β/α repeat are elaborated further by the occurrence of short secondary-structural elements, which have also been observed in the other family 1 β-glycosidase structures. It exists as a dimer that has a dimension of 103 Å (long) × 51 Å (wide) × 47 Å (high) (Fig. 1B), while each monomer has overall dimension of about 52 Å (long) × 51 Å (wide) × 47 Å (high). The dimer interface is formed by residues and peptide spans that are located in the mid-section (Thr-270-Pro-400) of the polypeptide chain.

β-Glucosidase of Bacillus circulans *sp.* alkalophilus

β-Glucosidase from *B. circulans* sp. *alkalophilus* optimally growing at pH of above 9 has optimum activity in the pH range of 6-9 and is fairly stable at temperatures up to 50°C.

This cellulolytic enzyme is of great interest because of their industrial applications due to the stability at high temperature and alkaline pH. The monomeric structure shows a similar $(\beta/\alpha)_8$ barrel structure to those observed within other members of family 1 β-glucosidase (Fig. 2) [22]. It exists *in vivo* as an octamer that is formed by four dimers, and the octamer is able to form long polymeric assemblies only in the *in vitro* crystalline state. When two octamers are viewed along the fourfold axis, in the middle of the molecule there exits a long central tunnel which is mainly filled by the layers of loops from Leu364 to Arg369. The diameter of the narrowest entry of the tunnel is below 5 Å. When the enzymatic function considered, the more important tunnel may be the one between the dimers in the octameric form. The diameters of these tunnels are about 20 Å and active site cavities open into these tunnels. The active site of the upper monomer of one dimer faces to the tunnel, as does also the active site of the lower monomer of the other dimer.

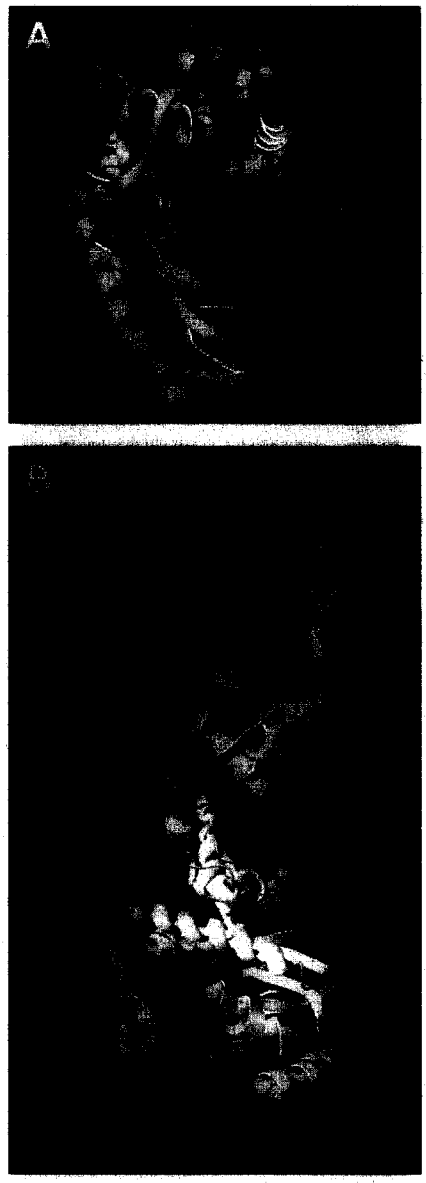

Fig. 1. Structure of maize β-gludosidase. PDB sever is used for the three dimensional protein structures of maize β-gludosidase. Figure can be handled with the Swiss-PdbViewer. The 3-dimensional structure is generated based on the coordinates of 1ELE. Monomeric structure (A) and dimeric structure (B) of maize β-gludosidase.

Oat β-glucosidase

Oat β-glucosidase is the only known enzyme that exists and functions in cells in the form of a supramolecular assembly of long fibrillar multimers. The long fibrillar multimer assembly is distinct from the oligomeric structure of the other β-glycosidases. The multimers exist as two isomeric forms, type I and type II, which are a homomultimer of As-Glu 1 and a heteromultimer of As-Glu 1 and As-Glu 2, respectively [27]. As-Glu 1 monomer plays a crucial role in the formation of

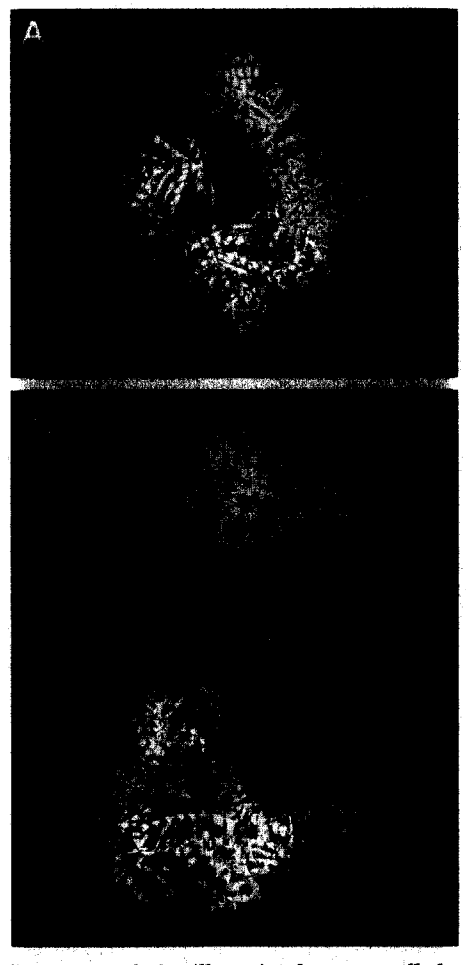

Fig. 2. Structure of *Bacillus circulans* sp. *alkalophilus* β-gludosidase. For three dimensional protein structures of *Bacillus circulans* sp. *alkalophilus* β-Gludosidase, PDB sever is used. Figure can be handled with the Swiss-PdbViewer. The three dimensional structure is generated based on the coordinates of 1QOX. Top-view (A) and side-view (B) of the oligomeric structure of *Bacillus circulans* sp. *alkalophilus* β-gludosidase.

multimers, because it assembles into long fibrillar multimers by itself and also helps As-Glu 2 to form the heteromultimer, whereas As-Glu 2 monomer alone forms a dimer [38].

The unique fibrillar assembly of oat type I β-glucosidase is elucidated by means of cryo-electron microscopy (Fig. 3) [39]. Two electron microscopy approaches, the tilt series and 'single particle' reconstruction are used in order to elucidate the quaternary structure of the β-glucosidase. Oat type I β-glucosidase is assembled by linear stacking of hollow trimeric units and the resulting fibril has a long central tunnel connecting to the outer medium via regularly distributed side fenestrations.

The length of two stacked trimers within the long multimers is 9.0 nm, while 12.7 nm in the free hexamers. This result indicates that the packing within the assemblies are much tighter when the trimers assemble into multimers and less tight when the assemblies are chopped up into hexamers. Tighter packing into the multimers also involves slight rotation between the trimers, as well as an increase in the outer dimensions of the basic unit. When the hexamer is projected down to the longitudinal axis, it results in pure six-fold symmetry in the projection, indicating that two trimers are rotated by 60 degrees to each other upon stacking. On the other hand, the multimers digress from this canonical arrangement, and the trimeric blocks are rotated by 38 degrees to each other. Tighter packing of subunits also results in slight change in the diameter of the multimer: The diameter changes from 10.8 nm in hexameric ring, which is uniform around the circumference of the assembly, to 11.4 nm in one direction and 9.0 nm perpendicular to it.

Enzyme kinetic studies of oat β-glucosidase multimers reveal that larger multimers have higher affinity to substrates than the smaller multimers, and that the largest multimers hydrolyse the β-glycosidic linkage at a lower rate than the hexamer. The experiment of active site inactivation shows that the active site is located on the inner of fibrillar multimers [39]. The results suggests that the oligomerisation of the oat type I â-glucosidase enhances the affinity of the enzyme to the avenacosides, against the competing substrates.

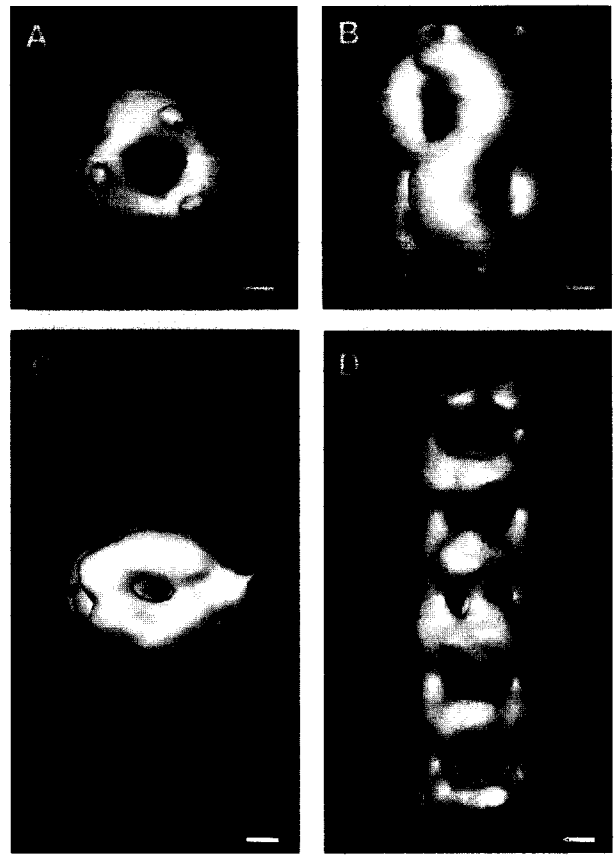

Fig. 3. Fibrillar multimeric structure of oat β-gludosidase. The three dimensional reconstruction was calculated from tomographic tilt series generated by cryo-electron microscopy. A and B are the top and side views of the structure of the trimeric dimer of oat β-gludosidase, respectively, computed from the projection images of randomly oriented particles. C and D are the top and side views of the structure of the fibrillar multimer, respectively, computed from tomographic tilt series and subsequent averaging of extracted volumes.

CONCLUSION

The β-glucosidases occur widely in all living organisms and has in general a tendency to form oligomers of varying numbers of subunits or aggregates, although the functional implications of such diverse oligomerization schemes remain unclear. In particular, the assembly mode of the oat β-glucosidase is very unique in that it multimerizes by linear stacking of a hexameric building block to form long fibrillar multimers. Some structural proteins such as actin and tubulin assemble into long fibrils in a helical fashion and several enzymes such as GroEL and *Pyrodictium* ATPase functional complexes, 20S proteasome of the archaebacterium *Thermoplasma acidophilum*, and glutamine synthetase from blue-green algae, assemble into discrete oligomers upto 4 stacked rings to maintain their enzymatic activities. In particular, oat β-glucosidase exists *in vivo* as a discrete long fibrillar multimer assembly that is a novel structure for enzyme protein. It is assembled by linear stacking of hollow trimeric units. The fibril has a long central tunnel connecting to the outer medium via regularly distributed side fenestrations. The enzyme active sites are located within the central tunnel and multimerization increases enzyme affinity to the substrates and catalytic efficiency of the enzyme. Although it is suggested that oligomerization may contribute to the enzyme stability and catalytic efficiency of β-glycosidases, the functional implications of such diverse oligomerization schemes remain unclear so far.

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