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Endo-*N*-acetyl-β-D-glucosaminidases and peptide-N^4-(*N*-acetyl-β-D-glucosaminyl) asparagine amidases: More than just tools

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Abstract: Since the discovery of endo-N-acetyl-β-D-glucosaminidases (ENGase) and peptide-N4-(N-acetyl-β-D-glucosaminyl) asparagine amidases (PNGase) most of the published work described their use for structural studies. Less attention was given to the potential roles of those enzymes in the physiology of the cells/organisms they produced them. The scope of this review is firstly to analyse the data on the occurrence and characteristics of murein-, chitin-, and N-glycan-ENGases acting on GlcNAc-containing polymers in three structural families, namely murein, chitin, and N-glycosylproteins, and of PNGases, only acting on N-glycosylproteins, and secondly to discuss the biological roles of the enzymes in the producing cells. The analysis demonstrates the remarkable diversity of the enzymes, and simultaneously the interest of studying their substrate specificity and their structural features. Many examples illustrate the importance of the structure/function relationships studies. Diverse biological roles were anticipated, e.g. they are useful for feeding purposes, are implicated in pathogenesis processes, modulate the activity of macromolecules, and help in the destruction of misfolded proteins. Their effect can be direct or indirect, through the reaction products. Current knowledge only partially explains the biological roles of ENGases and PNGases, thus further studies are expected for determining novel possibilities and elucidating other cell pathways.

Keywords: Engase, Pngase, Biological Functions, Murein, Chitin, *N*-Glycosyl Proteins

1. Introduction

Endo-*N*-acetyl-*β*-D-glucosaminidases (ENGases) are endoglycosidases within subclass EC 3.2.1 (glycosidases or glycosyl hydrolases) that cleave the *O*-glycosyl linkage between the *N*-acetyl-*β*-D-glucosamine (GlcNAc) residue and the adjacent monosaccharide within an oligosaccharide chain. Three types can be distinguished among the various ENGases that have been isolated and characterised [1], namely, the enzymes acting on murein (murein-ENGase), those acting on chitin (chitin-ENGase) and those acting on *N*-glycans (*N*-glycan-ENGase). The murein-ENGases hydrolyse murein by splitting the glycosidic bond *β*(1,4) between GlcNAc and *N*-acetyl-muramic acid (MurNAc) to yield a polymer with a reducing GlcNAc. Chitin-ENGases include endochitinases, which split within the chitin polymer, and exochitinases, which release chitobiose. *N*-glycan-ENGases hydrolyse the linkage between the two GlcNAc residues of the invariant pentasaccharide inner core of *N*-glycans. The abbreviation "Endo" was initially used for *N*-glycan cleaving enzymes and it was only on 1994 that the abbreviation "ENGase" was introduced by the author's group [2] and was used in all the subsequent publications by the group and others. The purpose was to invent an abbreviation comparable to the one used for peptide-N^4-(*N*-acetyl-*β*-D-glucosaminyl) asparagine amidases (PNGases).

PNGases have, in common with *N*-glycan-ENGases, the ability to release oligosaccharides from *N*-glycosylproteins. As well as other glycosidases, *N*-glycan-ENGases and PNGases were widely used for the characterisation of *N*-glycosylproteins. In this context, PNGases, which in fact are amidases, were also considered as endoglycosidases since the latter were defined as the enzymes cleaving oligosaccharide chains and releasing a disaccharide or larger molecules during the course of their action [3]. Both *N*-glycan-ENGases and PNGases were mainly studied with the aim of offering new endoglycosidases which are the

"restriction enzymes of the carbohydrate world" to the community of glycobiologists [3].

The literature survey on ENGases and PNGases using the "PubMed Advanced Search Builder" on December 2013 retrieved approximately 1550 and 1950 articles respectively, publishing starting from 1969 [4] for ENGase and 1981 [5] for PNGase. The exact start for PNGases was 1977 [6]. The results of the survey are presented in Figure 1. The maximum number of articles on ENGases was reached in 1986 while PNGase started to increase. Ten years later, articles on PNGase reached a maximum while those for ENGase had decreased significantly. References dealing with the characterization and the biology of the enzymes represented a limited number of articles (less than 300) the remaining corresponding to their use as tools.

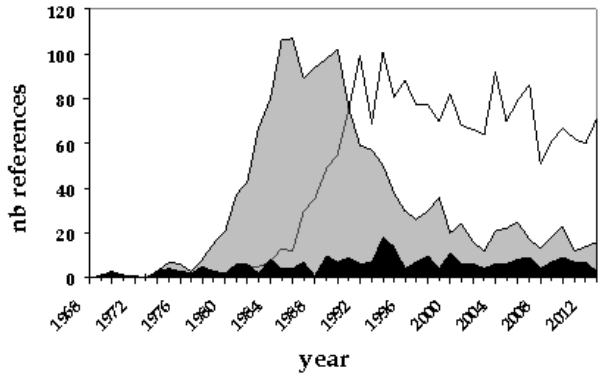

Figure 1. *Literature survey on ENGases and PNGases. The "PubMed Advanced Search Builder" was used with the following query: 1) ((endo-N-Acetyl-β-D-glucosaminidase) OR Engase) OR endo-beta-N-acetylglucosaminidase (grey area) and 2) (((peptide : N-glycosidase)) OR (PNGase)) OR (Peptide-N4- AND (N-acetyl-beta-glucosaminyl) AND asparagine amidase) (white area). References dealing with the characterization and the biology of ENGases and PNGases represented a limited number (black area) the remaining corresponding to their use as tools.*

The purpose of this review is to analyse the data on occurrence and characteristics of murein-, chitin-, and N-glycan-ENGases acting on GlcNAc-containing polymers in three structural families, namely murein, chitin, and N-glycosylproteins, and of PNGases, only acting on N-glycosylproteins. It is also to discuss the biological roles of the enzymes in the producing cells.

2. Potential Substrates for ENGases and PNGases

All the potential substrates for ENGases contain an internal *N*,*N*'-diacetylchitobiose except in the case of murein where the MurNAc is the 3-O-lactyl derivative of GlcNAc [1] (Figure 2). The structure of murein includes a linear chain of alternating *β*(1,4) linked GlcNAc and MurNAc (the O-lactyl group is depicted by R'' in Figure 2B). Chitin is a homopolymer of *β*(1,4) linked GlcNAc residues. Nodulation (Nod) factors, that could be considered as chitin-like oligomers, carry an *N*-linked acyl group (depicted by R in Figure 2B) on the terminal non-reducing glucosamine residue on the *β*(1,4) linked GlcNAc backbone that could be diversely substituted depending on the rhizobial species [7]. *N*-glycans are characterised by an invariant pentasaccharide core [8] which contains an *N*,*N*'-diacetylchitobiosyl moiety and three mannose residues. This core pentasaccharide can be substituted by additional mannose, GlcNAc, galactose, xylose, fucose and/or *N*-acetylneuraminic acid residues.

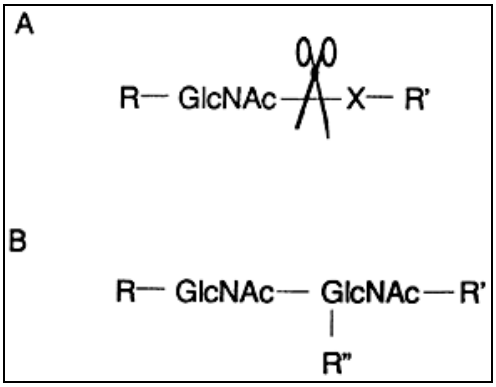

Figure 2. *(A) All the enzymes cleaving the GlcNAc-X linkage could be considered as ENGases. (B) The minimal structure of all the known substrate. R=monosaccharide; R'=monosaccharide or Asn-peptide; R"=H or lactyl group.*

The action on *N*-glycans of PNGases and of *N*-glycan-ENGases, which are both considered as de-*N*-glycosylation enzymes [9], is depicted in Figure 3, in which is also reported the action of "parent" enzymes and of proteases. ENGases cleave between the distal - GlcNAc1 - and the proximal - GlcNAc2 - residues, leaving the proximal GlcNAc1 residue linked to Asn alone, in a peptide or in a protein, and liberating the *N*-glycan with the distal GlcNAc2 in reducing position. *N*-glycans are also substrates for PNGases, which cleaves the amide linkage between the proximal GlcNAc1 and Asn to yield a substituted *N*-acetyl-*β*-D-glucosaminylamine and a peptide containing an Asp residue.

PNGase requires the presence of at least two amino-acid residues in the substrate. The N^4-(*β*-*N*-acetylglucosaminyl)-L-asparaginase (NGase) is a lysosomal enzyme that hydrolyses the amide bond between the proximal GlcNAc1 and Asn only when the α-amino and α-carboxyl groups of the Asn residue have become free [10] (first described in 1968 [11]). The di-*N*-acetylchitobiase activity was demonstrated to be involved in the lysosomal catabolism of *N*-glycosylproteins in rats and humans [12] (first described in 1974 [13]) and was also reported as ENGase [14] or endo-chitobiase [15]. Later it was shown that this chitobiase was a true reducing-end glycoside hydrolase and acts as well on chito-oligomers [16]. It is a GH18 family member and is distinguished from exochitinases, which in fact release chitobiose [17]. In the catabolic pathway for sialoglycoasparagines the action of NGase is mandatory before the action of di-*N*-acetylchitobiase [12,14].

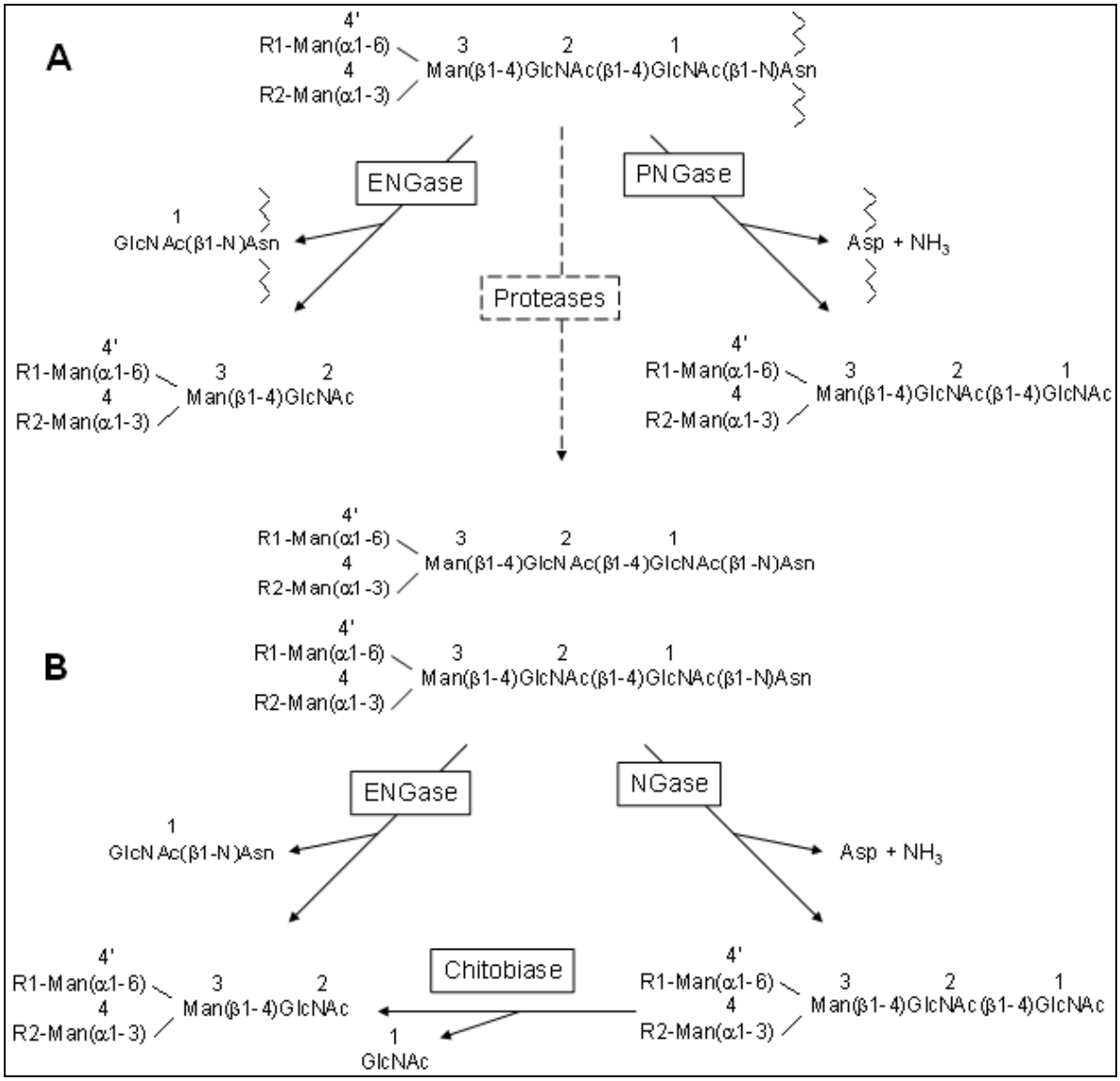

Figure 3. *Action of ENGases and PNGases and their "parent" enzymes, di-N-acetylchitobiase (chitobiase) and N4-(β-N-acetylglucosaminyl)-L-asparaginase (NGase) on asparagine linked glycans. (A) Action of ENGase and PNGase. Proteases can covert the substrate to glycoasparagines. (B) Action of ENGase, NGase and chitobiase. Zig zag depicts the peptide chain, when present. R1 and R2 represent Man or GlcNAc residues. GlcNAc1 and GlcNAc2 are referred to in the text as the proximal and distal GlcNAc residues respectively.*

3. Aspects on the Nomenclature and Classification of Glycoside Hydrolases

Following the recommendations of the Nomenclature Committee of the International Union of Biochemistry and Molecular Biology (NC-IUBMB) on nomenclature and classification of enzymes by the reaction they catalyse, the classification of glycoside hydrolases is mainly based on their substrate specificity [18] and does not reflect the structure of the enzymes. This aspect is covered by the Carbohydrate Active Enzymes (CAZy) database (http://www.cazy.org/) [19] which describes the families of structurally-related catalytic and carbohydrate binding modules (or functional domains) that degrade, modify or create glycosidic bonds. Glycoside Hydrolases (GH) correspond to a widespread group of enzymes which hydrolyse the glycosidic bond between two or more carbohydrates or between a carbohydrate and a non-carbohydrate moiety [20]. ENGases are classified within the GH families whereas PNGases are not included in the CAZy database. Currently 132 families of GH were described, among which 52 were grouped in 14 clans of related families [21,22]. The GH-K clan, in which ENGases are found, includes three related families, all displaying an hydrolysis mechanism with net retention of configuration (Table 1).

Table 1. *Members of the Glycoside Hydrolases superfamily K (clan GH-K)*

GH family	concerned enzymes or proteins
GH18	chitinases (EC 3.2.1.14) of classes III and V
	endo-β-N-acetylglucosaminidases (EC 3.2.1.96)
	xylanase inhibitor, concanavalin B (non-catalytic [23])
	narbonin (non-catalytic [24])
GH20	β-hexosaminidase (EC 3.2.1.52)
	lacto-N-biosidase (EC 3.2.1.140)
	β-1,6-N-acetylglucosaminidase (EC 3.2.1.-)
	β-6-S03-N-acetylglucosaminidase (EC 3.2.1.-)
GH85	endo-β-N-acetylglucosaminidase (EC 3.2.1.96)

In addition, the GH73 family concerns the peptidoglycan hydrolases with endo-*β*-*N*-acetylglucosaminidase specificity (EC 3.2.1.-). A classification is also available in the CAZy database for the carbohydrate binding modules (CBM) [25] which are defined as contiguous amino acid sequences within a carbohydrate-active enzyme with a discreet fold having carbohydrate-binding activity and sometimes displaying catalytic activity.

4. Occurrence, Characteristics and Functions of ENGases

4.1. Murein-ENGases

Among the numerous sequences (>2500) included in the CAZy GH73 family, which is described as peptidoglycan hydrolases with ENGase specificity (EC 3.2.1.-), 20 were characterised from the following bacteria: *Bacillus subtilis* [26], *Clostridium difficile* [27], *Clostridium perfringens* [28], *Enterococcus feacalis* [29], *Enterococcus hirae* [30], *Lactobacillus plantarum* [31], *Lactobacillus lactis* [32,33], *Listeria monocytogenes* [34], *Sphingomonas* sp [35], *Staphylococcus aureus* [36], *Staphylococcus lugdunensis* [37], *Staphylococcus warneri* [38], *Streptococcus pneumoniae* [39].

Most of the characterised proteins were described either as peptidoglycan hydrolases, as autolysins - often bi-functional, combining an *N*-acetylmuramoyl-L-alanine amidase with a *β*-*N*-acetylglucosaminidase -, or as ENGases that are associated with EC 3.2.1.96 corresponding to the *N*-glycan-ENGases. This situation is quite confusing and it is difficult, when analysing the literature, to attest that the isolated enzymes display activities cleaving the *β*(1,4) linkage between the GlcNAc and MurNAc residues by acting on the polymer and thus correspond to an ENGase, or if they display an exo-*β*-*N*-acetylglucosaminidase activity which means acting after lysozyme. Among the isolated bacteriolytic enzymes, certain were found to act as murein-ENGases such as the *S. aureus* autolysin [40], and the *B. subtilis* endolysin [41,42]. A possible role was suggested concerning symbiosis and synergism, the neighbouring species being able to use the degradation products and in return cross-feed the strain that is releasing the degraded cell wall [26]. It is also possible that the released peptidoglycan components or glucosamine might correspond to signals as it was suggested for aggregation and sporulation of *Myxococcus xanthus* cells [43]. The murein-ENGase from *S. aureus* (SaG) interferes with the immune response in mammals [44] and could represent an example of pathogenicity determinant. A murein-ENGase was characterised in *Streptococcus pneumoniae* [45]. The *lytB* gene was cloned, and LytB purified and localized at the cell poles. The polar position of this enzyme might be indicative of its critical role in cell separation. A recent study demonstrated that the LytB and LytC (lysozyme) of *S. pneumoniae* are essential virulence factors involved in the colonization of the nasopharynx and in the progress of invasive disease by avoiding host immunity [46]. The *atlC* gene, encoding an autolysin which binds fibronectin, was isolated from the chromosome of an infectious isolate of *Staphylococcus caprae* and sequenced [47]. AtlC is similar to other staphylococcal autolysins such as Atl [40], AtlE [48], and Aas [49], which are necessary for cluster dispersion during cell division and are located outside the cell membrane [50]. It contains a putative signal peptide of 29 amino acids and two enzymatic centres, *N*-acetylmuramoyl-L-alanine amidase and endo-*β*-*N*-acetylglucosaminidase, interconnected by three imperfect fibronectin-binding repeats [47].

4.2. Chitin-ENGases

Chitinases (chitin-ENGases) and chitin binding proteins (CBPs) have been identified in several organisms and have displayed diverse physiological and biological roles depending on the organism. Chitin and chitinolytic enzymes are gaining importance for their biotechnological applications, especially the chitinases exploited in agriculture to control pathogens [51].

More than 5000 entries are currently included in the CAZy database in the GH18 family, found in a great variety of organisms, and around 1500 in the GH19 family only found in some plants and in bacteria. Bacterial chitinases serve to degrade chitin for carbon and nitrogen sources to support growth [52]. It has recently become clear that representatives of both Gram-positive and Gram-negative bacterial pathogens encode chitinases and CBPs that support infection of non-chitinous mammalian hosts [53]. Chitinases are also produced by various bacteria which are unable to use chitin as a sole carbon source and seem to be involved in bacterial pathogenesis [54]. In fungi and insects, such enzymes are needed to break down chitin components of cell walls and old cuticle respectively, processes that are essential for moulting, growth and development of cell walls and cuticles [55]. In fungi they are considered as one of the virulence factors [56]. Plant chitinases play a role in plant resistance against distinct pathogens, and regulate plant growth and development [57]. They are also associated with stress responses both biotic and abiotic. In addition to the functions of regulating cellular growth and proliferation, chitinases also play an important biological role in

defending the host cells or by increasing interaction with pathogens due to their ability to hydrolyze and/or interact with chitin present in pathogens or host cells. The roles of plant chitinases in defending against the infectious pathogens, including viruses, bacteria, fungi and insects, have been well understood [55,57] and have been extensively studied in the context of plant-pathogen interactions [58]. In general, leguminous and non-leguminous chitinases that belong to the same chitinase class have similar substrate specificities. Chitinases cleave, and thus inactivate, Nod factors. The local induction, or down-regulation of these enzymes in leguminous roots, would modulate the local lipo-oligosaccharides concentrations. Since Nod factor levels seem to be host-range determinants, plant hydrolases might help tailor host specificity [58].

In another study, Nod factor cleaving pea enzymes were analyzed using *Sinorhizobium meliloti* Nod factors (NodSm) as substrates [59]. When intact roots, or protein extracts, were incubated with pentameric NodSm, two hydrolytic activities were observed. One activity released the lipo-disaccharide, whereas the other activity formed the lipo-trisaccharide as it was described for a chitinase lysozyme in alfalfa roots [60]. The lipo-disaccharide-forming pea hydrolase was specifically induced by Nod factors from *Rhizobium leguminosarum* bv *viciae* [59,61], whereas the lipodisaccharide-forming enzyme from alfalfa was induced by NodSm factors [62]. In pea plants, the degradation of Nod factors correlated with a chitinase which was stimulated by high doses of Nod factors as well as stress-related signals.

Hevamine, a chitinase from the rubber tree *Hevea brasiliensis* belongs to the GH18 family. Its cleavage specificity for peptidoglycan was studied by HPLC and MS analysis of enzymatic digests [63]. The results clearly indicated that the enzyme cleaves between the C-1 of a GlcNAc and the C-4 of a MurNAc. Hevamine was the first enzyme found with this cleavage specificity.

Until late '90s it was generally assumed that man lacks the ability to produce a functional chitinase. The observation of an elevated chitotriosidase activity in plasma of symptomatic Gaucher patients formed the basis for the subsequent identification of a human phagocyte-specific chitinase, named chitotriosidase, which is remarkably homologous to chitinases from plants, bacteria, fungi, nematodes and insects [64,65]. It has been found to inhibit hyphal growth of chitin-containing fungi such as *Candida* and *Aspergillus* species. The specific expression by phagocytes also suggests a physiological role in defence against chitin-containing pathogens. The discovery of a second mammalian chitinolytic enzyme, the mouse acidic mammalian chitinase (AMCase) was described later [66]. In rodents and man, the enzyme is relatively abundant in the gastrointestinal tract and is found to a lesser extent in the lung. In contrast to chitotriosidase, the enzyme is extremely acid stable and shows a distinct second pH optimum around pH 2. It has been assumed that the enzyme plays a role in digestion, and has a protective role in the lungs [66]. A correlation has been observed between AMCase and asthma in several studies on mice and human [67,68]. The level of upregulation of AMCase in asthmatic mice was reduced in a dose-dependent manner when the mice were treated with glucocorticoids [69]. This indicated that one effect of glucocorticoid treatment of asthma may be a reduction in the level of AMCase. AMCase breaks down chitin to very small chitin micro-particles, CMPs (<2 μm) that have been proven to have no effect on the immune system [70], whereas medium-sized CPMs (40-70 μm) induce immune responses. Together, these results indicated that AMCase might be considered as a potential therapeutic target in the treatment of asthma and considered as a novel target for anti-asthma drug design [71]. The potential roles of chitinases during the development of acute and chronic intestinal inflammatory conditions were recently pointed out [54].

4.3. N-Glycan-ENGases

4.3.1. Bacterial N-Glycan-ENGases

The first *N*-glycan-ENGase (Endo D) to be described was the endoglycosidase isolated in the culture medium of *Diplococcus (Streptococcus) pneumoniae* [72] and consecutively partially purified and characterised [73]. Its limited specificity, especially by the fact that it was unable to remove glycans from intact glycoproteins [74], restricted its utility [3]. Meanwhile, Endo H from *Streptomyces plicatus (griseus)* was discovered in attempts to define the structure of the ovalbumin glycans [75]; it displayed a large substrate specificity except if fucose substituted the proximal GlcNAc1 residue. In the same study a second enzyme, Endo L, was evidenced which when further characterised, was shown to be able to cleave $Man(GlcNAc)_2Asn$ [76] and also to hydrolyse chitin oligosaccharides carrying at least three unmodified GlcNAc residues. Endo H, acting even in the presence of intersecting GlcNAc [77], and removing *N*-glycans from intact glycoproteins, became rapidly the endoglycosidase of choice for structure/function studies of *N*-glycans.

Two ENGases were highly purified from the culture medium of *Clostridium perfringens* [78]. Endo CI had the substrate specificity identical to Endo D; Endo CII showed specificity similar to Endo H except concerning their action on ovalbumin glycans. Similar substrate specificity to Endo CII was also shown for Endo S, found in vegetative cells of the cellular slime mold *Dictyostelium discoideum* [79] that was neither secreted during vegetative growth or development nor was it developmentally regulated.

Arthrobacter protophormiae produces a high level of extracellular *N*-glycan-ENGase, EndoA, when cells are grown in a medium containing ovalbumin [80] and this is due to the glycopeptide fraction of the glycoprotein and its production is also induced by yeast invertase and bovine ribonuclease B (RNase B) but not by monosaccharides. The enzyme induction seems to have a close relation to its substrate specificity, very similar to that of Endo CII. The gene encoding Endo A was cloned later [81]. The primary

structure of Endo A, composed of a 24 amino acid signal peptide and of a mature 621 amino acid protein, exhibited significant homology with a gene product from *Caenorhabditis elegans*, of unknown function at that moment, but later characterised [82], and no significant homology with any previously reported ENGases.

Endo F was detected in an endoglycosidase activity produced by *Flavobacterium meningosepticum* [83]. This bacterium was later reclassified as *Chrysobacterium meningosepticum* [84] before being transferred into *Elisabethkingia meningoseptica* comb. nov. [85]. Endo F was shown to cleave *N*-glycans of both the high-mannose and the complex type. It was also noticed that it can act on denatured intact glycoproteins. In the course of defining the substrate specificity of this enzyme, the presence of PNGase F was evidenced [86]. When hydrolysed by glycerol-containing preparations of Endo F, *N*-glycans were found to have glycerol attached to their reducing ends [87]. Glycerol was linked via its C-1(3) to the C-1 of the distal GlcNAc2. This phenomenon was avoided when no glycerol was added to the preparation. In a second, less favoured reaction, the glycerol glycoside was hydrolyzed by Endo F using water as the terminal nucleophile, thus regenerating the GlcNAc2 reducing end. Later, three distinct ENGase activities, Endo F1, Endo F2, and Endo F3, were identified in the Endo F preparations [88]. In the same study it was also shown that Endo F2 was able to cleave complex multiantennary glycans, that neither Endo F1 nor Endo H could hydrolyse. The corresponding genes were cloned [89,90]. The three enzymes are members of the GH18 family.

The complete amino acid sequence of Endo Fsp from *Flavobacterium sp*. has been determined by analysis of peptides after cleavage with lysyl-endopeptidase, pepsin and chymotrypsin [91]. The protein consists of a single polypeptide chain made of of 267 amino acid residues. The sequence of Endo Fsp has 60% similarity and very similar hydropathy profiles to Endo H. The authors also found similarities between Endo Fsp and chitinases from *Bacillus circulans*, *Serratia marcescens* and *Phaseolus vulgaris*.

The genome sequencing project on alkaliphilic *Bacillus halodurans* revealed a putative *N*-glycan-ENGase, Endo BH [92,93], which consists of a 24 amino acid signal peptide, a catalytic region of 634 amino acids exhibiting 50.1 % identity with Endo A, and a C-terminal tail of 220 amino acids. Recombinant Endo BH hydrolyzed high-mannose type oligosaccharides and hybrid type oligosaccharides, and showed transglycosylation activity. On deletion of 219 C-terminal amino acid residues of Endo BH, the wild type level of activity was retained, whereas with deletions of the Endo A homolog domain, the proteins were expressed as inclusion bodies and their activities were reduced. These results suggest that the enzymatic properties of Endo BH are similar to those of Endo A, and that the C-terminal tail does not affect the enzyme activity. Although the C-terminal tail region is not essential for enzyme activity, the sequence is also conserved among ENGases of various origins.

Streptococcus pyogenes is an important human pathogen that selectively interacts with proteins involved in the humoral defense system, has the ability to hydrolyze the chitobiose core of *N*-glycan of immunoglobulins G (IgG) when bacteria are grown in the presence of human plasma [94]. This activity is associated with the secretion of an ENGase, denoted Endo S, which acts on purified soluble IgG as well as IgG bound to the bacterial surface. Endo S is required for the activity on IgG, as an isogenic Endo S mutant could not hydrolyze the glycan on IgG. In addition, the secreted streptococcal cysteine proteinase SpeB cleaves IgG in the hinge region in a papain-like manner. This was the first example of a bacterial pathogen ENGase that selectively hydrolyzes human IgG, and revealed a mechanism which possibly contributes to *S. pyogenes* pathogenesis.

Based on the fact that the human pathogen *Enterococcus faecalis* can degrade the *N*-glycans of human RNase B to acquire nutrients, an 88-kDa secreted protein was identified, which is most likely responsible for this activity [95]. This *N*-glycan-ENGase, Endo E, consists of a α-domain with a GH18 family motif and a β-domain similar to GH20 family. Phylogenetic analysis of Endo E indicates that the α-domain is related to human chitobiase, and the β-domain is related to bacterial and human hexosaminidase. Recombinant expression of full-length Endo E or Endo Eα, site-directed mutagenesis of the catalytic residues, mass spectroscopy, and homology modelling shows that Endo Eα hydrolyzes the glycan on human RNase B, whereas Endo Eβ hydrolyzes the conserved glycan on IgG. Both activities could be important for the molecular pathogenesis and persistence of *E. faecalis* during human infections. Another ENGase from *E. faecalis* was analyzed, and displayed a single domain GH18 protein [96], with an activity similar to Endo H.

Capnocytophaga canimorsus is a Gram-negative bacterium from the normal oral flora of pets that causes septicaemia and peripheral gangrene with a high mortality in humans who have been bitten or simply licked. A surprising feature of *C. canimorsus* is the capacity to feed by foraging the glycan moieties of glycoproteins from animal cells, including phagocytes [97] and can also de-*N*-glycosylate human IgG, reinforcing the idea that this property of harvesting host glycoproteins may contribute to pathogenesis. This first characterised complete de-*N*-glycosylation system belongs to a large family of systems devoted to foraging complex glycans, found exclusively in the *Capnocytophaga-Flavobacteria-Bacteroides* group [97].

Infant-borne bifidobacteria have developed various molecular strategies for utilizing human milk oligosaccharides as a carbon source. It was hypothesised that they interact with *N*-glycans found in host glycoproteins that are structurally similar to free oligosaccharides in human milk. ENGases were identified in certain isolates of *Bifidobacterium longum subsp. longum*, *B. longum subsp. infantis*, and *Bifidobacterium breve*, and their presence correlated with the ability of these strains to

de-*N*-glycosylate glycoproteins [98]. A phylogenetic tree presented in this study classified these protein sequences in three types. One group was exclusively found in *B. infantis* strains, termed GH18a (corresponding to Endo BI-1) and distantly related to EndoE from *E. faecalis*. Another set of sequences found in strains of *B. infantis*, *B. breve*, and *B. longum* shared 60% amino acid identity with Endo BI-1, and it was termed GH18Bb (corresponding to Endo BI-2). Sequences belonging to GH85 (corresponding to Endo BB) were almost exclusively found in fecal *B. breve* isolates. Endo BI-1 was active toward all major types of *N*-glycans found in glycosylated proteins. Its activity was not affected by core fucosylation or extensive fucosylation, antenna number, or sialylation, releasing *N*-glycans from human lactoferrin, IgA and IgG. Extensive de-*N*-glycosylation of whole breast milk was also observed after co-incubation with this enzyme. Mutation of the active site of Endo BI-1 did not abolish binding to *N*-glycosylproteins, and this mutant specifically recognized the fucosylated core pentasaccharide, $Man_3GlcNAc(\alpha 1\text{-}6Fuc)GlcNAc$, of human *N*-glycans. Endo BI-1 is constitutively expressed in *B. infantis*, and incubation of the bacterium with human or bovine lactoferrin led to the induction of genes associated to the import and consumption of human milk oligosaccharides, suggesting linked regulatory mechanisms among these glycans.

4.3.2. Animal N-Glycan-ENGases

The first evidence of the occurrence of an *N*-glycan-ENGase activity in a mammalian tissue was reported in 1974 [99] but the enzyme was not simultaneously purified. A similar enzyme was partially purified from hen oviduct [100] and later purified to homogeneity and further characterised [101]. In the meantime an ENGase activity was demonstrated in rat liver [102,103] and then the enzyme characterised in rat liver [104] and in human tissues [105] indicating that *N*-glycan-ENGases occur widely in the animal kingdom. It was also proposed [106] that the cytosolic exoglycosidases and/or ENGases could play an important role in the control of *N*-glycosylprotein biosynthesis. The function of exoglycosidases could be to destroy unfinished glycans but ENGases could be at the origin of free *N*-glycans (FNGs) carrying oligosaccharin-like activity [107].

Formation of FNGs occurs both in the cytosol and in the lumen of the endoplasmic reticulum (ER). Luminal FNGs are transported into the cytosol to ensure that they do not interfere with proper functioning of the glycan-dependent quality control machinery in the lumen of the ER for newly synthesized glycoproteins [108]. Once in the cytosol, FNGs are catabolised, possibly to maximize the reutilization of the component sugars and ENGase is a key enzyme involved in their processing.

In 1989, two enzymes were found in human kidney [109] and referred to as E-β-GNase 1 and 2. The E-β-GNase 1 activity was shown to cleave glycopeptides containing high mannose-type glycans but not those containing the complex-type sugar chains and can be considered as *N*-glycan-ENGase. E-β-GNase 2 showed the same characteristics as the lysosomal di-*N*-acetylchitobiase previously reported [12,14,15]. More recently, on the basis of partial amino acid sequences of the ENGase purified from hen oviduct and the expressed sequence tag (EST) database analysis, the cDNA of a human orthologue of the cytosolic ENGase has been identified [110]. The human protein consists of 743 amino acids and is localized in the cytosol. The subsequent gene database survey revealed the occurrence of ENGase homologues in *Drosophila melanogaster*, *Caenorhabditis elegans*, and *Arabidopsis thaliana*, indicating the broad occurrence of ENGase in higher eukaryotes [110]. This gene was expressed in a variety of human tissues, suggesting that the enzyme is involved in basic biological processes in eukaryotic cells.

It was also noticed, at that moment, that one interesting structural feature of the cytosolic ENGase is that this protein shares reduced, but significant, homology with ENGases such as Endo M [111,112] or Endo A [81]. These ENGases (Endo M, A, and cytosolic ENGases), which the authors [110] proposed should be called class II ENGases are structurally distant from class I ENGase (Endo H and its structurally related enzymes), for which extensive structural studies have been carried out. Interestingly the *N*-glycan-ENGases in the proposed class II are all members of the GH85 family in the CAZy database (Table 2).

Those in class I are members of the GH18 family (Table 3) in which is also found the parent enzyme di-*N*-acetylchitobiase.

Table 2. *Characterised N-glycan-ENGases members of the GH85 family*

Organism	Abbreviation	Reference
Bacterial N-glycan-ENGases		
Arthrobacter protoformiae	Endo A	[81]
Bacillus halodurans	Endo BH	[93]
Bifidobacterium longum	Endo BB	[98,180]
Streptococcus pneumoniae	Endo D	[181,182]
Animal N-glycan-ENGases		
Caenorabditis elegans	Endo CE	[82]
Homo sapiens	Engase 1, ENGase	[110]
Plant N-glycan-ENGases		
Arabidopsis thaliana	ENGase	[183]
Solanum lycopersicum	Endo LE	[121]
Fungal N-glycan-ENGases		
Mucor hiemalis	Endo M	[134]
Ogataea minuta	Endo Om	[135]

The ENGase HS that was partially purified from human saliva [113] is apparently different from those enzymes and since it was found associated to the outer surface of

epithelial cell membrane of human oral cavity epithelium, it probably takes part in other pathways of metabolism.

4.3.3. Plant N-glycan-ENGases

ENGase was involved in the production of high-mannose type FNGs, during plant development and fruit maturation [114,115]. *N*-glycans that are not linked to a protein or peptide, were found to occur in various plant cells during differentiation, growth and ripening stages [116–119]. They were reported as "unconjugated *N*-glycans" [120] and as FNGs in plants, and also as "free oligosaccharides" in mammals [110].

Table 3. *Characterised N-glycan-ENGases members of the GH18 family*

Organism	Abbreviation	Reference
Bacterial N-glycan-ENGases		
Bacteroides thetaiotaomicron	Endo BT	[184]
Bifidobacterium longum subsp infantis	Endo BI	[98,185]
Capnocytophaga canimorsus	-	[186]
Elisabethkingia meningoseptica comb	endo F1	[90]
	Endo F2	[89]
	Endo F3	[89]
Enterococcus faecalis	Endo E	[95]
Flavobacterium sp.	-	[91]
Streptococcus pyogenes	Endo S	[94]
Streptomyces plicatus	Endo H	[187]
Fungal N-glycan-ENGases		
Flammulina velutipes	Endo FV	[137]
Hypocrea jecorina (Trichoderma reesei)	Endo T	[136]

A gene encoding tomato ENGase LE was identified and expressed in *E. coli* in which the substrate specificity remained identical to the native enzyme [121]. Moreover, real-time PCR demonstrated that the gene expression did not vary during ripening and thus that the ENGase LE activity was ubiquitously expressed. Previously, it was demonstrated that the activities of ENGase and PNGase were ubiquitously found in tomato plants [122]. PNGase activities were consistently higher than ENGase activities. Both activities were associated with high levels of proteins and protease activities. During fruit growth and ripening, these three parameters decreased notably. The ubiquitous detection of these enzyme activities in the different organs is probably linked to the previously characterized FNGs in tomato. The significant increase in high-mannose type FNGs at the late stage of fruit ripening [115] must be due to an increase in endogenous substrates or to a decrease in the degradation rate of FNGs by cytosolic α-mannosidase [121].

The structural analysis of FNGs accumulated in rice cultured cells [123] revealed two types of FNGs, (i) high-mannose carrying the distal GlcNAc2 residue at the reducing end, and (ii) truncated complex type with the proximal GlcNAc1 residue at the reducing end, which was 10 fold less abundant. This result suggested the action of cytosolic ENGase and intracellular acidic PNGase for the production of FNGs in rice cells. This was confirmed in other plant cells. The presence in the culture medium of high-mannose type and of complex type FNGs, substituted with Fuc(*α*1-4) (Le^a epitope), carrying the proximal GlcNAc1 residue, suggested that an extracellular acidic PNGase was involved in the release of *N*-glycans from mature *N*-glycosylproteins in the extracellular space. The presence of unusual FNGs with the distal GlcNAc2 at the reducing end, and of a biantennary complex type structure harbouring the Le^a epitope, suggested that the cytosolic ENGase, and other Golgi *N*-glycan-processing enzymes, were involved in the production of this type of FNGs.

An *Arabidopsis thaliana* double-knockout mutant was constructed in which the expression of two putative ENGase genes was suppressed [124,125] and no ENGase activity was detected. In the mutant the high mannose FNGs carried the proximal GlcNAc1 at reducing end, revealing the probable action of endogenous PNGase. The double-knockout cell line was viable and shown no obvious morphological phenotype under standard growth conditions.

The action of the cytoplasmic ENGases can potentially be at the origin of various proteins bearing a unique *N*-linked GlcNAc (*N*-GlcNAc). Those *N*-GlcNAc modifications were previously shown to occur in fungi [126] and were recently discovered in animals [127]. In the meantime it was also found on ribosome-inactivating proteins from sponge gourd (*Luffa cylindrica*) and pokeweed (*Phytolacca americana*) seeds [128,129]. The latter is a ribosome-inactivating protein, containing three well defined *N*-GlcNAc modifications, at N10, N44, and N255. The *N*-GlcNAc at N44 is in the *α*- conformation whereas the two others in the *β*- conformation [129]. Is this type of *N*-GlcNAc modification due to a glycosyl-transferase? During a study of antibodies expressed in transgenic plants MALDI-TOF analysis allowed the detection of fragments with masses consistent with *N*-GlcNAc modification [130]. Recently *Arabidopsis thaliana* proteins that are predicted to transit through the endoplasmic reticulum and Golgi were identified with such *N*-GlcNAc modifications [131]. During this study a mass labelling method was developed and used to study *N*-GlcNAc modification of two myrosinases (thioglucoside glucohydrolases, TGG), TGG1 and TGG2, that are also substituted by high-mannose (Man)-type glycans. *N*-GlcNAc and high-Man-type glycans can occur at the same site. It has been hypothesized that *N*-GlcNAc modifications are generated when ENGase cleaves *N*-glycans. The effects of mutations affecting the two known *Arabidopsis* ENGases were examined on *N*-GlcNAc modification of myrosinase. The modification of TGG2 was greatly reduced in one of the single mutants and absent in the

double mutant. Surprisingly, *N*-GlcNAc modification of TGG1 was not affected in any of the mutants. These data support the hypothesis that ENGases hydrolyze high-Man glycans to produce some of the *N*-GlcNAc modifications but also suggest that some modifications are generated by another mechanism. It was finally concluded that, since the modification was detected at only one site on each myrosinase, the production of the *N*-GlcNAc modification may be regulated [131]. This prompts the investigation of the anomeric configuration of the GlcNAc linkage, and also the occurrence of enzymes that transfer GlcNAc to consensus Asn-X-Ser/Thr sites, equivalent to the *Haemophilus influenzae* glycosyltransferase, able to transfer hexose (Gal or Glc) residues with no requirement for a lipid donor intermediate that has been identified recently [132].

4.3.4. Fungal N-glycan-ENGases

The fungal N-glycan-ENGase isolated from *Mucor hiemalis*, EndoM, displayed a specificity similar to Endo F but was shown able to hydrolyse biantennary complex-type *N*-glycans that Endo F cannot cleave and could even act on triantennary structures while Endo F could not [111,112]. However, Endo M is essentially known as a useful enzyme for the synthesis of neoglycopeptides due to its transglycosylation activity [133]. The EndoM gene encodes a putative 744 amino acid protein, which shows high identity to ENGases in the GH85 family [134]. The first yeast ENGase, from *Ogataea minuta* (Endo Om), was very recently reported [135]. Endo Om encoding gene was directly amplified from *O. minuta* genomic DNA and sequenced. The deduced amino acid sequence indicated that the putative protein belonged to GH85 family. Homologous ENGase sequences were identified by database searches; in other yeast strains, *Candida parapolymorpha*, *Pichia anomala* and *Zygosaccharomyces rouxii* and corresponding enzyme activities were also confirmed in crude cell extracts from those strains. Neither ENGase sequence nor activity were present in *S. cerevisiae* and *P. pastoris* [135]. Endo Om exhibited substrate preference for high-mannose *N*-glycans, and was able to cleave hybrid, biantennary and (2,6)-branched triantennary *N*-glycans, but not tetraantennary, (2,4)-branched triantennary, bisecting GlcNAc containing and core-fucosylated biantennary *N*-glycans. Endo Om also was able to hydrolyze *N*-glycans attached to RNase B and human transferrin under both denaturing and non-denaturing conditions. It was proposed that Endo Om acts *in vivo* to trim the proximal GlcNAc1 residue (chitobiose activity) and/or to directly digest *N*-glycans on misfolded glycoproteins (ENGase activity).

A new fungal subgroup of ENGases belonging to GH18 family has recently been discovered. The first biochemically characterized representatives, Endo T from the ascomycete *Hypocrea jecorina* [136], and Endo FV from the basidiomycete *Flammulina velutipes* [137] show low sequence homology with the bacterial ENGases and with the fungal chitinases. However, this de-N-glycosylation activity is widely distributed [137] and several highly homologous proteins or gene products are found among ascomycetes [136]. Both Endo T and Endo FV hydrolyze high-mannose type structures as observed in fungal and yeast glycoproteins, but do not release complex type *N*-glycans [136,137]. Endo T was shown to be responsible for the microheterogeneity observed for *H. jecorina* cellulases and hemicellulases [138]. The genome of *H. jecorina* does not contain other de-*N*-glycosylation enzymes (such as PNGases or ENGases in GH85 family) that could play this important role in the cell.

4.4. Catalytic Mechanism of ENGases

The ENGases included in the GH-K clan (superfamily) display a retaining mechanism that is hydrolysis with net retention of configuration. In general, the hydrolysis of the glycosidic bond is catalysed by two amino acid residues of the enzyme: a general acid (proton donor) and a nucleophile/base [139]. Concerning the chitin- and *N*-glycan-ENGases reviewed here, the catalytic nucleophile is not always borne by the enzyme, and is replaced by the acetamido group at C-2 of the substrate [140]. The general acid seems to be always a Glu residue. The details of the catalytic mechanism of ENGases in the GH18, 20 and 85 families are presented in CAZypedia (www.cazypedia.org) [141–143]. The mechanism for enzymes in GH73 family is not known. The catalytic general acid is Glu, but the identification of the catalytic nucleophile/base is not conclusive [144].

5. Occurrence, Characteristics and Functions of PNGases

When the first PNGase activity (PNGase A) was detected in plants the term “amidase” was used [6], followed by “glycopeptidase” for the enzyme partially purified from almond emulsin [145,146]. Later the enzyme was characterised as a homogenous peptide [147]. The term Peptide:*N*-glycosidase, and its abbreviation PNGase, was introduced in 1981 [5].With the ^{1}H NMR spectroscopy evidence for the presence of the intermediate 1-*β*-amino-*N*-acetylglucosamine-oligosaccharide, it was demonstrated that PNGase A was an amidase [148], the enzyme catalysing the hydrolysis of the glycopeptide to form an aspartic acid-containing polypeptide and an intermediate oligosaccharide amine which is hydrolysed, non-enzymatically, to yield the final oligosaccharide product. Thus, the enzyme is in fact an amidohydrolase (amidase, EC 3.5.1.52) and not an *N*-glycosidase [148]. The trivial name glycopeptidylamidase was suggested [148] but the term PNGase was adopted and is still largely in use. After the detection of PNGase A, several other PNGases were purified and characterised (Table 3).

A few biological roles were suggested for the plant enzymes (reviewed in [9]). PNGase J can contribute to the process of activation of the lectin Concanavalin A (Con A) produced by *Canavalia ensiformis* and found in significant

amounts in seeds. This lectin is synthesized as a glycosylated precursor that is incapable of interacting with carbohydrate, while the active (mature) protein is not a glycoprotein [150]. Using metabolic labelling and pulse-chase experiments it was suggested that the de-*N*-glycosylation precedes the proteolysis events leading to the mature lectin [151]. The fact that de-*N*-glycosylation is necessary was confirmed when de-*N*-glycosylated proCon A was detected in immature jack bean seeds [152] and it was also concluded that this event was not sufficient since a decrease in the pH, allowing proper folding of the proprotein, was necessary.

Table 3. *PNGases*

Organism	Abbreviation	Reference
Bacterial PNGases		
Elisabethkingia meningoseptica comb	PNGase F	[86]
Animal PNGases		
Oryzias latipes	-	[153]
L929 fibroblasts	-	[154,155]
Plant PNGases		
Almond	PNGase A	[6,145,146]
Canavalia ensiformis (jack bean)	PNGase J	[188,189]
Pisum sativum	PNGase P	[190]
Silene alba	PNGase Se	[191,192]
Raphanus sativus	PNGase R	[193]
Arabidopsis thaliana	AtPng1	[170]
Lycopersicum esculantum	PNGase LE	[194]
Fungal PNGases		
Saccharomyces cerevisiae	Png1p	[157]

The bacterial PNGase F [149] was the first to be subjected to crystallographic analysis and site-directed mutagenesis in order to identify the catalytic, and oligosaccharide recognition, amino acid residues. Mutagenesis has shown that three acidic residues, D60, E206, and E118, located in a cleft at the interface between the two domains of the protein, are essential for its activity. The crystallographic analysis indicated that D60 is the primary catalytic residue, while E206 probably is important for stabilization of reaction intermediates. E118 forms a hydrogen bond with O-6 of the second GlcNAc residue of the substrate.

The presence of PNGase activity in animals was first reported in Medaka fish (*Oryzias latipes*) [153], and later in various mammalian-derived cultured cells [154,155]. While the fish enzyme is believed to be of lysosomal origin [156], mammalian PNGase is a cytoplasmic enzyme and its optimal activity is at neutral pH [154]. A gene encoding cytoplasmic PNGase (*PNG1*) was identified in *Saccharomyces cerevisiae* and mapped to the left arm of chromosome XVI [157]. *PNG1* encodes a soluble protein (Png1p) and when expressed in *E. coli*, exhibited notable PNGase activity.

The amount of Png1p in yeast cells is very limited and thus it was difficult to detect it without a very sensitive assay [158]. Png1p has been reported to be a member of the transglutaminase (TGase) superfamily based on sequence alignment and its structure-function relationships was investigated by site-directed mutagenesis [159]. Point mutations of C191, H218, and D235 residues in Png1p resulted in complete loss of activity. These residues are conserved in the sequence of factor XIIIa, where these amino acids constitute a catalytic triad. Other conserved amino acid residues, Y220, Y231, R210, and E222, were revealed to be important for folding and structure stability of the enzyme. An elongated substrate-binding groove centred on the active site C191 was visualized in the crystal structure of apo-PNGase [160]. Its complex with the peptide-based inhibitor of PNGase, Z-VAD-fmk (benzyloxycarbonyl-Val-Ala-Asp (OMe) fluoromethylketone), revealed that the inhibitor occupied one end of the substrate-binding groove while being covalently linked to the active site Cys. The N,N'-diacetylchitobiose binds on the side opposite to the peptide binding site with the active site C191 being located approximately midway between the carbohydrate and peptide binding sites. Mutagenesis studies suggested that efficient oligosaccharide binding is required for PNGase activity and that C-terminal residues might be involved in binding of the mannose residues of the glycan chain. Endoplasmic reticulum (ER)-associated degradation (ERAD) is a component of the quality control system for newly synthesised proteins [161]. Cytoplasmic PNGase is involved in the efficient degradation of some ERAD substrates. However, some of the PNGase orthologues were reported to be catalytically inactive [162], raising the possibility that, in addition to its enzyme activity, PNGase orthologues might have significant enzyme-independent roles. It was recently elucidated that PNGases in animal and plant kingdoms are playing important roles in the ERAD pathway [163–165].

During biosynthesis, all the *N*-glycans are added 'en bloc', as Glc3·Man9·GlcNAc2, to the Asn residue in the consensus Asn-X-Thr (or Ser) sequence of the polypeptide chain while being translated in the rough endoplasmic reticulum [166]. It is converted to GlcMan9GlcNAc2 by the action of glucosidases I and II, while the protein is still present in the endoplasmic reticulum. This oligosaccharide is trimmed to Man5GlcNAc2 until the polypeptide reaches the Golgi. Under this form, it plays an important role as a ligand for Calnexin and Calreticulin, which retain the polypeptide within ER for proper folding [167]. If the protein is misfolded, it will be released into the cytoplasm and degraded by the proteasome. The cytoplasmic PNGase plays an important role in removing *N*-glycans from the misfolded nascent proteins and helps its entry into proteasomes. The oligosaccharides are then brought into lysosomes after being

partially hydrolysed by cytoplasmic mannosidase, and completely hydrolyzed into monosaccharides by the action of lysosomal exoglycosidases. PNGase is considered to play a main role in the removal of *N*-glycans in the catabolism of glycoproteins. We re-iterate here that cytoplasmic ENGases can potentially act on the glycoproteins and this could result on the production of various proteins bearing a unique *N*-linked GlcNAc (*N*-GlcNAc) and that PNGase cannot release those residues [154]. Therefore, it was predicted that biological actions of such modified glycoproteins are interesting targets for investigation [168].

From an evolutionary point of view, the cytoplasmic PNGase is an interesting protein with a diverse structural arrangement [165,169]. The core catalytic domain of cytoplasmic PNGase is highly conserved throughout eukaryotes and, has homology with TGases. In fact, the plant orthologue of cytoplasmic PNGase was first identified in *Arabidopsis thaliana* (AtPNG1), based on the homology of a TGase domain [170], while the regions outside the TGase domain are quite unique, and show no apparent homology with the primary structures of animal/fungal orthologues. This indicated that the plant cytoplasmic PNGase may have followed a distinct evolutional route from others to acquire its unique function. Interestingly, AtPng1 was reported to possess TGase activity [171], besides PNGase activity [172], *in vitro*. However, the functional role of AtPng1, *in vivo,* remains poorly understood. In a recent study, it was demonstrated that when the gene is expressed in yeast, AtPng1 can act as a de-*N*-glycosylation enzyme *in vivo* and also facilitate the degradation of ERAD substrate in an *N*-glycan-dependent fashion [173]. The animal enzymes could be at the origin of the accumulation of FNGs which retain the *N*,*N*'-diacetylchitobiosyl part at their reducing termini during oogenesis, vitellogenesis and embryogenesis [174,175].

The function of the only characterised bacterial PNGase, PNGase F, is still unknown. It has been suggested [9] that one of the possible functions of this bacterial de-*N*-glycosylation enzyme could be to participate, together with proteases, in the degradation of foreign *N*-glycosylproteins, for nutrition purposes. It is indeed possible that, after the separation of the oligosaccharide and protein moieties, a better accessibility to the protein could be offered to proteases and this could result in an optimised nutrient supply.

6. Concluding Remarks and Future Prospects

The analysis of the occurrence and characteristics, on the one hand of the known ENGases acting on different structural families of GlcNAc-containing polymers, namely murein, chitin and *N*-glycosylproteins, and on the other of the PNGases acting on *N*-glycosylproteins, demonstrated their remarkable diversity and simultaneously the interest in studying their substrate specificity and their structural features.

I remind here that murein serves as a mechanical framework for the cell wall of most eubacteria [176] and largely determines cell shape. Chitin is the main structural component of the exoskeletons of invertebrates such as crustaceans, insects and spiders, and is also present in the cell wall of most fungi and many algae. It is as abundant as cellulose. *N*-glycans represent the oligosaccharide moieties of *N*-glycosylproteins which are major components of all animal, plant and fungal cells and many viruses. *N*-glycan-ENGases were shown to be produced by many bacteria and also to be generally secreted but seeing that *N*-glycosylproteins were not identified and characterised in bacteria, we have hypothesised [177] that the enzymes were probably devoted to exogenous functions, such as degrading macromolecules for feeding purposes and also stated that the bacterial *N*-glycan-ENGases could be considered as markers of cell events. *N*-glycosylproteins were also described in bacteria and over the last decade considerable progress has been made in the understanding of bacterial *N*-glycosylation systems [178]. Despite this, the utility of bacterial *N*-glycan-ENGases and PNGase cannot be demonstrated in the physiology of the producing cells, since the currently known bacterial *N*-glycans do not contain the *N*,*N*'-diacetylchitobiosyl unit and are linked through di-*N*-acetylbacillosamine (di-NacBac). The structure of the bacterial *N*-glycans was first described in *Campylobacter jejuni* [179] and correspond to the following structure:

GalNAc(α1,4)GalNAc(α1,4)[Glcβ1,3-]GalNAc-α1,4)
GalNAcçα1,4)GalNAc(α1,3)di-NacBac(β1,N)Asn-Xaa

The first two or three reducing-end monosaccharides, including the terminal di-NacBac, are conserved in many *Campylobacter* species. Indeed, di-NAcBac is a GlcNAc derivative but no evidence has illustrated the possibility that ENGases and/or PNGase could cleave this type of *N*-glycans.

Many examples illustrated the importance of the structure/function relationships studies for the enzymes, and one example also highlighted the possibility of cross-reactions, i.e. the possibility of hydrolysing substrates of another structural family.

Since their discovery, ENGases and PNGases have greatly contributed to a better knowledge of the structural features of the macromolecules they hydrolyse and have empowered the evaluation of their structure/function relationships. Initially tracked for the structural help they offer and still representing powerful analytical tools, these enzymes are certainly produced by the cells for functional purposes. Indeed, a number of studies attempted to elucidate the biological functions of the enzymes in the context of the producing cells and/or their effects on their environment. The published data highlighted prominent aspects in this field, in particular concerning their role in feeding, their assistance in the destruction of misfolded proteins and in the modulation of the activity of macromolecules, as well as their contribution in pathogenesis but many grey areas are still remaining. As an example, it will be necessary, to

investigate the role of *N*-glycan-ENGases in the intriguing *N*-GlcNAc modification of proteins and also to address the possibility *N*-GlcNAc modification being independent of a de-*N*-glycosylation mechanism. To conclude, about 40 years from their discovery, current knowledge only partially explains and covers the biological roles of ENGases and PNGases. Further studies are expected for determining novel possibilities and elucidating other cell pathways

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Nomenclature

CBM, carbohydrate binding modules; CBPs, chitin binding proteins; ENGases, Endo-*N*-acetyl-*β*-D-glucosaminidases; ER, endoplasmic reticulum; ERAD, endoplasmic reticulum-associated degradation;; FNGs, free *N*-glycans; GH, Glycoside Hydrolases; GlcNAc, *N*-acetyl-*β*-D -glucosamine; MurNAc, *N*-acetyl-muramic acid; PNGases, peptide-*N*4-(*N*-acetyl-*β*-D-glucosaminyl)asparagine amidases; TGase, transglutaminase; TGG, thioglucoside glucohydrolase;

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