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Review

Enzymatic release of oligosaccharides from glycoproteins for chromatographic and electrophoretic analysis

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Abstract

For most separations-based analyses of glycoprotein oligosaccharides, the first step is release of the oligosaccharides from the polypeptide. Historically, O-linked and N-linked oligosaccharides have been released from glycoproteins using chemical means, such as alkaline degradation (β-elimination) or hydrazinolysis. In the last two decades, a growing repertoire of enzymes, including endoglycosidases and glycoamidases, able to release glycoprotein oligosaccharides under mild conditions, have become available. This review traces the discovery, characterization and use of these glycoprotein oligosaccharide releasing enzymes. Emphasis is placed on providing information of practical value for the researcher wishing to incorporate enzymatic oligosaccharide release into their study of glycoprotein oligosaccharide structure and function.

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1. Introduction

Oligosaccharides, and in some cases monosaccharides, are found attached to eukaryotic glycoproteins primarily through amide linkage to asparagine residues (N-linked oligosaccharides), and through O-glycosidic linkage to serine and threonine residues (O-linked carbohydrates). Analysis of glycoprotein oligosaccharides by means of analytical separations, whether chromatographic or electrophoretic, requires in most cases the release of these oligosaccharides from the polypeptide. This review focuses on the enzymes available for glycoprotein oligosaccharide release. Following enzymatic release, the free oligosaccharide products can either be directly applied to certain types of analytical separation systems, or can be labeled by any of a variety of techniques facilitating not only their detection, but also their separation. For details of such labeling and separation techniques the reader is directed to the many excellent papers specifically addressing these topics within this special thematic issue of *The Journal of Chromatography*. Given the preponderance of such papers on labeling and separation techniques within this volume and elsewhere, these topics are beyond the intended scope of this particular review.

Reviews on the synthesis and structures of N-linked [1] and O-linked [2,3] oligosaccharides are available. Virtually all N-linked carbohydrates in eukaryotes are oligosaccharides that share a common trimannosyl-chitobiose core structure in which N-acetylglucosamine is the glycosyl residue that is attached to asparagine (Fig. 1). However, an apparently rare N-linked glucosyl residue, attached to asparagine, has been reported recently in a mammalian protein [4].

O-linked carbohydrates, while tending to be smaller and less complex than N-linked oligosaccharides, are at the same time more variable in that they do not as consistently share a common core structure. The most common structural motif among O-linked carbohydrates is the disaccharide Gal-β-1,3-GalNAc, attached to serine or threonine through the GalNAc residue.

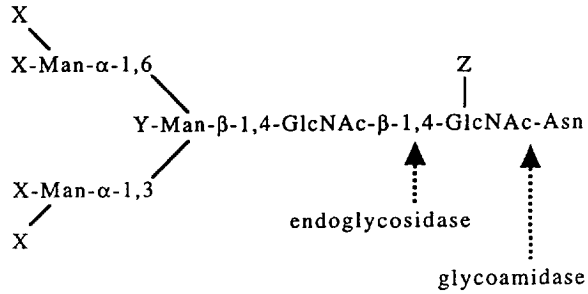

Fig. 1. Generalized diagram of an N-linked oligosaccharide attached to an asparagine residue. The core structure consists of the three mannose residues and two N-acetylglucosamine residues. This core structure is common to essentially all N-linked oligosaccharides. Positions of possible additional sugar linkages are shown by 'X' (many possible sugars and linkages), 'Y' (site of bisecting GlcNAc, when present) and 'Z' (site of core fucosylation, when present). In high mannose type oligosaccharides, all 'X' substituents are one or more mannosyl residues, except for those where no sugar substituent exists at a given 'X' position; for complex type oligosaccharides, all sugar substituents in 'X' positions are saccharides other than mannosyl residues; in hybrid type oligosaccharides, some 'X' substituents are one or more mannosyl residues, while other 'X' substituents are saccharides other than mannosyl residues (see Ref. [1] for review). Points of cleavage by endoglycosidases and glycoamidases are indicated by arrows, as labeled.

This is a core structure common to many O-linked oligosaccharides. However, a variety of O-linked sugars have now been identified in which this core disaccharide is lacking, and where sugars other than N-acetylgalactosamine are attached directly to serine or threonine residues. Among these are O-linked xylose, commonly initiating glycosaminoglycan chains attached to glycoproteins [5], O-linked N-acetylglucosamine [6], and O-linked fucose [7].

An additional type of protein glycosylation is the attachment of a glycosylphosphatidylinositol (GPI) anchor to the C-terminal α-carboxyl group of a protein, serving to anchor the protein to a cell membrane. Methods for release and analysis of these structures are available elsewhere [8], and are outside of the scope of this review. Non-specific, non-enzymatic attachment of sugars to various amino acids in glycoproteins (glycation) can also occur [9], but is also outside of the scope of this review.

Historically, chemical methods have been used

to release O-linked and N-linked oligosaccharides. Chief among these are alkaline borohydride treatment (β-elimination) and hydrazinolysis. Alkaline degradation can be highly effective for the release of O-linked oligosaccharides, provided sufficient sodium borohydride is included in the reaction mixture, converting released oligosaccharides to sugar alcohols (alditols) upon release [10]. Reduction to the alditol is necessary, as without it, a "peeling reaction" occurs, progressively degrading the released carbohydrates beginning at the reducing end. The drawback to this reduction is that the only unique group available on an oligosaccharide for labeling subsequent to release is the reducing end aldehyde. Upon reduction, this unique feature is eliminated.

Hydrazinolysis and re-N-acetylation as a method for the release and recovery of carbohydrates from glycoproteins has been practiced for nearly three decades [11]. A significant advantage of this method is that it can be used for both N-linked and O-linked carbohydrates. Recent advances have allowed release of oligosaccharides by use of anhydrous hydrazine with moderately high selectivity for O-linked oligosaccharides [12,13, and in this volume]. Some limitations of the use of hydrazinolysis for the release of carbohydrates from glycoproteins should be noted, including the loss of acetyl and glycolyl groups from N-acetyl and N-glycolyl substituted sugars, rendering determination of the ratio of N-acetylated to N-glycolylated sugars in hydrazine-released carbohydrates impossible. O-acyl substitutions of monosaccharides, particularly common in sialic acids, are also not expected to be retained upon hydrazinolysis [13]. Earlier studies on hydrazinolysis [14–16] reported that a substantial proportion of the carbohydrates released may be in a variety of modified forms, although recent refinements of the process, if carefully followed, may significantly reduce the production of undesired side products [12]. Another group has reported that oligosaccharides containing 3-linked substituents on the reducing end sugar (such as occurs in N-linked oligosaccharides from plants containing 3-linked core fucosylation, and in the common O-linked core structure Gal-α-1,3-GalNAc) may be degraded upon hydrazinolysis [17].

While chemical methods for the release of carbohydrates from glycoproteins may in many cases fit our needs, it is often desirable to perform oligosaccharide release under mild conditions where modification of the released sugars is unlikely, and recovery of the deglycosylated protein may be desired. The use of carbohydrate releasing enzymes would seem to be a nearly ideal approach in this regard. Additionally, these enzymatic digestions are generally easier to perform in the biochemical laboratory than the relatively laborious chemical release methods.

In the last two decades, a wide variety of endoglycosidase and glycoamidase enzymes able to release oligosaccharides from glycoproteins have been discovered and characterized. Using these enzymes it is now possible to conveniently and non-selectively release N-linked oligosaccharides from glycoproteins. Additionally, enzymes that exhibit a surprising degree of specificity with respect to the type of N-linked oligosaccharides released have now been described and well characterized. Several of these enzymes have been cloned, and a number of them are available commercially.

Enzymatic release of O-linked sugars is much more limited. Endoglycosidases that are able to perform this task for a commonly occurring O-linked structure have been identified, characterized, and made commercially available. However, their very restricted substrate specificity limits their utility. Another enzyme with a more relaxed specificity towards O-linked structures has been identified and partially characterized [18,19]. However, the specificity of this enzyme is at this point not completely characterized, and appears to be more limited than would be ideal.

This review attempts to summarize the current state of our knowledge of enzymes able to release N-linked and O-linked oligosaccharides from glycoproteins. An historical overview of the discovery and characterization of these enzymes is presented. The focus of this work is on the description of the enzymes available today, with emphasis on their practical applications. Wherever possible, details of practical methods and

conditions for enzymatic deglycosylation of proteins will be given, and references that detail the use of these enzymes are provided.

2. Endoglycosidases that release N-linked oligosaccharides

In the early 1970's the first descriptions of enzymes able to efficiently cleave oligosaccharides from glycoproteins appeared in the literature [20,21]. Since that time, a wide variety of endoglycosidase enzymes able to release N-linked oligosaccharides from glycoproteins, some with remarkable specificity for certain oligosaccharide structural types, have been isolated and characterized, as reviewed by Maley et al. [22]. Several of these enzymes have been cloned and heterologously expressed [23–26]. It is now possible to cleave high mannose oligosaccharides, hybrid type oligosaccharides, and complex type oligosaccharides from glycoproteins with remarkable selectivity, depending on the endoglycosidase enzymes used [22,26,27].

All of the endoglycosidases in this class cleave N-linked oligosaccharides within the chitobiosyl core, producing a released oligosaccharide truncated by one N-acetylglucosamine (GlcNAc) residue, and leaving one GlcNAc still attached to the peptide or protein substrate (Fig. 1). Several of these glycoprotein-specific endoglycosidases are now commercially available, several as cloned, expressed proteins. Papers presenting strategies for the analytical use of endoglycosidases in glycoprotein studies are available [22,28,29]. Specific information about individual N-linked oligosaccharide-releasing endoglycosidases is given below, and is summarized in Table 1.

2.1. Endoglycosidase from Diplococcus (Streptococcus) pneumoniae; endo D

It appears that the first described endo-β-N-acetylglucosaminidase able to release N-linked oligosaccharides from glycoproteins was endo D [20]. This enzyme acquired its name from the genus *Diplococcus*, of the bacterium in which the enzyme was found. The Genus for this bacterium has since been changed to *Streptococcus*.

The specificity of this enzyme is relatively narrow compared to several other endoglycosidases available for N-linked oligosaccharide release [30–32]. It is probably for this reason that this enzyme has not come into common use for glycoprotein analysis. The enzyme is able to cleave some high mannose type oligosaccharides (those for which the Man-α-1,3 residue is not substituted at C-2) [33]. However, most high mannose, hybrid and complex type oligosaccharides are not cleaved, as most of them contain a substituent at C-2 of the trimannosyl core Man-α-1,3 residue, which abolishes activity of this enzyme [34].

Endoglycosidase D is commercially available from Boehringer Mannheim (Indianapolis, IN, USA) and Genzyme (Boston, MA, USA).

2.2. Endoglycosidase from Streptomyces plicatus; endo H

Shortly after the first description of endo D, a similar enzyme was reported to have been found in culture filtrates from *Streptomyces plicatus* [21]. This enzyme, named endoglycosidase H for the fact that it cleaved high molecular weight glycosides, was purified and characterized in 1974 [35]. Since that time, endo H has been cloned [23] and expressed in high yield [24]. Endo H has found widespread use in glycoprotein research, e.g. [22] for review.

Endoglycosidase H, like many of the endoglycosidases active on N-linked glycoprotein oligosaccharides, is highly stable [22]. It is able to cleave all high mannose type oligosaccharides from glycoproteins, as well as most hybrid type oligosaccharides [31,36,37]. It is also able to cleave structures with a bisecting GlcNAc residue [36], and core fucosylated structures [37]. It is inactive on complex type oligosaccharides [22,37,38].

The utility of endo H is perhaps best realized in working with glycoprotein substrates that are known not to contain complex oligsaccharides, or in cases where selective removal of oligosaccharides is desired. For example, in recombi-

Table 1
Glycoprotein oligosaccharide-releasing enzymes

Enzyme	Source	Susceptible N-glycans[a]	References
Endo D	*Diplococcus pneumoniae*	Some high mannose Some (few) hybrids	30,31,32,33
Endo H	*Streptomyces plicatus*	High mannose Most hybrids	31,36,37
Endo C_I	*Clostridium perfringens*	Similar to endo D	36,40
Endo C_{II}	*Clostridium perfringens*	Similar to endo H (narrower)	36,40
Endo F_1	*Flavobacterium meningosepticum*	Similar to endo H (narrower)	27,37
Endo F_2	*Flavobacterium meningosepticum*	High mannose Biantennary complex	26,27
Endo F_3	*Flavobacterium meningosepticum*	Biantennary complex Triantennary complex	26,27
Endoglycosidase	*Arthrobacter protophormiae*	Similar to endo C_{II}	44
Endoglycosidase	*Bacillus circulans*	High mannose[b]	45
Endoglycosidase	*Bacillus alvei*	Similar to endo C_{II}	46
Endo S	*Dictyostelium discoideum*	Similar to endo C_{II}	47
Endo M	*Mucor Hiemalis*	Similar to endo F_2	48
Endo B	*Sporotrichum dimorphosporum*	Similar to endo F_2	49
Endoglycosidase	*Aspergillus oryzae*	Similar to endo H	50
Endoglycosidase	Fig (enzyme 1)	Similar to endo C_{II}	51
Endoglycosidase	Fig (enzyme 2)	Similar to endo D	52
Endoglycosidase	Jack bean	Similar to endo C_{II}	53
Endoglycosidase	Human kidney (enzyme 1)	Mostly high mannose	54
Endoglycosidase	Human kidney (enzyme 2)	High mannose and complex (free oligosaccharides only)	54
Endoglycosidase	Rat liver	Similar to endo F_2	55,56
Glycoamidase A	Almond	Similar to Glycoamidase F	69,70,71,72,73,74
Glycoamidase F	*Flavobacterium meningosepticum*	All N-linked (except 1,3-core Fuc)	27,42,78,79,82
Glycoamidase	Jack bean	Similar to Glycoamidase F)	68
EndoGalNacase D	*Diplococcus*	Gal-β-1,3-GalNAc only	89,93,94
EndoGalNacase A	*Alcaligenes*	Gal-β-1,3-GalNAc only	94,95
EndoGalNacase S	*Streptomyces*	Gal-β-1,3-GalNAc (plus larger structures)	18,19

[a] N-Glycan substrate specificities given are brief. Further details of most specificities are given in the text.
[b] Substrate specificity not fully characterized.

nant tissue plasminogen activator (rt-PA) Asn 117 is known to contain exclusively high mannose type oligosaccharides, while other glycosylation sites contain hybrid and complex type oligosaccharides [39]. By using endo H to deglycosylate this glycoprotein, it would be possible to study the effects of elimination of high mannose glycosylation at Asn 117.

Endo H is commercially available from a number of sources, including Boehringer Mannheim (Indianapolis, IN, USA) and Genzyme (Boston, MA, USA).

2.3. Endoglycosidases from Clostridium perfringens; endo-β-N-acetylglucosaminidases C_I and C_{II}

In 1975, two endoglycosidase enzymes, endo C_I and endo C_{II}, were purified from culture fluid of *Clostridium perfringens*. These enzymes are

very similar in their substrate specificities to endo D and endo H, respectively [40]. While endo D and endo C_I are indistinguishable with respect to their substrate specificities, endo C_{II} exhibits a subtly narrower specificity than endo H [36]. This difference is due to the requirement for endo C_{II} that the Man-α-1,3 residue in the trimannosyl core not be substituted at C-4. Therefore, while endo H is able to cleave all high mannose type oligosaccharides, endo C_{II} is able to cleave only a subset of these structures. Additionally following from this structural requirement of endo C_{II}, most hybrid structures would not be cleaved by this enzyme. Endo C_I and endo C_{II} have seen only limited use in glycoprotein structural studies.

2.4. *Endoglycosidases from Flavobacterium meningosepticum; endoglycosidases F_1, F_2 and F_3*

A potentially very useful class of endoglycosidase enzymes active on N-linked glycoprotein oligosaccharides are those produced by *Flavobacterium meningosepticum*. The first report of such an activity from this organism recognized that a very broad range of N-linked oligosaccharide substrates was being cleaved [41]. It is now known that the glycoprotein oligosaccharide releasing enzymes produced by this organism include several endoglycosidases, as well as a glycoamidase that is distinct from these [25–27,37,42,43]. The glycoamidase from this species will be discussed in a separate section, below. The endoglycosidases of *Flavobacterium* have now been isolated as three separate species; endo F_1, F_2 and F_3, each with different substrate specificities [37]. Each of these three related enzymes has been cloned [25,26].

The specificity of endo F_1 is now well understood, and that of endo F_2 and F_3 is becoming increasingly well defined [27]. Endo F_1 is similar to endo H in its substrate specificity, cleaving both high mannose type and hybrid type oligosaccharides. Endo H and endo F_1 in fact exhibit considerable amino acid sequence homology [25]. The main difference between endo H and endo F_1 appears to be the effect of core fucosylation of hybrid type structures; endo H is little effected by core fucosylation, whereas the rate of hydrolysis of hybrids by endo F_1 is greatly reduced by core fucosylation [27,37].

Endo F_2 and endo F_3, while exhibiting moderate sequence homology between each other, are relatively less similar in this respect to endo F_1 or endo H [26]. The substrate specificities of endo F_2 and endo F_3, while not yet fully elucidated, are becoming clear [27]. Endo F_2 is active on high mannose type oligosaccharides, but exhibits a strong preference for biantennary type oligosaccharides. It is unable to cleave the hybrid, tri-antennary and tetra-antennary type oligosaccharides that have been tested thus far. Endo F_3, in contrast, is inactive against high mannose type oligosaccharides, but able to cleave triantennary oligosaccharides. Like endo F_2, endo F_3 is active against biantennary oligosaccharides, and unable to cleave the hybrid type and tetraantennary oligosaccharides that have been tested thus far. These specificities are summarized in Table 1.

Preparations of endoglycosidase from *Flavobacterium meningosepticum* are currently commercially available, generally being sold as "endoglycosidase F." These preparations are primarily endo F_1, with relatively small and variable amounts of endo F_2 and endo F_3 being present [37]. Commercial suppliers include Boehringer Mannheim (Indianapolis, IN, USA) and Genzyme (Boston, MA, USA).

2.5. *Endoglycosidase from other bacterial sources; Arthrobacter protophormiae, Bacillus circulans and Bacillus alvei enzymes*

Endoglycosidases specific for N-linked oligosaccharides of glycoproteins have now been reported from a variety of bacterial sources. These include in addition to those described above, endo-β-N-acetylglucosaminidases from *Arthrobacter protophormiae* [44], *Bacillus circulans* [45] *and Bacillus alvei* [46]. In most cases where the activities of these enzymes have been examined in any detail, their substrate specificities appear to be generally similar to enzymes

discussed above (see Table 1), and therefore their use does not offer any general advantage over the use of the enzymes described previously. It appears that none of these enzymes is commercially available in purified form.

2.6. Endoglycosidase from non-bacterial sources

In addition to the many endoglycosidases that have been described from prokaryotes, a variety of eukaryotic endoglycosidases specific for N-linked glycoprotein oligosaccharides have been reported. The cellular slime mold *Dictyostellium discoideum* has been reported to produce an endoglycosidase, termed endo S, with substrate specificity similar to that of endo C_{II} from *Clostridium* [47].

Several fungal endoglycosidases reported to date include those from *Mucor hiemalis*, termed endo M [48], *Sporotrichum dimorphosporum*, termed endo B [49] and *Aspergillus oryzae* [50]. Endo M and endo B are similar to endo F_2 in that they cleave high mannose and some complex type oligosaccharides. These enzymes also cleave some hybrid type oligosaccharides. The enzyme from *Aspergillus* appears to be very similar in its substrate specificity to endo H.

Endoglycosidases have also been reported in higher plants. These include two fig enzymes; one with activity reported to be similar to endo C_{II} of *clostridium* [51] and the other with activity similar to endo D [52]. An enzyme from jack bean, also with similarity to endo C_{II}, has been reported [53].

Mammals also possess endoglycosidases active on N-linked oligosaccharides. Two such endoglycosidases from human kidney, one with good activity towards high mannose oligosaccharides and little activity towards complex type oligosaccharides, have been reported [54]. The second enzyme identified by this group was reported to be unusual in that it cleaved high mannose and complex type oligosaccharides, but only for oligosaccharides in which the chitobiose core was in reducing form, not attached to asparagine. An endoglycosidase from rat liver with specificity similar to that of endo F_2, has also been reported [55,56].

It is interesting to note the relative ubiquity of N-linked oligosaccharide-specific endoglycosidases, and the fact that these enzymes have in some cases been shown to be inducible by their substrates [57,58]. These considerations suggest a function for these enzymes that is common to many organisms, but as yet, no clear biological role for these endoglycosidases has been demonstrated.

2.7. Conditions for release of N-linked oligosaccharides from glycoproteins using endoglycosidases

The endoglycosidases, cleaving glycoprotein oligosaccharides at a point one sugar away from the N-acetylglucosamine-asparagine linkage (Fig. 1), are less susceptible to the steric hindrance that is often apparent for glycoamidases (see below). For this reason, a wider variety of glycoproteins can have their oligosaccharides effectively removed using endoglycosidases, without denaturation of the glycoprotein substrate, than can be achieved using glycoamidases. However, it should be noted that while we refer below to endoglycosidase-mediated *deglycosylation*, in fact these enzymes leave a rudimentary glycosylation (a single GlcNAc residue attached to asparagine) still attached. Examples of non-denatured glycoproteins that can be effectively deglycosylated (with the above noted qualifier) by endoglycosidases from *Flavobacterium* include ribonuclease B, yeast carboxypeptidase, *Ricinus* lectin, [59] and ovalbumin [59,60]. Native (non-denatured) yeast invertase was shown to be completely deglycosylated (with the exception of the remaining GlcNAc-Asn) by endo H in the absence of chaotropic or other agents intended to extend the protein structure [61]. Intact asialotransferrin was substantially but not completely deglycosylated with endo M [48]. Notwithstanding the above examples, as with the glycoamidases (see below), most glycoproteins are more effectively deglycosylated when they are first denatured or otherwise treated to increase exposure of attached oligosaccharides to endoglycosidases.

Effective strategies for increasing the suscep-

tibility of glycoprotein oligosaccharides to endoglycosidase digestion include treatment with the chaotropic agent sodium thiocyanate (NaSCN), effective for thyroglobulin deglycosylation by endo H [62]. Sulfitolysis has been shown to be effective in increasing the susceptibility to hydrolysis by endo H of DNase A and ovalbumin [61]. Combined reduction, carboxymethylation, and SDS denaturation were found to greatly increase the rate of deglycosylation of yeast invertase by endo H [63,64].

While some endoglycosidases, such as endo H, are comparatively stable to SDS for a few hours at moderate temperatures, others such as endo F are quite instable to SDS. Given that many glycoproteins are made more susceptible to oligosaccharide cleavage by SDS denaturation, a general strategy involving SDS denaturation followed by addition of enzyme protecting agents is effective. For denaturation of the substrate glycoprotein, boiling in a 1.2 fold weight excess of SDS is effective [62]. Addition of 2-mercaptoethanol or dithiothreitol, generally at about 0.1 *M*, is well tolerated by both endo H (personal experience) and endo F [65]. Protection of the enzyme from SDS can be achieved by addition, prior to enzyme addition, of BSA, presumably adsorbing free SDS [62], or by addition of any of several nonionic detergents, such as Nonidet P-40, Triton X-100 or octylglucoside [65]. Dilution of the reaction mixture prior to enzyme addition, to reduce the concentration of SDS, may also be helpful [62], but introduction of the protecting agents listed above may obviate this. The enzyme reaction mixture, with enzyme added, is generally incubated for a few hours to 24 hours, at 37°C. Protease inhibitors, if desired, are generally well tolerated by these endoglycosidases [22]. Selection of buffer for use during deglycosylation should be made depending on the pH optima and tested buffers reported in the specific papers cited above for each of the endoglycosidases discussed.

One note that should be considered is from an observation by Trimble et al. [66], that endo F cleavage of oligosaccharides in the presence of glycerol can result in the attachment of glycerol to the reducing end carbon of N-acetylglucosamine. In time, endo F replaces the glycerol with water, regenerating the reducing end of the oligosaccharide [22]. Endo H can also cause the attachment of glycerol to the released oligosaccharide, but at a slower rate and to a lesser degree [22].

3. Glycoamidase-based release of N-linked oligosaccharides

The endoglycosidases discussed above can collectively cleave most types of N-linked oligosaccharides of glycoproteins, with the exception of tetraantennary oligosaccharides. They can also be particularly useful where their specificities towards certain types of N-linked structures can be informative as to the nature of those structures released. However, where the nature of the N-linked oligosaccharides on a glycoprotein of interest is unknown, and a general, non-selective enzymatic release method is desired, the endoglycosidases are inappropriate. For this purpose another class of N-linked oligosaccharide releasing enzymes, the glycoamidases, are highly effective.

A glycoprotein specific glycoamidase active on N-linked glycoproteins was first discovered in almond nut nearly two decades ago [67]. Since that time, numerous reports have appeared on this type of enzyme, now including another enzyme of eukaryotic origin, from jack bean [68], as well as an enzyme originating from the prokaryote *Flavobacterium meningosepticum* [42]. These enzymes cleave N-linked oligosaccharides directly between the asparaginyl residue and the reducing end N-acetylglucosamine residue of the oligosaccharide (Fig. 1), by cleavage of the amide linkage. In this process, the asparaginyl residue is converted to an aspartyl residue, and the oligosaccharide is released as a glycosylamine. The amine group at C-1 of N-acetylglucosamine is spontaneously lost as ammonia following the initial cleavage reaction. This reaction mechanism is illustrated by Takahashi [69]. These enzymes have variably become known as glycopeptidases, peptide:N-glycosidases, PNGases, N-glycohydrolases, and

N-Glycanase. The EC name for this type of enzyme is peptide-N^4-(N-acetyl-β-D-glucosaminyl)asparagine amidase (EC 3.5.1.52). It has been suggested that the trivial name glycoamidase be adopted [69].

The glycoamidases from all three sources listed above appear to be very similar in their substrate specificities, being able to cleave all types of N-linked glycoprotein oligosaccharides; high mannose, hybrid and complex. Those differences that do exist are subtle, but important for practical use in deglycosylation reactions, and are elaborated below.

3.1. Glycoamidase from almond; glycoamidase A

The glycoamidase from almond, glycoamidase A, was first purified to homogeneity by Taga et al. [70]. It has been shown to be able to release all types of N-linked oligosaccharides found on glycoproteins [69–74]. One of the most distinguishing features of glycoamidase A is its ability to release single N-linked N-acetylglucosamine residues that are left on glycopeptides or glycoproteins following endoglycosidase mediated oligosaccharide cleavage [71]. Also distinguishing glycoamidase A from glycoamidase F, described below, is its substantially lower pH optimum between 4 and 6 [70].

For practical application, glycoamidase A is more restrictive than glycoamidase F, exhibiting much poorer activity towards larger peptides and intact proteins than towards smaller glycopeptides [70,75,76]. It is speculated that this relatively narrower substrate specificity is due to the larger size of glycoamidase A, at nearly 80 000 mw (vs. 35 500 mw for glycoamidase F), causing steric hindrance in interactions with larger glycopeptides or with intact glycoproteins [22,69]. Accessibility of substrates is improved by denaturation [70,77].

One aspect in which the specificity of glycoamidase A is actually broader than that of glycoamidase F is in its ability to release oligosaccharides from substrates containing core fucosylation at C-3 of the GlcNAc residue attached to asparagine [78]. In contrast, α-1,3 core fucosylation prevents oligosaccharide release by glycoamidase F, as noted below.

Similar to glycoamidase F, glycoamidase A is unable to cleave glycosyl asparagine, and is unable to cleave or greatly reduced in activity towards glycosyl-peptides in which the N-linked carbohydrate is attached at or near the peptide carboxy or amino terminus [71]. Glycoamidase A is particularly sensitive to SDS, but relatively insensitive to NaSCN, non-ionic detergents, and EDTA [70].

Glycoamidase A is commercially available from Siekagaku Kogyo Co., Tokyo, Japan.

3.2. Glycoamidase from Flavobacterium meningosepticum; glycoamidase F

The most versatile and widely used enzyme for releasing N-linked oligosaccharides from glycoproteins and glycopeptides is glycoamidase F (also known as PNGase F, peptide:N-glycosidase F, and N-Glycanase). This enzyme was first recognized by Plummer et al. [42] as a distinct activity within a preparation of endoglycosidases from the prokaryotic source *Flavobacterium meningosepticum*. Shortly thereafter, detailed characterization of this activity made clear that the enzyme was not only in fact a glycoamidase, distinct in mechanism of action from the endoglycosidases produced by *Flavobacterium*, but that the broad substrate specificity of this glycoamidase would make it useful in release of all classed of N-linked oligosaccharides from glycoproteins [79].

Glycoamidase F, in contrast to glycoamidase A, is unable to release individual N-acetylglucosamine residues from peptides or proteins [27]. Glycoamidase F is, however, able to release all classes of N-linked oligosaccharides, including high mannose, hybrid and multiply sialylated complex oligosaccharides up to and including tetraantennary structures [27,79,80], as well as oligosaccharides containing sulfate-substituted residues [81]. Oligosaccharide release by glycoamidase F is prevented by the carbohydrate structure being present on the amino-terminal or carboxy-terminal amino acid residue (Asn) of a glycopeptide or glycoprotein [79]. Interestingly,

location of the oligosaccharide structure on a penultimate asparagine residue of a glycopeptide can also prevent release by this enzyme [82]. The only feature of an oligosaccharide that appears able to prevent its release by glycoamidase F is the presence of an α-1,3-linked fucosyl residue on the reducing end N-acetylglucosamine [83], as occurs in some plant glycoproteins. The pH optimum of glycoamidase F, at 8.6 [79], is much higher than that of glycoamidase A. The major advantage in using glycoamidase F vs. glycoamidase A is that the former is very effective in releasing oligosaccharides from glycoproteins (see recommended conditions below), whereas the later is often ineffective in treating glycoprotein or large glycopeptide substrates [70,75,76].

Glycoamidase F has been cloned and heterologously expressed by a variety of workers, seemingly all working independently at about the same time [84–86]. This enzyme is commercially available both as a recombinant product and as the naturally occurring product recovered by purification from cultures of *Flavobacterium meningosepticum*. Suppliers include Boehringer Mannheim (Indianapolis, IN, USA), Genzyme (Boston, MA, USA), Glyko (Novato, CA, USA), New England Biolabs (Beverly, MA, USA), and Takara Shuzo (Kyoto, Japan).

3.3. Glycoamidase from jack bean

A glycoamidase activity from jack bean that was able to release N-linked oligosaccharides from glycoproteins was first reported by Sugiyama et al. [68]. Unlike glycoamidase A, and similar to glycoamidase F in this respect, the jack bean enzyme was unable to cleave a single N-acetylglucosamine residue N-linked to a peptide. Similar to both glycoamidases A and F, the jack bean enzyme was able to release multiply-sialylated complex type oligosaccharides, as well as Hybrid and high mannose type structures. The enzyme exhibited a pH optimum of 6.5. Purification of this activity was reported by Yet and Wold [53]. Glycoamidase from jack bean is not currently commercially available.

3.4. Conditions for release of N-linked oligosaccharides using glycoamidases

General, non-selective release of asparagine-linked oligosaccharides can be effectively achieved using any of the glycoamidases discussed above. However, due to its greater ability to release oligosaccharides from glycoproteins and larger glycopeptide substrates, and its commercial availability, glycoamidase F is most commonly used. Glycoamidase A is much less effective for glycoprotein substrates [70,75,76].

A number of papers have been written detailing methods used in releasing oligosaccharides from glycoproteins using these enzymes [e.g. 65,87,88]. Most glycopeptides are effectively deglycosylated with either glycoamidase F or A without any particular treatment other than incubation of the substrate and enzyme in an appropriate buffer (e.g. 20 to 200 m*M* sodium phosphate, pH 7 to 8.6, for glycoamidase F, or 10 to 20 m*M* sodium acetate, pH 4.5 to 5, for glycoamidase A).

The main problem of glycoprotein deglycosylation by glycoamidases is accessibility to the carbohydrate structure by the enzyme. Some glycoproteins are effectively deglycosylated by glycoamidase F in their native form, but reduction with a sulfhydryl reducing reagent, such as β-mercaptoethanol or dithiothreitol, and denaturation with an ionic detergent such as SDS, generally greatly improves oligosaccharide release [79,87]. Because glycoamidase F is highly susceptible to denaturation by ionic detergents, and glycoamidase A even more so [70], it is necessary before addition of the enzyme to the denatured protein sample to add one or more non-ionic detergents to the mixture. The function of these non-ionic detergents, examples of which are Nonidet P-40 and Triton X-100, is to protect the enzyme from the ionic detergents used for denaturation.

It is interesting to note that many published accounts of the use of glycoamidase F for glycoprotein deglycosylation advocate incubation with the enzyme for periods of up to 72 h. Work in our own laboratory has shown that under the best of the conditions recommended in the

literature for protecting glycoamidase F from denaturing conditions, the enzyme has a lifetime of less than 3 h when treating glycoproteins [82]. For this reason, we recommend that when following literature procedures for reduction, denaturation, protection with non-ionic detergents and finally glycoamidase F treatment of glycoproteins [e.g. 65,87,88], sufficient enzyme be added to complete the deglycosylation rapidly, in two hours or less. Longer treatments are simply not productive, and will not allow an initially inadequate amount of glycoamidase F to do the job over a long incubation time.

Instead of addition of non-ionic detergents to protect glycoamidases from SDS-mediated denaturation, non-glycosylated proteins such as BSA can be added to the denatured substrate mixture, prior to enzyme addition, to adsorb excess ionic detergent [65]. Further, alternatively to protocols involving SDS denaturation, it is possible to deglycosylate resistant glycoproteins in the absence of detergents, taking advantage of the relatively good stability of glycoamidases to high concentrations of urea (2 to 5 *M*) and NaSCN (typically 0.25 *M*) [22,77].

For some particularly recalcitrant glycoproteins it may be necessary to take further measures beyond reduction and denaturation to expose the oligosaccharide chains to glycoamidase F attack. Such treatments may include reduction and acetylation of the glycoprotein [39] or digestion to smaller peptides and glycopeptides with trypsin [69] or other proteases. In many cases, digestion to glycopeptides will be the only method effective for releasing oligosaccharides using glycoamidase A.

4. Enzymatic release of O-linked carbohydrates; endo-α-N-acetylgalactosaminidases

As we have seen, release of N-linked glycoprotein oligosaccharides is well achieved by enzymatic means. Enzymatic release of O-linked carbohydrates is more problematic. Probably the most commonly occurring O-linked structure found on glycoproteins is the disaccharide Gal-β-1,3-GalNAc [2,3]. This structure can be cleaved by an enzyme activity available from either of two microbial sources; endo-GalNAc-ase D from *Diplococcus pneumoniae* [89], and endo-GalNAc-ase A from *Alcaligenes* sp. [90]. The difficulty with these enzymes is that their activity towards O-linked glycosides is limited to the Gal-β-1,3-GalNAc disaccharide; no extension of this structure appears to be tolerated by the endo-GalNAc-ase enzymes described above. O-linked oligosaccharides are, however, quite variable in structure; the disaccharide chain described above is often extended with additional sugars [2,3,91], and recently, O-linked GlcNAc [6] and O-linked fucose [7] have been reported. Given the restricted substrate specificity of endo-GalNAc-ase D and endo-GalNAc-ase A, these enzymes are of only very limited utility to those interested in using them to release O-linked oligosaccharides for subsequent analysis.

Recently, reports have appeared describing an as yet poorly defined enzyme from *Streptomyces* sp., designated endo-GalNAc-ase S, that is able to release larger O-linked structures than the disaccharide described above [18,19]. This enzyme would presumably be much more useful than endo-GalNAc-ase D and endo-GalNAc-ase A, as some of the most interesting O-linked oligosaccharides are larger structures not cleaved by the disaccharide-releasing enzymes. However, the substrate specificity of endo-GalNAc-ase S is as yet poorly understood, and it does appear to be limited to those O-linked oligosaccharides containing the Gal-β-1,3-GalNAc core structure. Additionally, even among this class of O-linked structures, the specificity of endo-GalNAc-ase S appears to be quite limited. Additional details of this and the other endo-GalNAc-ase enzymes are given below.

4.1. Endo-α-N-acetylgalactosaminidase from Diplococcus; endo-GalNAc-ase-D

The endo-α-N-acetylgalactosaminidase from *Diplococcus* was first reported in 1976 by two independent groups [89,92]. Like endo-GalNAc-ase-A, the *Diplococcus* enzyme is specific for the

disaccharide Gal-β-1,3-GalNAc O-linked to either serine or threonine residues [89,93]. A comparison of the activity of Endo-GalNAc-ase-D to that of Endo-GalNAc-ase-A, using substrates consisting of Gal-β-1,3-GalNAc disaccharide O-linked to the otherwise free amino acids serine or threonine suggest that the *Diplococcus* enzyme prefers glycopeptide substrates over individual glyco-amino acids [94]. pH optima ranging from 6 [89] to 7.6 [93] have been reported for endo-GalNAc-ase-D, appearing to depend at least in part on the buffer used.

Endo-GalNAc-ase-D is commercially available from Genzyme Corporation (Boston, MA, USA) as "O-Glycanase".

4.2. Endo-α-N-acetylgalactosaminidase from Alcaligenes; endo-GalNAc-ase-A

An endo-α-N-acetylgalactosaminidase from *Alcaligenes* sp., with very similar substrate specificity to that of endo-GalNAc-ase D, was first described by Yamamoto et al. [95]. Like the *Diplococcus* enzyme, endo-GalNAc-ase A is apparently only able to cleave the Gal-β-1,3-GalNAc disaccharide from glycoprotein substrates. Studies with this enzyme using the glyco-amino acid substrates described above, indicated a strong preference by this enzyme for the disaccharide attached to serine over the analogous substrate containing threonine in place of serine [94]. A pH optimum of 4.5 has been reported for this enzyme [90,95].

Endo-GalNAc-ase-A is commercially available from Siekagaku Kogyo Co., Ltd. (Tokyo, Japan).

4.3. Endo-α-N-acetylgalactosaminidase from Streptomyces

As described briefly above, the endo-α-N-acetylgalactosaminidase from *Streptomyces*, Endo-GalNAc-ase-S, is able to cleave from glycoproteins O-linked oligosaccharide structures that are larger than the Gal-β-1,3-GalNAc core structure. The original paper describing this enzyme activity from *Streptomyces* showed that a crude preparation of this enzyme was able to liberate from porcine gastric mucin oligosaccharides with apparent d.p. corresponding to up to 10 or more monosaccharide units [18]. Analysis of one of these oligosaccharides eluted from a TLC plate contained GalNAc, GlcNAc, Gal and Fuc in the ratio 1:3:3:2, which combined with the apparent size of this species based on TLC, suggested that the molecule analyzed was a nonasaccharide.

In a later paper by this same laboratory, a more highly purified preparation of the endo-α-N-acetylgalactosaminidase activity from *Streptomyces* was shown to be capable of releasing a tetrasaccharide with the apparent structure Gal-β-1,3-(Gal-β-1,4-GlcNAc-β-1,6)-GalNAc from asialofetuin [19]. Some evidence reported in that paper suggests that endo-GalNAc-ase-S, like endo-GalNAc-ase-A, exhibits a preference for O-linked structures attached to serine residues over those attached to threonine. There has been no mention in the literature of this enzyme being able to release sialylated O-linked species. The pH optimum of endo-GalNAc-ase-S was reported to be 5.5.

4.4. Conditions for release of O-linked oligosaccharides using endo-GalNAc-ases

Conditions for the use of the endo-GalNAc-ase enzymes for the release of O-linked carbohydrates from glycoproteins are described in reviews by Iwase and Hotta [96,97]. Some specific recommendations follow.

Given the very limited substrate specificities of endo-GalNAc-ases D and A, and the probable limitations of endo-GalNAc-ase S noted above, it is typical to first desialylate substrates to be treated with any of these enzymes [98–100]. It should also be noted that endo-GalNAc-ase D has been reported to transglycosylate hydroxyl group containing materials such as buffer components (Tris), glycerol (often added to stabilize enzymes), hydroxyl-containing amino acids, and sugars [101]. These materials should therefore be avoided in reaction mixtures, if possible. Bar-

dales and Bhavanandan suggested a 20 m*M* phosphate buffer system, pH 6.0 [101].

5. Conclusions

We have seen that enzymes are available that allow us to release either N-linked or O-linked oligosaccharides from glycoproteins and glycopeptides. In the case of N-linked oligosaccharides, virtually any structure that is found in nature can be released with an enzyme such as glycoamidase F. For release of O-linked oligosaccharides we are far more limited, as the enzymes available are able to release only a very limited subset of the carbohydrate structures we find linked to serine and threonine residues in glycoproteins.

5.1. Advantages of enzymatic methods

Notwithstanding current limitations for O-linked oligosaccharide release, the discovery and characterization in the last 20 years of a wide variety of glycoprotein oligosaccharide releasing enzymes offers significant advantages over former chemical methods. Chief among these advantages is the ability to release oligosaccharides under mild conditions using comparatively simple methods, as opposed to employing chemical methods that are comparatively complex, finicky, often require the use of highly reactive materials, and may result in modification of the released oligosaccharides. Additionally, enzymatic release, particularly of N-linked oligosaccharides, provides the opportunity to extract information about the oligosaccharides released by virtue of our knowledge of the substrate specificities of the enzymes being employed. Finally, the ability to recover the peptide or protein portion of the glycopeptide or glycoprotein substrate following enzymatic deglycosylation allows analysis of that material. Of interest to the biochemist is determination of the biological activity, functioning, or other properties of deglycosylated peptides or proteins, so as to determine the functional role of the oligosaccharides formerly present.

5.2. The search for additional enzymes

Probably the greatest limitation to the generalized enzymatic release of carbohydrates from glycoproteins is the substrate specificities of the enzymes available for O-linked oligosaccharide release. The commercially available enzymes, endo-GalNAc-ases D and A, are strictly limited to release of the disaccharide Gal-β-1,3-GalNAc. This can be useful for verification that these species are present, without particular need to perform any sophisticated analysis of the released species. It can also be useful where this disaccharide is known to exist on a glycoprotein of interest, and where it is desired to study the effect on the protein of elimination of this substituent. Additionally, as the amino acids serine and threonine are not altered by enzymatic release of O-linked oligosaccharides, it may be possible to obtain additional peptide sequence information by first de-O-glycosylating a peptide or protein.

Discovery of endo-GalNAc-ase S, with its apparently broader substrate specificity, gives hope that an enzyme will become available that is to O-linked structures what glycoamidase F is to N-linked structures; an enzyme able to non-selectively release all oligosaccharides within the class. While characterization of endo-GalNAc-ase S is not yet complete, it appears that this enzyme does release a broader array of O-linked structures than do the commercially available endo-GalNAc-ase enzymes. However, apparent limitations of endo-GalNAc-ase S suggest that this is far from our ideal of a non-selective, O-linked oligosaccharide releasing enzyme. It is hoped that in time, ongoing screening experiments with selective substrates will result in the identification of O-linked oligosaccharide releasing enzymes with broad specificity.

References

[1] R. Kornfeld and S. Kornfeld, Ann. Rev. Biochem., 54 (1985) 631.

[2] H. Schachter, Biochem. Cell. Biol., 64 (1986) 163.
[3] H. Schachter and I. Brockhausen, in H.J. Allen and E.C. Kisailus (Editors), Glycoconjugates, Marcel Dekker, New York, 1992, Ch. 9, p. 263.
[4] R. Schreiner, E. Schnabel and F. Wieland, J. Cell Biol., 124 (1994) 1071.
[5] B.M. Vertel, L.M. Walters, N. Flay, A.E. Kearns and N.B. Schwartz, J. Biol. Chem., 268 (1993) 11105.
[6] G.W. Hart, R.S. Haltiwanger, G.D. Holt and W.G. Kelly, Annu. Rev. Biochem., 58 (1989) 841.
[7] R.H. Harris and M.W. Spellman, Glycobiology, 3 (1993) 219.
[8] A.K. Menon, Meth. Enzymol., 230 (1994) 418.
[9] P. Nacharaju and A.S. Acharya, in R.H. Angeletti (Editor), Techniques in Prot. Chem. III, Academic Press, San Diego, 1992, p. 303.
[10] R.N. Iyer and D.M. Carlson, Arch. Biochem Biophys., 142 (1971) 101.
[11] Z. Yosizawa, T. Sato and K. Schmid, Biochim. Biophys. Acta, 121 (1966) 417.
[12] T. Patel, J. Bruce, A. Merry, C. Bigge, M. Wormald, A. Jaques and R. Parekh, Biochemistry, 32 (1993) 679.
[13] T. Patel and R. Parekh, Meth. Enzymol., 230 (1994) 57.
[14] P.W. Tang and J.M. Williams, Carbohydr. Res., 121 (1983) 89.
[15] B. Bendiak and D.A. Cumming, Carbohydr. Res., 144 (1985) 1.
[16] B. Bendiak and D.A. Cumming, Carbohydr. Res., 151 (1986) 89.
[17] S.R. Carter, J.M. Williams and J.R. Clamp, Carbohydr. Res., 205 (1990) 181.
[18] H. Iwase, I. Ishii, K. Ishihara, Y. Tanaka, S. Omura and K. Hotta, Biochem. Biophys. Res. Comm., 151 (1988) 422.
[19] I. Ishii-Karakasa, H. Iwase, K. Hotta, Y. Tanaka and S. Omura, Biochem. J., 288 (1992) 475.
[20] T. Maramatsu, J. Biol. Chem., 246 (1971) 5535.
[21] A.L. Tarentino, T.H. Plummer, Jr. and F. Maley, J. Biol. Chem., 247 (1972) 2629.
[22] F. Maley, R.B. Trimble, A.L. Tarentino and T.H. Plummer, Jr., Anal. Biochem., 180 (1989) 195.
[23] H. Shimatake and M. Rosenberg, Nature, 272 (1981) 128.
[24] R.J. Trumbly, P.W. Robbins, M. Belfort, F.D. Ziegler, F. Maley and R.B. Trimble, J. Biol. Chem., 260 (1985) 5683.
[25] A.L. Tarentino, G. Quinones, W.P. Schrader, L.-M. Changchien and T.H. Plummer, Jr., J. Biol. Chem., 267 (1992) 3868.
[26] A.L. Tarentino, G. Quinones, L.-M. Changchien and T.H. Plummer, Jr., J. Biol. Chem., 268 (1993) 9702.
[27] A.L. Tarentino and T.H. Plummer, Jr., Meth. Enzymol., 230 (1994) 44.
[28] A. Kobata, Anal. Biochem., 100 (1979) 1.
[29] K. Yamamoto, J. Biochem., 116 (1994) 229.
[30] N. Koiede and T. Muramatsu, J. Biol. Chem., 249 (1974) 4897.
[31] A.L. Tarentino and F. Maley, Biochem. Biophys. Res. Commun., 67 (1975) 455.
[32] T. Mizuochi, J. Amano and A. Kobata, J. Biochem., 95 (1984) 1209.
[33] T. Maramatsu, Meth. Enzymol., 50 (1988) 555.
[34] T. Muramatsu, in N. Takahashi and T. Maramatsu (Editors), CRC Handbook of Endoglycosidases and Glycoamidases, CRC Press, Boca Raton, 1992, p. 13.
[35] A.L. Tarentino and F. Maley, J. Biol. Chem., 249 (1974) 811.
[36] T. Tai, K. Yamashita, S. Ito and A. Kobata, J. Biol. Chem., 252 (1977) 6687.
[37] R.B. Trimble and A.L. Tarentino, J. Biol. Chem., 266 (1991) 1646.
[38] M. Arakawa and T. Muramatsu, J. Biochem., 76 (1974) 307.
[39] M.W. Spellman, L.J. Basa, C.K. Leonard, J.A. Chakel, J.V. O'Connor, S. Wilson and H. van Halbeek, J. Biol. Chem., 264 (1989) 14100.
[40] S. Ito, T. Maramatsu and A. Kobata, Arch. Biochem. Biophys., 171 (1975) 78.
[41] J.H. Elder and S. Alexander, P.N.A.S. USA, 79 (1982) 4540.
[42] T.H. Plummer, Jr., J.H. Elder, S. Alexander, A.W. Phelan and A.L. Tarentino, J. Biol. Chem., 259 (1984) 10700.
[43] T.H. Plummer, Jr. and A.L. Tarentino, Glycobiology, 1 (1991) 257.
[44] K. Takegawa, M. Nakoshi, S. Iwahara, K. Yamamoto and T. Tochikura, Appl. Env. Microbiol., 55 (1989) 3107.
[45] R.E. Cohen, W.-J. Zhang and C.E. Ballou, J. Biol. Chem., 257 (1982) 5730.
[46] T. Moringa, M. Kitamikado, H. Iwase, S.-C. Li and Y.-T. Li, Biochim. Biophys. Acta., 749 (1983) 211.
[47] H.H. Freeze and J.R. Etchison, Arch. Biochem. Biophys., 232 (1984) 414.
[48] S. Kadowaki, K. Yamamoto, M. Fujisaki, K. Izumi, T. Tochikura and T. Yokoyama, Agric. Biol. Chem., 54 (1990) 97.
[49] O. Kol, C. Brassart, G. Spik, J. Montreuil and S. Bouquelet, Glycoconjugate J., 6 (1989) 333.
[50] J. Hitomi, Y. Murakami, F. Saitoh, N. Shigemitsu and H. Yamaguchi, J. Biochem., 98 (1985) 527.
[51] M. Ogata-Arakawa, T. Muramatsu and A. Kobata, J. Biochem., 82 (1977) 611.
[52] S.-F. Chien, R. Weinburg, S.-C. Li and Y.-T. Li, Biochem. Biophys. Res. Commun., 76 (1977) 317.
[53] M.-G. Yet and F. Wold, J. Biol. Chem., 263 (1988) 118.
[54] R. DeGasperi, Y.-T. Li and S.-C. Li, J. Biol. Chem., 264 (1989) 9329.
[55] Y. Tachibana, K. Yamashita and A. Kobata, Arch. Biochem. Biophys., 214 (1982) 199.
[56] J.J.W. Lisman, C.J. Van der Wall and B. Overdijk, Biochem. J., 229 (1985) 379.
[57] S. Kadowaki, K. Takegawa, K. Yamamoto, H. Kumagai and T. Tochikura, Agric. Biol. Chem., 52 (1988) 2105.

[58] K. Takegawa, M. Nakoshi, S. Iwahara, K. Yamamoto and T. Tochikura, Appl. Env. Microbiol., 55 (1989) 3107.
[59] K. Yamamoto, K. Takegawa, J. Fan, H. Kumagai and T. Tochikura, Ferment. Technol., 64 (1986) 397.
[60] N. Kitabatake, A. Ishida, K. Yamamoto, T. Tchikura and E. Doi, Agric. Biol. Chem., 52 (1988) 2511.
[61] A.L. Tarrentino, T.H. Plummer, Jr. and F. Maley, J. Biol. Chem., 249 (1974) 818.
[62] R.B. Trimble and F. Maley, Anal. Biochem., 141 (1984) 515.
[63] R.B. Trimble and F. Maley, J. Biol. Chem., 252 (1977) 4409.
[64] R.B. Trimble, F. Maley and F.K. Chu, J. Biol. Chem., 258 (1983) 2562.
[65] A. Haselbeck and W. Hosel, Topics. Biochem., 8 (1988) 1.
[66] R.B. Trimble, P.H. Atkinson, A.L. Tarentino, T.H. Plummer, Jr., F. Maley and K.B. Tomer, J. Biol. Chem., 261 (1986) 12000.
[67] N. Takahashi, Biochem. Biophys. Res. Commun., 76 (1977) 1194.
[68] K. Sugiyama, H. Ishihara, S. Tejima and N. Takahishi, Biochem. and Biophys. Res. Comm., 112 (1983) 155.
[69] N. Takahashi, in N. Takahashi and T. Muramatsu (Editors), Handbook of Endoglycosidases and Glycoamidases, CRC Press, Boca Raton, 1992, p. 183.
[70] E.M. Taga, A. Waheed and R.L. Van Etten, Biochemistry, 23 (1984) 815.
[71] T.H. Plummer, Jr. and A.L. Tarentino, J. Biol. Chem., 256 (1981) 10243.
[72] N. Takahashi and H. Nishibe, Biochim. Biophys. Acta., 657 (1981) 457.
[73] H. Ishihara, N. Takahashi, J. Ito, E. Takeuchi and S. Tejima, Biochim. Biophys. Acta., 669 (1981) 216.
[74] N. Tomiya, M. Kurono, H. Ishihara, S. Tijima, S. Endo, Y. Arata and N. Takahashi, Anal. Biochem., 163 (1987) 489.
[75] H. Nishibe and N. Takahashi, Biochim. Biophys. Acta., 661 (1981) 274.
[76] N. Takahashi, H. Toda, H. Nishibe and K. Yamamoto, Biochim. Biophys. Acta., 707 (1982) 236.
[77] A.L. Tarentino and T.H. Plummer, Jr., J. Biol. Chem., 257 (1982) 10776.
[78] E. Staudacher, F. Altmann, L. Marz, K. Hard, J.P. Kamerling and J.F.G. Vliegenthart, Glycoconj. J., 9 (1992) 82.
[79] A.L. Tarentino, C.M. Gomez and T.H. Plummer, Jr., Biochemistry, 24 (1985) 4665.
[80] R.D. McCoy and F.A. Troy, Meth. Enzymol., 138 (1987) 627.
[81] H.H. Freeze, R. Yeh, A.L. Miller and S. Kornfeld, J. Biol. Chem., 258 (1983) 14874.
[82] S. Yuen, L.R. Zieske, I. Zaidi, D. Fu and R.A. O'Neill, Techniques in Protein Chem. V., Academic Press, New York, 1994, p. 275.
[83] V. Tretter, F. Altmann and L. Marz, Eur. J. Biochem., 199 (1991) 647.
[84] A.L. Tarentino, G. Quinones, A. Trumble, L.-M. Changchien, B. Duceman, F. Maley and T.H. Plummer, Jr., J. Biol. Chem., 265 (1990) 6961.
[85] G.D. Barsomian, T.L. Johnson, M. Borowski, J. Denman, J.F. Ollington, S. Hirani, D.S. McNeilly and J.R. Rasmussen, J. Biol. Chem., 265 (1990) 6967.
[86] D. Lemp., A. Haselbeck and F. Klebl, J. Biol. Chem., 265 (1990) 15606.
[87] S. Hirani, R.J. Bernasconi and J.R. Rasmussen, Anal. Biochem., 162 (1987) 485.
[88] R. Nuck, M. Zimmerman, D Sauvageot, D. Josic and W. Reutter, Glycoconjugate J., 7 (1990) 279.
[89] Y. Endo and A. Kobata, J. Biochem., 80 (1976) 1.
[90] J.-Q. Fan, S. Kadowaki, K. Yamamoto, H. Kumagai and T. Tochikura, Agric. Biol. Chem., 52 (1988) 1715.
[91] J.C. Paulson, T.I.B.S., (1989) 272.
[92] V.P. Bhavanandan, J. Umemoto and E.A. Davidson, Biochem. Biophys. Res. Comm., 70 (1976) 738.
[93] J. Umemoto, V.P. Bhavanandan and E.A. Davidson, J. Biol. Chem., 252 (1977) 8609.
[94] J.-Q. Fan, K. Yamamoto, H. Kumagai and T. Tochikura, Agric. Biol. Chem., 54 (1990) 233.
[95] K. Yamamoto, J.-Q. Fan, S. Kadowaki, H. Kumagai and T. Tochikura, Agric. Biol. Chem., 51 (1987) 3169.
[96] H. Iwase and K. Hotta, in N. Takahashi and T. Maramatsu (Editors), CRC Handbook of Endoglycosidases and Glycoamidases, CRC Press, Boca Raton, 1992, p. 41.
[97] H. Iwase and K. Hotta, in E.F. Hounsel (Editor), Meth. Mol. Biol., 14: Glycoprotein Analysis in Biomedicine, Humana Press, Totowa, N.J., U.S.A., 1993, p. 151.
[98] D.M. Lublin, J. Krsek-Staples, M.K. Pangburn and J.P. Atkinson, J. Immunol., 137 (1986) 1629.
[99] D.M. Lambert, Virology, 164 (1988) 458.
[100] F. Aubrit, C. Gelin, D. Pham, B. Raynal and A. Bernard, Eur. J. Immunol., 19 (1989) 1431.
[101] R.M. Bardales and V.P. Bhavanandan, J. Biol. Chem., 264 (1989) 19893.