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*BİLİMSEL TARAMALAR / SCIENTIFIC REVIEWS*

# The Characterisation of the Carbohydrate Moieties of Glycoproteins and the Role of Hydrazinolysis

Sibel SÜZEN*°

***The Characterisation of the Carbohydrate Moieties of Glycoproteins and the Role of Hydrazinolysis***

***Summary :*** *The characterisation of the carbohydrate moieties of glycoproteins requires the prior release of the oligosaccharides from the glycoprotein. The use of hydrazinolysis allows chemical release from glycoproteins of N-linked oligosaccharides but hydrazinolysis of glycopeptides containing serine (or threonine) bound oligosaccharides causes degradation of the oligosaccharides. Although it has been found that by using a lower temperature the degradation can be avoided, complete release of oligosaccharides from mucus glycoproteins which contain large numbers of oligosaccharides chains is still difficult to achieve.*

***Key words:*** *Hydrazinolysis, Glycoprotein, Carbohydrate*

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***Glikoproteinlerde karbohidrat yapılarının tanımlanması ve hidrazinolizin rolü***

***Özet :*** *Glikoproteinlerin taşıdıkları karbohidratların yapılarının tanımlanabilmesi, oligosakkaritlerin glikoproteinlerden ayrılmalarını gerektirmektedir. Hidrazinoliz N köprüsü ile glikoproteine bağlı oligosakkaritlerin kimyasal ayrılmasını gerçekleştirebilmekte fakat serin (yada treonin) içeren glikopeptidlerde oligosakkaritlerin degradasyonuna neden olmaktadır. Bu degradasyon düşük ısı kullanarak engellenebilse de, çok sayıda oligosakkarit zincirleri içeren mukus glikoproteinleri gibi yapılarda halen uygulanması güçtür.*

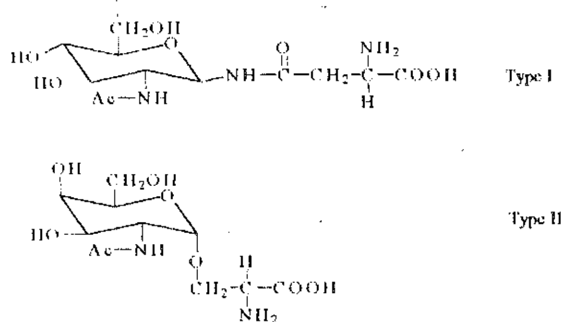
***Anahtar kelimeler:*** *Hidrazinoliz, Glikoprotein, Karbohidrat*

## INTRODUCTION

Glycoproteins are very important components of nature, being found in the extra and intracellular fluids, connective tissue and cellular membranes[1]. The glycoproteins, which contain carbohydrate groups attached covalently to the polypeptide chain, represent a large group having wide distribution and considerable biological significance, and are involved in diverse physiological functions. They seem to have a great variety of biological functions. Since glycoproteins are essential components of all mucous secretions and endow these secretions with their characteristic physicochemical properties, the functions of the mucous secretions may be taken as a reflection of the functions of glycoproteins. The covalent linkage of saccharides to the peptide chain represents a central aspect of glycoprotein structure. The glycans of glycoprotein are typically either N-linked (attached to asparagine) or O-linked (attached to serine or threonine)[2]. These two main saccharide-amino acid linkages present in glycoproteins are shown in (Scheme 1).

* Ankara Üniversitesi, Eczacılık Fakültesi, Farmasötik Kimya Anabilim Dalı, 06100 Ankara-Türkiye

° Correspondence




**Scheme 1.** The main linkages of the saccharides to the peptide chain

Type I linkages involve asparagine side chain groups and the sugar residue N-acetyl-D-glucosamine. Asparagine-linked carbohydrate chains fall into two classes. One is composed only of mannose and N-acetylglucosamine residues and is frequently branched. The second class contains a wider variety of carbohydrate residues in branched structures. Type II structures involve glycosidic carbohydrate linkages to serine or threonine alcoholic groups and are found in glycoproteins in mucus secretions, including those from the submaxillary glands, the epithelial cells of the gastrointestinal tract, the respiratory tract, and the female genital tract[3].

## RELEASE OF OLIGOSACCHARIDES

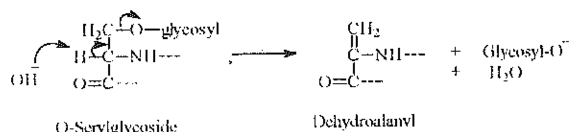
Analysis of the protein part of the molecule can be affected in one or more ways by the nonprotein moiety. Carbohydrate and lipids especially may increase the amount of destruction of certain amino acids during acid hydrolysis, and removal of these constituents is necessary[2,3]. Characterisation of the carbohydrate portion of glycoprotein requires prior release of the oligosaccharide portion from the molecule. This procedure is very important in clinical studies such as cancer research. Comparison of glycoproteins from normal mammary glands with glycoproteins from a tumour needs purification of the tissue samples[4]. In structural studies of the carbohydrate moieties of glycoproteins, one of the biggest problems is the heterogeneity of samples. Because most glycoproteins contain several sugar chains in one molecule, these sugar chains must be fractionated before starting the structural analysis[5].

Different strategies have been developed for the isolation and characterisation of the carbohydrate moieties of each type of glycoconjugate, and a very wide range of approaches and techniques have been adopted for the study of protein glycosylation[6]. It is not possible to determine the primary structure of carbohydrate chains on an intact glycoprotein. As a result, the characterisation of these oligosaccharides is carried out after their release using either enzymatic or chemical methods.

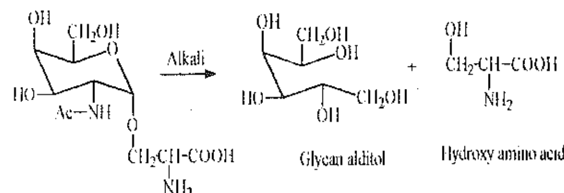
## AVAILABLE METHODS TO ISOLATE GLYCANS FROM GLYCOPROTEIN SOURCES

Several methods have been used to release the carbohydrate moieties from protein. The recent available methods that are especially related to O-linked oligosaccharides[7] are discussed.

1. Alkaline borohydride method : Glycosidic linkages that are β to a carbonyl group are alkali-labile, and are split to give a carbohydrate residue and an unsaturated aglycon (Scheme 2). Later studies showed that glycoproteins of animal origin have glycoside linkages involving the hydroxyl groups of serine and threonine, as treatment with alkali released the sugar component simultaneously with the loss of an equimolecular amount of hydroxyamino acid (Scheme 3). Effects of dilute alkali such as sodium borohydride on synthetic O-seryl and O-threonyl derivatives of monosaccharides were reported by others[8,9]. In order to protect the carbohydrate chain from degradation during hydrolysis, reduction with sodium borohydride was introduced to the study of O-glycoproteins[10]. Of the two major types, only the O-glycosidic linkage to serine or threonine is labile to alkali cleavage. The N-glycosidic bonds of β-aspartyl glycosides are relatively stable to mild alkaline solutions at room temperature, but they are unstable at elevated temperature[11,12]. This cleavage using sodium borohydride conditions occurs by a β-elimination reaction[13,14] for the O-glycosidic bonds.



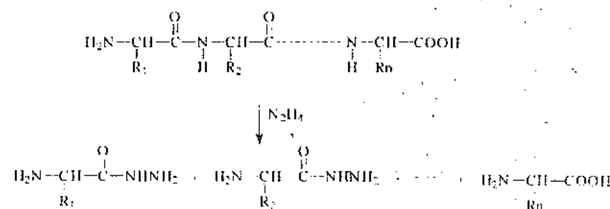

**Scheme 2.** The mechanism of β-elimination reaction




**Scheme 3.** Alkali treatment of a typical O-glycan

2. Enzymatic methods : Enzymic release of N-glycans can be achieved with peptide N-glycosidases and endoglycosidases[15], but fewer enzymes are available for removal of O-glycans[16]. While endoglycosidase enzymes[6] such as Endo-H (30) and PNGase-F(31) are specific for the release of N-linked oligosaccharides, there are no corresponding enzymatic procedures that are available for the selective release of the intact O-linked oligosaccharides. Although the enzyme O-glycosidase from *Streptococcus pneumoniae* does release O-linked oligosaccharides[17], it is hampered by the fact that it is only effective after the removal of sialic acid. This enzyme has narrow substrate specificity and so is of limited use in characterising intact glycoproteins or glycopeptides[16].

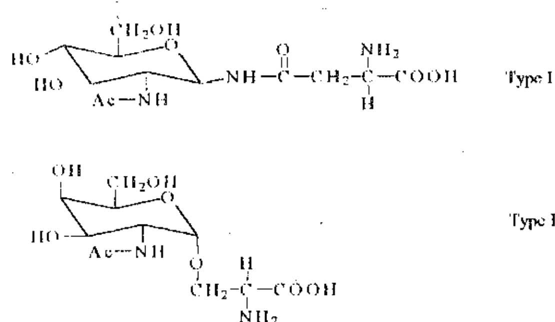
3. Hydrazinolysis : In 1952, Akabori *et al*[18] found that when proteins were heated with anhydrous hydrazine, only the carboxyl-terminal amino acids were liberated as free amino acids, all the other residues being converted into amino acid hydrazides as shown[19] (Scheme 4). When Yosizawa and Sato[20] utilised the method in an attempt to release the oligosaccharides from glycoproteins, after the hydrazinolysis, they found a complete loss of sialic acid, with partial loss of 2-amino-2-deoxy-D-hexoses, D-galactose and L-fucose. Later in their studies[21] they found that treatment of $\alpha_1$-acid glycoprotein with hydrazine in the presence of hydrazinium sulphate at an elevated temperature cleaved the protein-carbohydrate linkage and the recovery of carbohydrate material was almost quantitative.



**Scheme 4.** Hydrazinolysis of protein

Hydrazinolysis was studied by Matsushima and Fujii[22] and later developed by Bayard and Montreuil[23] specifically to cleave the GlcNAc→Asn linkage and N-acetylglucosaminyl linkages. The use of hydrazine to release N- and O-linked oligosaccharides from glycoproteins has now been investigated using several standard glycoproteins of which the glycosylation is known[24]. The release of O-linked oligosaccharides occurs with a lower temperature-dependence than the release of N-linked oligosaccharides. The generality of the hydrazinolysis reaction and the mechanism by which it releases O-linked oligosaccharides are under investigation. Preliminary experiments indicate the applicability of the reaction to the release of intact O-linked oligosaccharides from mucin[6]. Anhydrous hydrazine can therefore be used to release selectively O-linked oligosaccharides or to release both N- and O-linked oligosaccharides. Excessively vigorous conditions can cause degradation during the hydrazinolysis of O-glycans from glycoprotein.

Montreuil[25], in 1975, reported that O-linked oligosaccharides of glycoproteins were not released by hydrazinolysis, and Amerongen *et al* [26] used hydrazinolysis to isolate the N-linked oligosaccharides from mouse submandibular mucin which also contained O-linked oligosaccharides. However, the lability of O-galactosylserine linkages in extensin to hydrazinolysis was reported in 1973 by Lamport *et al* [27]. Use of a mixture of hydrazine and hydrazine sulfate as suggested by Lehninger[28] is no better than using plain anhydrous hydrazine. The work of Patel

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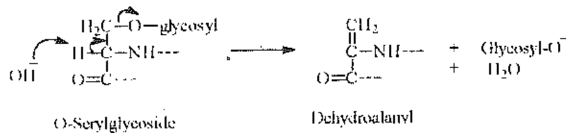


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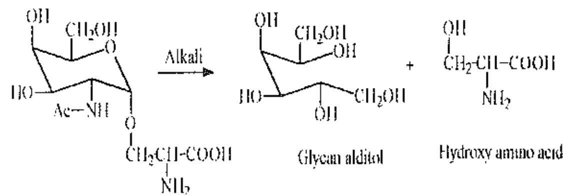


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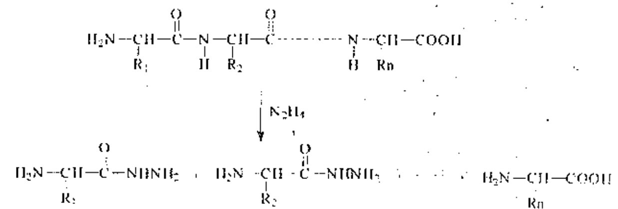

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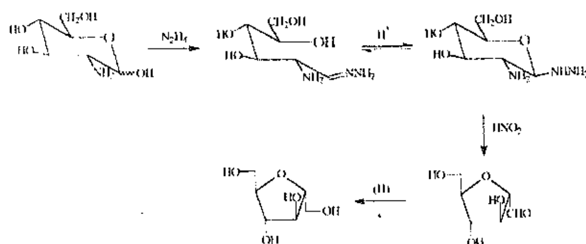
and co-workers[24] was an important development. Their results indicated that hydrazinolysis, when performed under controlled and optimised conditions, can be used to release intact unreduced N- and O-linked oligosaccharides in high yield.

## NITROSATION OF OLIGOSACCHARIDES

Many investigators[29,30] used nitrosation of the hydrazinolysis products to achieve regiospecific degradation which formed smaller oligosaccharides that were easier to characterise. When the N-glycosidic linkage between 2-acetamido-2-deoxy-D-glucose and asparagine is cleaved, an N-deacetylated oligosaccharide terminating with an acyclic 2-amino-2-deoxy-D-glucose hydrazone is obtained. This tautomerizes into a (-D-glucopyranosylhydrazine containing oligosaccharide in a weakly acidic solution[30]. Sequential nitrosation and reduction then gave 2,5-anhydro-D-mannose[29,30] as seen in Scheme 5.



**Scheme 5.** Hydrazinolysis of the glycosidic linkage between 2-acetamido-2-deoxy-D-glucose and asparagine

## HYDRAZINOLYSIS CONDITIONS

Two sets of conditions had been used for such hydrazinolysis until Patel *et al* [24] described milder conditions: A. the hydrazinium sulphate-catalysed reaction[21] as modified by Kochetkov and his co-workers[31], which involves heating at 105°C for 10 h. B. the uncatalysed reaction used by Bayard and Montreuil[23], which involves heating at 100°C for 30 h.

Tang and Williams[29] found that 8-12 h at 100°C is sufficient to effect the complete release of the oligosaccharides in glycoproteins and glycopeptides. In the recent detailed study of Parekh *et al* [24] it was found that anhydrous hydrazinolysis can be used to selectively release O-linked oligosaccharides at 60°C for 5 h or release both N- and O-linked oligosaccharides at 95°C for 4 h in high yield from all glycoproteins investigated. During the hydrazinolysis of N-glycans from glycoprotein, the formation of hydrazide is a possible intermediate in the reaction[32]. Participation by the hydrazide nitrogen atom is then possible as shown in Figure 1.

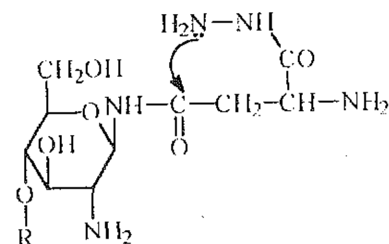



**Figure 1.** Participation by the hydrazide

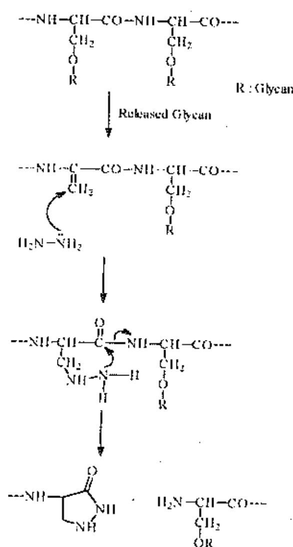
## THE DEGRADATION PROBLEM

Hydrazinolysis is a reaction that has been used successfully to cleave the asparagine bound oligosaccharides, but Tang and Williams[29,32] found degradation in the hydrazinolysis of GlcAc-Asn under excessively vigorous conditions. Saeed and Williams[30] established that the major products of the hydrazinolysis of 2-acetamido-1-N-acetyl-2-deoxy-β-D-glucopyranosylamine, under Kochetkov conditions, was 2-amino-2-deoxy-D-glucose hydrazone. This requires hydrolysis but glycosyl hydrazines are resistant to mild acid hydrolysis, and vigorous hydrolysis would cleave the glycosidic linkages. Mild hydrolysis is possible after N-acetylation; also some investigators have used copper salts as catalysts[21,24]. Vigorous conditions were used to optimise the de-N-acetylation as well as the cleavage of the GlcNAc-Asn linkage. Carter and Williams[33] pointed out that the degradation of the released O-glycans was due to the fact that the reducing GalNAc end group was substituted at po-

sition 3 and was most suspectible to the "peeling reaction", which is analogous to the elimination reaction which released the oligosaccharide from serine (or threonine). Carter and Williams[33] found that analyses of the oligosaccharides released from pig gastric mucus glycopeptides by hydrazinolysis showed that degradation had occurred. They also pointed out that the hydrazinolysis method has two potential advantages, namely (a) polypeptides are degraded to amino acid hydrazides, and (b) N-deacetylation of N-acetylhexosamine and N-acetylneuraminic acid residues will give a product amenable to degradation by nitrosation. Their results implied that the O-linkages in mucus glycopeptides were cleaved during hydrazinolysis by a β-elimination, and that the resulting oligosaccharides are degraded from the reducing hydrazone end-group.

## POSSIBLE REACTION MECHANISM OF HYDRAZINOLYSIS OF O-LINKED OLIGOSACCHARIDES

The generality of the hydrazinolysis reaction and the mechanism by which it releases O-linked oligosaccharides are still under investigation. During the hydrazinolysis of an oligosaccharide chain a β-elimination reaction possibly takes place (Scheme 6).




**Scheme 6.** Possible reaction scheme for the hydrazinolysis of a highly O-glycosylated glycoprotein

When the glycan is released from the glycoprotein by β-elimination, a dehydroalanine moiety would form. After the addition of hydrazine to the alkene, pyrazolidinone formation may result from the attack of the nitrogen on the carbonyl. This would cleave the peptide chain and form an N-terminal serine (or threonine) residue. If the different oligosaccharides are released at slightly different rates, an N-terminal glycosylated serine (or threonine) residue would be formed. Because of the absence of the activating N-acyl substituent this would be expected to undergo elimination less readily[34]

## THE LOW YIELD PROBLEM

As discussed earlier, hydrazinolysis is a convenient reaction for the isolation of the asparagine-bound carbohydrates of glycoproteins[5]. However hydrazinolysis of highly glycosylated glycoproteins and those that contain regions with many adjacent serine and/or threonine residues causes degradation of the oligosaccharides. Using lower temperatures such degradation can be avoided but a low yield may result. If pyrazolidinone formation is possible (Scheme 6) during the hydrazinolysis, the β-elimination might less efficient. Since many of the linkages within the mucin-type sugar chains are 1→3, a type that is very sensitive to the alkaline peeling reaction, they might be degraded by the effect of moisture, once they are released from the polypeptide core as oligosaccharides. Sugar chains released from asparagine may resist this mild peeling reaction because the N,N'-diacetylchitobiose group, which is located at their reducing termini, is relatively alkali stable. In any event, care must be taken in the presence of mucin-type sugar chains, especially with those of large size, such as blood group substances[5].

## CONCLUSION

Hydrazinolysis of glycoproteins and glycopeptides has become a popular method for isolation of asparagine-linked oligosaccharides. A number of investigators[30,35] have used the procedure for identification of oligosaccharide structures from a wide variety of glycoproteins or glycoprotein mix-

tures[36,37]. O-linked oligosaccharides of glycoproteins have received relatively little attention compared to N-glycosides, despite the fact that they are involved in some important biological functions such as stabilisation of the extended form of polypeptide chains of mucin-like glycoproteins, protection of polypeptide from proteolytic enzymes, and involvement in cell/cell and cell matrix interaction of normal and tumour cells[38,39]. Since some of these functions are ultimately related to the oligosaccharide structures, and the O-glycoside structures are usually quite heterogeneous, it is important to have an efficient method for structure characterization[38] . To solve the low yield problem and identify the intermediates the cleavage process and possible side reactions which could interfere and reduce the yield of released oligosaccharide, hydrazinolyzis of serine-bound or threonine-bound carbohydrate in glycoproteins has to be estabilished. This includes the synthesis and behaviour towards hydrazinolysis of dehydroalanine derivatives. A recent study[7] has shown a clear difference in reactivity between the dehydroalanine ester and amides. The results imply that dehydroalanine moieties formed during the hydrazinolysis of glycoproteins or glycopeptides are likely to undergo conjugate addition of hydrazine, but subsequent cyclisation to form pyrazolidinone derivatives with contaminant cleavage of the peptide chain is unlikely to release O-linked oligosaccharides.

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