INVESTIGATION OF OLIGOSACCHARIDE STRUCTURES IN HUMAN SERUM α1-ACID GLYCOPROTEIN

Ph.D. Theses

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1. Introduction, objectives

Natural glycoconjugates (glycolipids and glycoproteins) play an important role in physiological processes like metabolism, transport, communication between cells and immunological symptoms. Oligosaccharides and their derivatives play an important role in the structure and biological function of glycoconjugates. Nowadays investigation of the structure of carbohydrates, namely glycoproteins microheterogeneity is a very important field of current research (proteomics, glycobiology), focusing on the relationship between structure and function. Bioanalytical (chromatographic) and structure exploratory techniques (mass spectrometry, NMR, CD) are frequently used in these examinations.

Acute phase proteins are mainly glycoprotein components of the human serum. Their concentration can increase in pathophysiological processes like injuries, changes in hormone system and tumorous diseases. The human serum α1-acid glycoprotein (AGP) contains glycan chains of branching (antennary) structure connecting to five definite positions of the polypeptide backbone. The aim of this study was to investigate differences in the oligosaccharide structure of human serum AGP in cancer diseases (ovary tumorous and lymphoma) compared to healthy individuals by using modern analytical methods.

The goal of my study was to develop a biocompatible sample preparation procedure for the extraction of AGP from human serum. Care was taken to yield samples of high purity and minimize degradation and desialyzation for analytical and mass spectrometric measurements.

Sialic acid content of human serum AGP samples was to be determined. The aim of the analysis was to detect changes in the relative proportion of sialic acid in diseased samples.

For the study of AGP-oligosaccharides, they were released with enzymatic hydrolysis. Proportion of antennary oligosaccharides was to be determined from healthy, ovary tumorous and lymphoma samples by liquid chromatographic analysis.

Exploration of AGP glycan structures was to be accomplished by preparing individual oligosaccharide samples for MALDI-TOF mass spectrometry. The aim was
to identify characteristic oligosaccharide structures and reveal significant differences between sera of healthy individuals and tumourous patients.

To investigate glycosylation sites on the polypeptide chain triptic digestion of AGP samples was performed. Liquid chromatographic and mass spectrometric measurements aimed at the exploration of oligosaccharide structures on each glycosylation site.

2. Methods

Preparation of AGP from human serum was accomplished using liquid/liquid extraction and precipitation with ethanol of the watery phase. Purification of the glycoprotein was made by ion-exchange, dye-ligand and gel chromatographic methods. The micropreparative processes were done by an FPLC liquid chromatograph apparatus (Pump-500, LCC-501 Plus Controller, Single path monitor UV-1, Pharmacia), and by Jasco HPLC system (PU-980 pump, LG-980-02 gradient mixer) with HP 1084B UV-detector. Identifying and purity testing of AGP samples was carried out with SDS-PAGE gel electrophoresis (Protean II™, Bio-Rad). The quality of the samples was also checked by MALDI-TOF mass spectrometry. The AGP content of the purified samples was measured by UV spectrophotometry (Hitachi U-2000).

Determination of sialic acid content in AGP samples was accomplished with their acidic hydrolysis and derivative formation, followed by reversed phase (RP-)HPLC analysis and fluorimetric detection (Merck-Hitachi HPLC apparatus: L-6200 Pump, AS-2000A Autosampler, D-2500 Chromato-Inegrator, and Jasco FP-1520 fluorescence detector).

To produce oligosaccharides AGP samples were digested with protein N-glycosidase F (PNGase F) enzyme. Elimination of the peptide was achieved by precipitation of the hydrolysate with ethanol.

Normal phase (NP-)HPLC analysis of the oligosaccharide samples was carried out following formation of fluorescent derivatives. Data was analysed statistically by the non-parametric Kruskal-Wallis test and Dunn’s post hoc test (GraphPad InStat 3.06 software).
Anthranilic derivatives of AGP-oligosaccharides were produced for mass spectrometric purposes. The samples were purified with gel chromatography from the excess of the derivatization agent and other impurities.

Hydrolysis of AGP peptide chain was carried out with trypsin. The hydrolysate was purified and concentrated with ultrafiltering and solid phase extraction. The peptide-glycopeptide mixture was fractionated by RP-HPLC method (ISCO 2350 pump, 2360 gradient mixer, V4 UV-detector).

Mass spectrometric experiments were performed by co-workers of the Department of Mass Spectrometry in Chemical Research Center, Hungarian Academy of Sciences, Budapest.

3. Theses

1. A selective, non-destructive sample preparation method was developed for the extraction of AGP from human serum. This method included non-miscible solvent extraction, precipitation with ethanol, anion-exchange, dye-ligand affinity and size exclusion chromatography. The resulting AGP samples were free from any impurities and suitable for HPLC and mass spectrometric measurements.

2. Sialic acid content of AGP samples from healthy and diseased subjects was determined. Results show that the amount of sialic acid was 20 (p<0.05) and 35 w/w % (p<0.01) higher in ovary tumorous and lymphoma samples compared to healthy individuals, respectively. This indicates a higher ratio of tri- and/or tetraantennary structures containing more sialic acid, in the diseased samples.

3. For the investigation of oligosaccharides PNGase F digestion of AGP samples was carried out, followed by sample preparation involving extraction of the glycans for further analytical and mass spectrometric examinations.
4. Anthranilic acid derivatives of enzymatically cleaved AGP glycans were separated by NP-HPLC. Bi-, tri- and tetraantennary oligosaccharides were detected. Based on relative peak areas a difference in the relative ratio of the four sialic acid containing tetraantennary fraction compared to other fractions was observed in diseased samples. This ratio was 35 % (p<0.01), and 45 % (p<0.001) higher in ovary tumorous and lymphoma compared to control samples, respectively. The components of oligosaccharide fractions were identified by injection of oligosaccharide standards and MALDI-TOF mass spectrometry.

5. A high number of individual oligosaccharide samples were produced for MALDI-TOF mass spectrometric investigations from serum AGP fractions of ovary tumorous, lymphoma and healthy subjects. Glycans were released by PNGase F digestion and the sensitivity of mass spectrometric tests was enhanced by derivatization with anthranilic acid. Bi-, tri- and tetraantennary oligosaccharides were detected in the measurements. A difference was found in the intensity of tetraantennary and fucose containing glycan structures in ovary carcinoma and lymphoma compared to normal control samples.

6. Tryptic hydrolysis and solid phase extraction purification of human serum AGP was carried out for the purpose of glycosylation site analysis.

7. The mixture of tryptic peptides and glycopeptides was separated by RP-HPLC method. Identification of glycopeptides was carried out by their PNGase F digestion followed by oligosaccharide analysis. Further HPLC-MS investigations of positional analysis of oligosaccharides are based on the previously described methods.

4. Conclusion

This study focused on the investigation of oligosaccharide structure in human serum α1-acid glycoprotein samples from healthy and tumorous subjects using modern analytical methods. Biocompatible extraction and correct sample preparation of AGP from serum is a crucial point in successful structure examinations. Derivatization allowed sensitive detection of oligosaccharides with fluorescence and soft ionization techniques.
Significant differences were found in the glycan structure of serum AGP in malignant diseases, suggesting the possibility of diagnostic application of these methods.

5. Publications

Papers used in this study:


Other papers on the topic:


Conferences:


