Borrower:

LIB. HAS v.11(1997) to present

131.156.159.48

Record 30 of 98

ILL pe Record 5 of 98

CAN YOU SUPPLY ? YES NO COND FUTUREDATE

:ILL: 7929590 :Borrower: JNA :ReqDate: 20040820 :NeedBefore: 20040930
:Status: IN PROCESS 20040823 :RecDate: :RenewalReq:
:OCLC: 13501997 :Source: Clio :DueDate: N/A :NewDueDate:
:Lender: ILU,WYU,*ORU
:CALLNO: *Lender's OCLC LDR: v.11- 1997-
:TITLE: Biomedical chromatography : BMC.
:IMPRINT: London : Heyden & Son, c1986-
:ARTICLE: T. Kremmer et al. "Liquid chromatographic and ..."
:VOL: 18 :NO: 5 :DATE: 2004 :PAGES: 323-329
:VERIFIED: OCLC ISSN: 0269-3879 [FORMAT: Serial]
:PATRON: Ryzhov, Victor
:SHIP TO:
NIU LibrariesAttn: IDS
Dekalb IL USA 60115

:BILL TO: Same

:SHIP VIA: Ariel :MAXCOST: $25IFM :COPYRT COMPLIANCE: CCG
:FAX: (815) 753-2003 ARIEL 131.156.159.48
:E-MAIL: illdept@niu.edu
:BORROWING NOTES: ClioID: Z0026662////
:AFFILIATION: LVIS, ILLINET

OCLC Number: 

NIU LibrariesAttn: IDS
Dekalb IL USA 60115

BIOMEDICAL CHROMATOGRAPHY
Biomed. Chromatogr. **18**: 323–329 (2004)
Published online in Wiley InterScience (www.interscience.wiley.com).
DOI: 10.1002/bmc.324

ORIGINAL RESEARCH

Liquid chromatographic and mass spectrometric analysis of human serum acid alpha-1-glycoprotein

T. Kremmer,[1]* É. Szöllösi,[1] M. Boldizsár,[1] B. Vincze,[1] K. Ludányi,[2] T. Imre,[2] G. Schlosser[2] and K. Vékey[2]

[1]National Institute of Oncology, Department of Biochemistry, PO Box 21, H 1122 Budapest, Hungary
[2]Chemical Research Center of the Hungarian Academy of Sciences, PO Box 17, H 1525 Budapest, Hungary

Received 15 July 2003; accepted 5 August 2003

ABSTRACT: Human serum acid alpha-1-glycoprotein (AGP, orosomucoid) content of healthy individuals and cancer patients was measured, isolated and purified using a protocol of fast and biocompatible sample preparation, ion exchange and dye-ligand affinity chromatographic methods. In comparison to the healthy individuals significantly higher serum AGP levels were found in a wide spectrum of cancer patients, indicating its diagnostic value in the malignant disease. Oligosaccharide content of AGP samples was separated following PNGase F enzyme digestion and analysed by RP-HPLC and MALDI-TOF mass spectrometry. RP-HPLC and MALDI-TOF mass spectrometric analysis of sugar constituents of AGP specimen originated from selected cancer patients with high serum AGP levels indicated the appearance of anomal distribution of bi-, tri- and tetra-antennary oligosaccharide structures compared to the healthy controls. Copyright © 2004 John Wiley & Sons, Ltd.

KEYWORDS: orosomucoid; oligosaccharide; RP-HPLC; MALDI-TOF MS

INTRODUCTION

Carbohydrate-rich complex proteins (e.g. sialoglycoproteins, proteoglycans, mucoproteins, etc.) represent a widely distributed family of natural compounds with particular structural and functional importance (Van Dijk *et al.*, 1995; Dall'Olio, 1996; Hounsell *et al.*, 1997; Taylor, 1998; Drickamer and Taylor, 1998; Rüdiger *et al.*, 2000). Among a vast number of glycoconjugates responsible for several basic cell membrane functions, (e.g. adhesion, communication, recognition, signal transduction, transport, immunological reactions, etc.), the sialoglycoprotein character of highly active biopolymers like antigens, interferons, erythropoietin and the great majority of conventional serological tumor markers (CEA, AFP, TPA, hCG, NSE, CA 125, CA 15-3, CA 72-4, CA 19-9, acute phase proteins, etc.) has been recognized (Hounsell *et al.*, 1997; Wu and Nakamura, 1997; Rüdiger *et al.*, 2000). It has been shown that the carbohydrate content of these glycoconjugates can be formulated by highly structured antennary oligosaccharide chains consisting of a wide variety of neutral sugars, hexosamines (*N*-acetyl glucose and galactose amines) and *N*-acetyl neuraminic (sialic) acids. Recent observations suggest that the exceptional variability in the length and branching of glycan chains, in the proportion and sequence of sugar constituents, as well as in the quantity and quality of terminal (sialyl) groups may constitute the basis of a new biological (glyco)system (sugar code) containing characteristic information on various physiological and/or pathological events (pregnancy, rheumatic arthritis, cancer, etc.; Van Dijk *et al.*, 1995; Dall'Olio, 1996; Hounsell *et al.*, 1997; Rüdiger *et al.*, 2000; Fournier *et al.*, 2000). Among the numerous circulating sialoglycoproteins, acute phase fractions (alpha-1-antitrypsin, haptoglobin, coeruloplasmin, C-reactive protein, IgG, A, M, etc.), and particularly one of the most heavily glycosylated serum acid alpha-1-glycoprotein (orosomucoid, AGP), attracts increasing interest from theoretical and practical aspects. Based on the analytical data concerning the glycan structure of sialoglycoproteins, elaboration and application of more sensitive and specific markers in the laboratory diagnosis and therapy of malignant diseases is proposed.

AGP is a highly soluble and acidic (pI ≈ 2–3) acute-phase protein fraction of human serum with exceptionally high (~45% w/w) carbohydrate content. The average molecular mass of human AGP in the range of 36–43 kDa is not defined accurately (Schmid, 1989; Fournier *et al.*, 2000). Physico-chemical characteristics, pathophysiological role and biological activity of human serum AGP are discussed in recent reviews (Kremer

*Correspondence to: T. Kremmer, National Institute of Oncology, Department of Biochemistry, P.O. Box 21, H 1122 Budapest, Hungary.
E-mail: kremmer@oncol.hu

Abbreviations used: AGP, alpha-1-glycoprotein; DMB, 1,2-diamino-4,4 methylenedioxy-benzene.

Contract/grant sponsor: Education Ministry of Hungary; Contract/grant number: NKFP MediChem 1/047/2001; FKPP 0075/2000; OTKA 030247.
Contract/grant sponsor: Italian-Hungarian Joint Project; Contract/grant number: MTA 707.
Contract/grant sponsor: Center of Excellence project; Contract/grant number: QLK2-CT-2002-90436.

Published online 8 March 2004

et al., 1988; Fournier et al., 2000). The carbohydrate content of AGP consists of five glycan units bound with *N*-glycosidic links to the distinct sites (Asn-15, -38, -54, -75, -85) of the linear peptide chain fixed by two disulfide bridges. Concerning the oligosaccharide moiety, glycan units commonly contain a variable number of di-, tri- and tetra-antennary structures, sialic acid at the end of chains, and fucose (0–3) attached to (1–3, 1–6) *N*-acetyl glucosamines (Fournier, et al., 2000). According to the antennary oligosaccharide structures, 8–10% galactose, 6–8% mannose, 1.0% fucose, 12–16% hexosamine (*N*-acetyl glucosamine) and 12–16% sialic acid (*N*-acetyl neuraminic acid) can be found in the biopolymer (w/w; Eggert and Jones, 1985; Kishino et al., 1995; Fournier et al., 2000). Owing to the molecular variabilities either in the oligosaccharide or in the peptide composition, AGP is heterogeneous (polimorph) and using various separation techniques (affinity, ion-exchange chromatography, isoelectric focusing) several molecule variants can be demonstrated (Moule et al., 1987; Schmid, 1989; Treuheit et al., 1992; Kishino et al., 1997; Elliott et al., 1997; Rüdiger et al., 2000; Duché et al., 2000; Iijima et al., 2000; Sei et al., 2002). Ion exchange chromatographic, colorimetric, turbidimetric, immuno-diffusion, -precipitation, RIA, ELISA etc. methods are extensively applied to the determination of serum AGP level, also demonstrating its applicability as a tumor marker in the laboratory diagnosis of malignant diseases (Harvey et al., 1981; Turner et al., 1985; Ganz et al., 1987; Kremer et al., 1988; Patel et al., 1994; Kremmer et al., 1995). Various combinations of gel and capillary electrophoretic, HPLC and mass spectrometric techniques have been extensively used to investigate the molecular structure of AGP (Küster et al., 1998; Charlwood et al., 2001). Owing to its high sensitivity MALDI is the preferred technique for AGP derived oligosaccharides after enzymatic cleavage of the links between the glycan and polipeptide chains (Harvey, 2001). Tandem mass spectrometry combined with MALDI ionization (post-source decay, PSD) proved to be useful for identifying sugar sequences, or giving informations on branching (Harvey et al., 1999). HPLC-coupled ES ionization is also favourably used to study oligosaccharides originated from AGP (Huang and Riggin, 2000). The widespread application of this technique is restricted by its much lower sensitivity. In order to apply MS analysis to the structural investigation of glycoconjugates sample preparation, gel-, ion-exchange and RP-HPLC techniques including enzymatic (tryptic, PNGase F) digestion and separation of oligosaccharides are frequently used.

Considering the theoretical and practical importance of glycoconjugates in various pathophysiological events the present study was aimed either to develop analytical and preparative methods for the isolation and purification of human serum AGP and its carbohydrate compartments, and/or to reveal the changes induced by cancer in the antennary structures of the glycan units compared with healthy controls.

EXPERIMENTAL

Materials. All reagents and solvents (NaCl, Na_2HPO_4, $CHCl_3$, methanol, ethanol, CH_3CN, H_2O) were of HPLC purity (Suprapur, LiChrosolv, gradient grade, Merck, Darmstadt, Germany). Fractogel EMD TMAE-650 S and Fractogel TSK AF-Blue were purchased from Merck, Darmstadt. Desalting procedures were made on Sephadex G-25 (superfine, Pharmacia, Uppsala). Trifluoroacetic acid from Aldrich Chemical Co. and bis–tris propane from Sigma were obtained. PNGase F (recombinant, glycerol free) was from Roche Diagnostics GmbH (Mannheim). Mass spectrometry was calibrated by oligosaccharide standards (A1, A2, A2F, A3) obtained from Oxford Glyco-Sciences (Abingdon, Oxfordshire, UK) and used without further purification. 2,5-Dihidroxybenzoic (2,5-DHB) and 2-aminobenzoic (anthranilic) acids were purchased from Aldrich Chemical Co. and Merck (Darmstadt), respectively.

Patients and serum sampling. Venous blood was taken without anticoagulants from volunteer healthy individuals (normal controls) as well as from hospitalized cancer patients fasting overnight. After spontaneous clotting at room temperature serum samples were isolated by centrifugation and kept at –20°C until use. Distribution and number of patients according to the type and localization of malignancy verified by imaging techniques, pathology and conventional laboratory markers are summarized in Table 1.

Preparation and analysis of AGP. Human serum AGP content was measured by microanalytical ion-exchange chromatography as described earlier (Kremmer et al., 1995). In the preparative procedure the protein content of water–methanol supernatant obtained by sample preparation with chloroform–methanol solvent extraction was precipitated with 2 vols of cold ethanol, kept at 0–4°C for 30 min and centrifuged (5000 rpm, 20 min, 0°C). Precipitate was dissolved in 25 mM bis–tris–propane (buffer A, pH 7.5) and applied to a Fractogel EMD TMAE-650 S column (Superformance, 15 × 1 cm, i.d.) using a simultaneous pH–NaCl gradient elution with 25 mM bis–tris-propane –350 mM NaCl (buffer B, pH 9.5). Fraction containing AGP was desalted on Sephadex G-25 column (30 × 1 cm) and liophylized. The traces of other serum protein contaminants (mostly albumin) were eliminated on a Fractogel TSK AF-Blue column (Superformance, 7 × 1 cm, i.d.) equilibrated with 10 mM Na-phosphate buffer (pH 5.8). AGP eluted first was desalted and liophylized. The purity of AGP samples was controlled by SDS polyacrylamide gel electrophoresis (Kremmer et al., 1995). Chromatographic procedures were performed with a JASCO PU-980 pump and LG-980-02 gradient unit equipped with a HP 79875A UV–VIS detector and 79850B LC terminal.

HPLC determination of sugar constituents. Sugar constituents of AGP were analysed using a Merck-Hitachi

Table 1. Human serum AGP levels in malignant diseases

Cancer (localization)	Patients (n)	Serum AGP,[a] mg/dl (mean)	Other markers[b] supporting the diagnosis
Ovary and cervix	104	157	CEA, CA-125
Gastrointestinal tract	30	170	CA 19-9, CA 72-4
Colorectal carcinomas	35	140	CEA, AFP, TPA
Melanoma malignum			S-100, 5-SCD
Primer	23	93	
Metastatized	79	142	
Thyroid gland cc (papillary, medullary)	24	134	HTG, anti-HTG Calcitonin
Lung carcinoma	21	136	CA-125, CEA,
Prostata carcinoma	9	111	PSA
Lymphomas (Hodgkin, non-Hodgkin)	130	153	LDH, β_2-μGlob

[a] Normal range in healthy individuals (n = 35): 40–80 mg AGP/dL; mean ± SD = 57.0 ± 15.5 mg/dL.
[b] CEA, carcinoembrionic antigen; CA-125, 19-9, 72-4, cancer antigens; AFP, alpha-fetoprotein; TPA, tissue polypeptide antigen; S-100, Ca-binding serum protein; 5-SCD, 5-*S*-cysteinyl DOPA; (anti) HTG, human (anti) thyreoglobin; PSA, prostate-specific antigen; LDH, Lactate dehydrogenase; β_2-μGlob, beta-2-microglobulin.

LiChrograph System consisting of an L-6200A Intelligent Pump, D-2500 Chromato-Integrator, AS-2000 Autosampler, L-4250 UV–VIS and a Shimadzu RF-530 fluorescence detector. Neutral and amino sugars were measured as anthranilate derivatives according to Anumula and Du (1999) after hydrolysis of samples with 4 M TFA (100°C, 6 h) using Supelcosil C_{18} column (25 × 0.46 cm i.d., 5 μm, flow rate 1 mL/min) at 15°C (thermostated) and isocratic elution with 20 mM *n*-butyl amine–phosphoric acid buffer (pH 1.8) containing 1% THF and 5% acetonitrile (v/v), respectively. Fluorescence detection was at excitation 360 nm and emission 425 nm. Sialic acid content was determined in the form of 1,2-diamino-4,4-methylenedioxy-benzene (DMB) derivatives according to Hara *et al.* (1987) following acid hydrolysis of samples (10–1000 μg sialic acid in 5–50 μL, 200 μL 12 mM H_2SO_4, 80°C, 2 h). Isocratic elution with methanol–acetonitrile–water (25:4:91 v/v) on a Nucleosil C_{18} column (25 × 0.46 cm i.d., 5 μm, flow rate 1.2 mL/min, temperature 25°C, thermostated) and fluorescence detection (excitation 373 nm, emission 448 nm) was used.

Enzymatic release of AGP oligosaccharides. PNGase F digestion of AGP was performed according to Elliott *et al.* (1997) modified as follows: 500–1000 μg of AGP solved in 500 μL 50 mM K_2HPO_4–2 mM EDTA buffer (pH 7.5) was treated with 10 μL 5% SDS and 10 μL beta-mercapto ethanol (freshly diluted 1:10 v/v) at 100°C for 5 min. The sample was cooled down for 10 min at –5°C and precipitated SDS was removed by centrifugation (10 min, 5000 *g*). The supernatant was transferred into glass stopped tube then 5 U PNGase F was added and incubated for 24–48 h at 37°C. Oligosaccharides were separated from the digest by 2 vols ice-cold ethanol (30 min, 0–4°C). The precipitate was centrifuged and the supernatant collected. The pellet was washed twice with ethanol and extracts were combined and freeze-dried under vacuum.

Mass spectrometry. Mass spectrometric measurements were carried out in linear mode with a Bruker Biflex™ MALDI-TOF mass spectrometer, and a Micromass MALDI-TOF mass spectrometer used in reflecton mode, both of them equipped with a 337 nm nitrogen laser. Accelerating voltage was 19.5 kV and 100–200 laser shots were applied for each spectrum. Aqueous solution of oligosaccharide samples (1 μl, 20–100 pmol) were mixed on the side-inlet probe with matrix [2,5-DHB dissolved in water–acetonitrile (2:1, v/v)] and allowed to dry in air. Fluorescent labeling of oligosaccharides was made according to the protocol of Anamula and Du (1999) supplemented with desalting of anthranilate derivatives on Sephadex G-25.

RESULTS

Recent developments in glycobiology provided convincing evidences on the structural or functional importance of natural glycoconjugates. In the particular case, recognizing and understanding the clinical applicability of human serum AGP, as well as the relationships between the structural changes in its oligosaccharide structure and cancer may have theoretical and practical importance as well. In this context the careful selection of well-characterized biological (serum) samples and the application of reliable preparative and analytical methods seem to be the primary requirements of these investigations. Earlier, we have worked out a micro-analytical ion-exchange chromatographic technique for the quantitative measurement of human serum AGP (Kremmer *et al.*, 1995), and during recent years a vast number of patients with different forms and localization of cancer was routinely investigated. Cumulative data of these observations are shown in Table 1. Results presented here demonstrate the applicability of serum AGP determination in the malignant diseases. It has to be noted that AGP is an aspecific marker

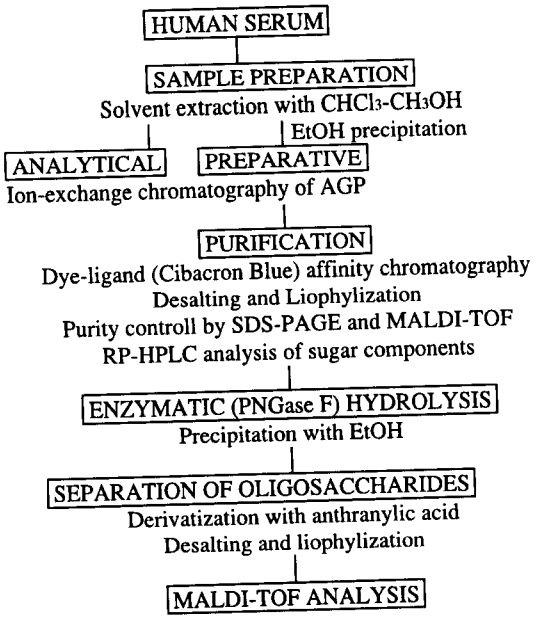


Figure 1. Working scheme for the measuring, isolation and purification of human serum AGP.

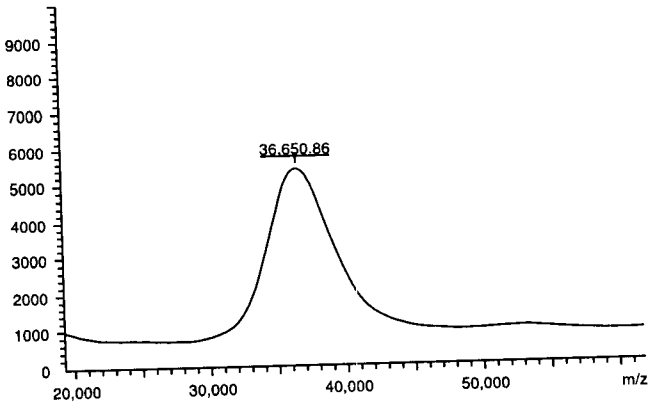


Figure 2. MALDI-TOF mass spectrum of human serum AGP.

Table 2. Sugar constituents of AGP isolated from the sera of healthy individuals and ovary cancer patients (in relative percentage of the total biopolymer)

Sugars	Healthy individuals ($n = 14$)	Cancer patients ($n = 18$)
Galactose	9.2 ± 1.1	8.9 ± 2.0
Mannose	5.1 ± 1.0	6.6 ± 1.5
Fucose	1.0 ± 0.2	1.7 ± 0.3
N-Acetyl glycosamine	13.2 ± 1.9	13.5 ± 2.7
Sialic acid	16.5 ± 1.6	15.6 ± 3.7

and temporary elevation of its serum level can also be found in many acute phase syndromes (inflammation, infections, burning, etc. see Kremer *et al.*, 1988; Fournier *et al.*, 2000). However, in agreement with the changes of other tumor markers and depending on the clinical status significantly higher serum AGP levels could be measured in cancer patients. On the other hand, this screening system gave an opportunity to select the appropriate serum specimen with high AGP levels (above 150 mg/dL) for further investigations. Applying various preparative and analytical techniques a protocol (Fig. 1) was developed for the fast and biocompatible separation, purification and analysis of human serum AGP. The solvent extraction of serum supplemented with the ethanol precipitation is one of the crucial points of sample preparation providing AGP specimen with the less degradation and desialylation. Following the preparative ion exchange chromatography the traces of serum protein contaminants were eliminated by dye-ligand (Cibacron Blue) affinity chromatography. This additional step resulted in AGP of high purity verified by SDS–polyacrylamide gel electrophoresis and MALDI TOF mass spectrometry. In rough agreement with previous estimates (Schmid, 1989; Fournier *et al.*, 2000) the MALDI spectra of AGP (Fig. 2) originated from healthy volunteers as well as from cancer patients showed a mean molecular mass of ca. 37 kDa. The MALDI spectrum also demonstrated that the molecular mass envelope is very wide (extends from ca. 31 to 43 kDa), indicating the considerable molecular heterogeneity of AGP. In accordance with other data (Eggert and Jones, 1985; Hara *et al.*, 1987; Kremer *et al.*, 1988), RP-HPLC analysis of the carbohydrate content of AGP verified the sugar composition in healthy individuals and demonstrated the changes induced by the cancerous state. Analytical data concerning the ovarian carcinoma patients compared to healthy controls shown in Table 2 indicated significant difference ($p < 0.01$) only in the fucose content. These characteristics are in accordance with the previous observations (Moule *et al.*, 1987; Van Dijk *et al.*, 1995; Kremmer *et al.*, 1995; Dall'Olio, 1996; Hounsell *et al.*, 1997; Elliott *et al.*, 1997) and with the results of our MALDI-TOF MS studies described below. In order to investigate the carbohydrate structure of AGP samples glycan units were released by endoglycosidase F hydrolysis and derivatized with anthranilic acid. Representative MALDI-TOF spectrum of the oligosaccharide derivatives originated from a healthy volunteer shown in Fig. 3 demonstrates a large number of individual oligosaccharides. In good agreement with studies published previously (Küster *et al.*, 1998), the most characteristic structures derived from healthy controls are listed in Table 3. Analysis of the MALDI spectra of compounds was performed on the base of relative peak abundances with a reproducibility within 20%. Distribution of oligosaccharide structures derived from healthy volunteers and cancerous patients showed various similarities and differences. In general, biantennary sequences were dominant in all samples (Fig. 4) and

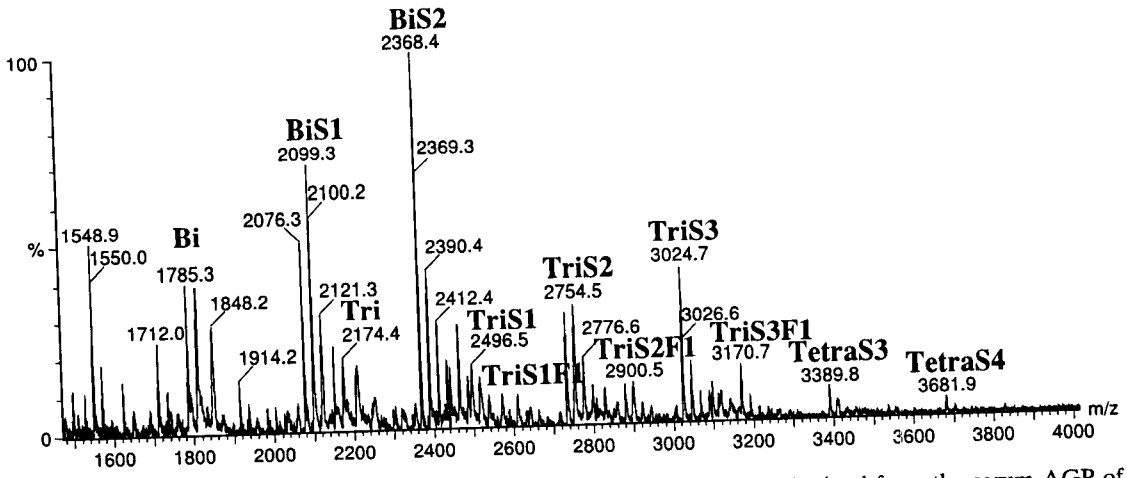
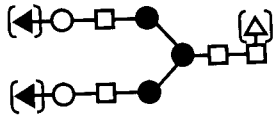
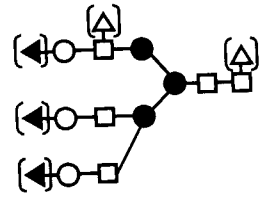
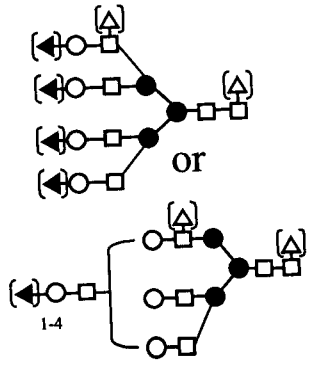


Figure 3. MALDI-TOF spectrum of antennary oligosaccharide structures obtained from the serum AGP of healthy individuals.

Table 3. Identified oligosaccharide structures in human serum AGP

Structure of oligosaccharide	Number of sialic acid	Number of fucose	Structure identifier
Biantennary	0	0	Bi
	1	0	BiS1
	2	0	BiS2
Triantennary	0	0	Tri
	0	1	TriF1
	1	0	TriS1
	1	1	TriS1F1
	2	0	TriS2
	2	1	TriS2F1
	3	0	TriS3
	3	1	TriS3F1
Tetraantennary	0	0	Tetra
	0	1	TetraF1
	1	0	TetraS1
	1	1	TetraS1F1
	2	0	TetraS2
	2	1	TetraS2F1
	3	0	TeraS3
	3	1	TetraS3F1
	3	2	TetraS3F2
	4	0	TetraS4
	4	1	TetraS4F1
	4	2	TetraS4F2

(◀) Sialic acid; (○) galactose; (□) (mannose; (●) *N*-acetyle glucosamine; (Δ) fucose.

the relative proportion of the total amount of bi-, tri- and tetraantennary sequences (1:0.8:0.3) was approximately the same in all specimen. A wide variability of sialylated oligosaccharides containing 0, 1, 2, 3 and 4 sialic acids were also detected. In spite of the predominance of bisialylated compounds differences between the healthy and tumorous MS spectra based on the ratio of all triantennary to all tetraantennary sequences were informative. Abundance of tetraantennary structures was higher in ovarium tumours compared either

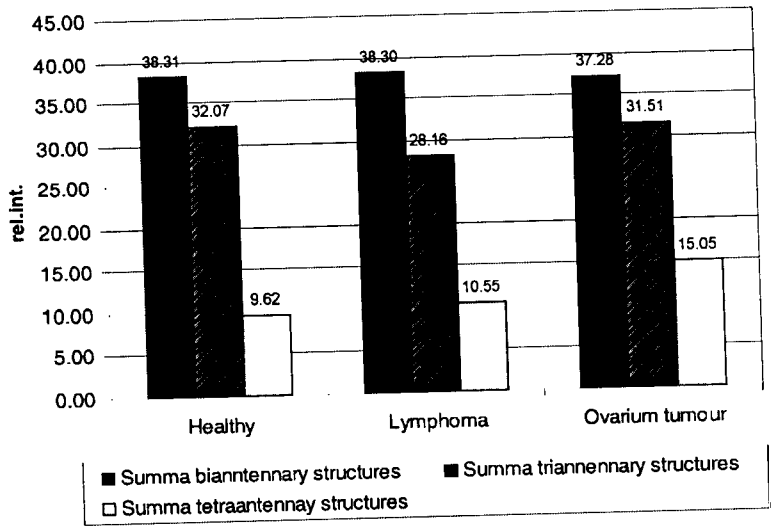


Figure 4. The relative proportion of the total amount of bi-, tri- and tetraantennary sequences.

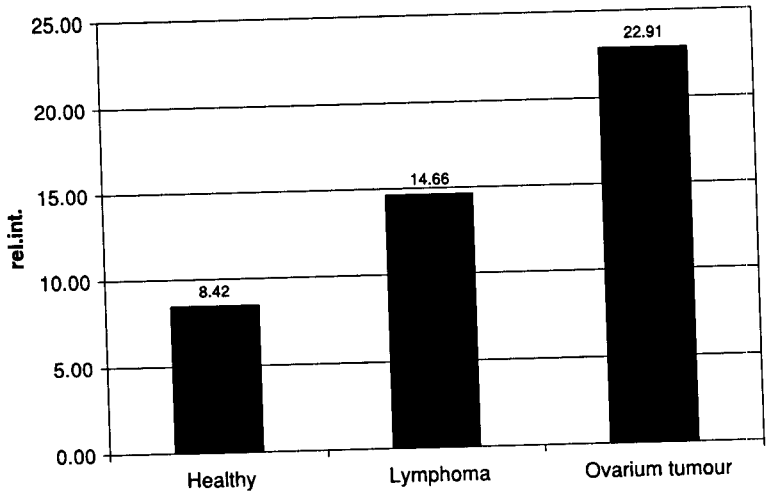


Figure 5. The relative proportion of the total amount of fucose containing structures.

to lymphomas, or healthy controls. Fucose-containing structures (Fig. 5) were 2–3 times higher in tumorous states than in healthy individuals. In conclusion, it can be stated that mass spectrometric observations concerning the changes induced by neoplastic diseases in the oligosaccharide structure of AGP correlate well with the results of RP-HPLC analysis (see Table 2) and with other data indicating the hypersialylation and hyperfucosylation of glycoconjugates in the various forms of cancer (Turner *et al.*, 1985; Patel *et al.*, 1994). The present study was not directed to identify the oligosaccharide composition in the distinct sites (I–V) of native AGP. Further investigations on a statistical evaluation of oligosaccharide composition derived from MALDI spectra of AGP in healthy and cancerous subjects and on the positional distribution of glycan chains in cancer are in progress.

Acknowledgements

Authors are grateful to Professor László Szepesy for the consultations and revising of the manuscript. The work was supported by the Education Ministry of Hungary NKFP MediChem 1/047/2001, FKFP 0075/2000, OTKA 030247 grants, Italian-Hungarian Joint Project (MTA 707) and Center of Excellence project (CBCH, no. QLK2-CT-2002-90436). We would like to express our sincere thanks to Gabriella Pócsfalvi and Professor Antonio Malorni for obtaining selected MALDI spectra of oligosaccharides.

REFERENCES

Anumula KR and Du P. Characterization of carbohydrates using highly fluorescent 2-aminobenzoic acid tag following gel electrophoresis of glycoproteins. *Analytical Biochemistry* 1999; **275**: 236.

Charlwood J, Bryant D, Skehel JM and Camilleri P. Analysis of *N*-linked oligosaccharides: progress towards the characterization of glycoprotein-linked carbohydrates. *Biomolecular Engineering* 2001; **18**: 229.

Dall'Olio E. Protein glycosylation in cancer biology: an overview. *Journal of Clinical Pathology and Molecular Pathology* 1996; **49M**: 126.

Drickamer K and Taylor M. Evolving views of protein glycosylation. *TIBS* 1998; **23**: 321.

Duché J-C, Urien S, Simon N, Malaurie E, Monnet I and Barré J. Expression of the genetic variants of human alpha-1-acid glycoprotein in cancer. *Clinical Biochemistry* 2000; **33**: 197.

Eggert FM and Jones M. Measurement of neutral sugars in glycoproteins as dansyl derivatives by automated high-performance liquid chromatography. *Journal of Chromatography* 1985; **333**: 123.

Elliott MA, Elliott HG, Gallagher K, McGuire J, Field M and Smith KD. Investigation into the Concanavalin A reactivity, fucosylation and oligosaccharide microheterogeneity of α_1-acid glycoprotein expressed in the sera of patients with rheumatoid arthritis. *Journal of Chromatography B* 1997; **688**: 229.

Fournier T, Medjoubi-N N and Porquet, D. Alpha-1-acid glycoprotein. *Biochemica Biophysica Acta* 2000; **1482**: 157.

Ganz PA, Ma PY, Wong HJ, Elashoff RM. Evaluation of three biochemical markers for serially monitoring the therapy of small-cell lung cancer. *Journal of Clinical Oncology* 1987; **5**: 472.

Hara S, Takemori Y, Yamaguchi M, Nakamura M and Ohkura Y. Fluorimetric high performance liquid chromatography of *N*-acetyl- and *N*-glycosil-neuraminic acids and its application to their microdetermination in human and animal sera, glycoproteins and glycolipids. *Analytical Biochemistry* 1987; **164**: 138.

Harvey DJ. Identification of protein-bound carbohydrates by mass spectrometry. *Proteomics* 2001; **1**: 311.

Harvey DJ, Hunter AP and Bateman RH. Matrix-assisted laser desorption/ionization mass spectrometry of carbohydrates. *Mass Spectrometry Reviews* 1999; **18**: 349.

Harvey HA, Lipton A, White D and Davidson E. Glycoproteins and human cancer. II. Correlation between circulating level and disease status. *Cancer* 1981; **47**: 324.

Hounsell EF, Young M and Davies MJ. Glycoprotein changes in tumours: a renaissance in clinical applications. *Clinical Science* 1997; **93**: 287.

Huang L and Riggin RM. Analysis of nonderivatized neutral and sialylated oligosaccharides by electrospray mass spectrometry. *Analytical Chemistry* 2000; **72**: 3539.

Iijima S, Shiba K, Kimura M, Nagai K and Iwai T. Changes of α_1-acid glycoprotein microheterogeneity in acute inflammation stages analyzed by isoelectric focusing using serum obtained postoperatively. *Electrophoresis* 2000; **21**: 753.

Kishino S, Nomura A, Sugawara M, Iseki K, Kakinoki S, Kitabatake A and Miyazaki K. Purification method for α-1-acid glycoprotein with subsequent high-performance liquid chromatographic determination of monosaccharides in plasma of healthy subjects and patients with renal insufficiency. *Journal of Chromatography B* 1995; **672**: 199.

Kishino S, Nomura A, Saitoh M, Sugawara M, Iseki K, Kitabatake A and Miyazaki K. Single-step isolation method for six glycoforms of human α_1-acid glycoprotein by hydroxylapatite chromatography and study of their binding capacities for disopyramide. *Journal of Chromatography B* 1997; **703**: 1.

Kremer JMH, Wilting J and Janssen LMH. Drug binding to human alpha-1-acid glycoprotein in health and disease. *Pharmacology Review* 1988; **40**: 1.

Kremmer T, Boldizsár M, Kovács J, Paulik E, Bencsik K and Szajáni B. Determination and analysis of human serum alpha-1-acid glycoprotein by liquid chromatographic methods. *Journal of Liquid Chromatograoly* 1995; **18**: 1207.

Küster B, Hunter AP, Wheeler SF, Dwek RA and Harvey DJ. Structural determination of N-lnked carbohydrates by matrix-assisted laser desorption/ionization-mass spectrometry following enzymatic release within sodium dodecyl sulphate-polyacrylamide electrophoresis gels: Application to species-specific glycosylation of α_1-acid glycoprotein. *Electrophoresis* 1998; **19**: 1850.

Moule SK, Peak M, Thompson S and Turner GA. Studies of the sialylation and microheterogeneity of human serum α_1-acid glycoprotein in health and disease. *Clinica Chimica Acta* 1987; **166**: 177.

Patel PS, Adhvaryu SB, Balar DB, Parikh BJ and Shah PM. Clinical application of serum levels of sialic acid, fucose and seromucoid fraction a tumor markers in human leukemias. *Anticancer Research* 1994; **14**: 747.

Rüdiger H, Siebert H-C, Solis D, Jiménez-Barbero J, Romero A, von der Lieth CW, Diaz-Maurino T and Gabius H-J. Medicinal chemistry based on the sugar code: fundamentals of lectinology and experimental strategies with lectins as targets. *Current Medical Chemistry* 2000; **7**: 389.

Schmid K. Human plasma alpha1-acid glycoprotein—biochemical properties, the amino acid sequence and the structure of the carbohydrate moiety, variants and polymorphism. In Baumann P, Müller WE, Eap CB and Tillement JP (eds) *Alphal-acid Glycoprotein. Genetics, Biochemistry, Physiological Functions, and Pharmacology*. Liss: New York, 1989; 7.

Sei K, Nakano M, Kinoshita M, Masuko T and Kakehi K. Collection of α_1-acid glycoprotein molecular species by capillary electrophoresis and the analysis of their molecular masses and carbohydrate chains. Basic studies on the analysis of glycoprotein glycoforms. *Journal of Chromatography A* 2002; **958**: 273.

Taylor CM. Glycopeptides and glycoproteins: focus on the glycosidic linkage. *Tetrahedron* 1998; **54**: 11317.

Treuheit MJ, Costello CE and Halsall HB. Analysis of the five glycosylation sites of human α_1-acid glycoprotein. *Biochemical Journal* 1992; **283**: 105.

Turner GA, Skillen AW, Buamah P, Guthrie D, Welsh J, Harrison J, Kowalski A. Relation between raised concentrations of fucose, sialic acid and acute phase proteins in serum from patients with cancer: choosing suitable serum glycoprotein markers. *Journal of Clinical Pathology* 1985; **38**: 588.

Van Dijk W, Havenaar EC and Brinkman-Van der Linden ECM. α_1-Acid glycoprotein (orosomucoid): pathophysiological changes in glycosylation in relation to its function. *Glycoconjugate Journal* 1995; **12**: 227.

Wu JT and Nakamura RM. *Human Circulating Tumor Markers. Current Concepts and Clinical Applications*. American Society of Clinical Pathologists Press: Chicago, IL, 1997.