How the sialylation level of serum N-acetyl-β-D-glucosaminidase A form in Type 1 diabetes mellitus influences their activity?

VESNA B. JOVANOVIĆ1#, JELENA M. AČIMOVIĆ1#, VESNA S. DIMITRIJEVIĆ2, SREČKOVIĆ2 and LJUBA M. MANDIĆ1*#

1Faculty of Chemistry, Chair of Biochemistry, University of Belgrade, Studentski trg 12–16, Belgrade 11158, Serbia and 2Institute of Endocrinology, Diabetes and Metabolic Diseases, Clinical Center of Serbia, Belgrade 11000, Dr Subotića 13, Serbia

Abstract: It was verified that the serum N-acetyl-β-D-glucosaminidase (NAG) activity is elevated in diabetes, but there are no reports about changes in the sialic acid (SA) content in the carbohydrate parts of the NAG A form and its influence on the total changes in NAG activity in type 1 diabetes mellitus patients with and without secondary complications. The NAG A form was isolated from the serum of 81 insulin-dependent diabetes mellitus (IDDM) patients with and without secondary complications (retinopathy, polyneuropathy and nephropathy) and 25 healthy persons, and purified and characterised. The content of α-2,6-bound SA, the isoenzyme patterns of the purified A form, and the total NAG and A form activities were determined. In all diabetic groups, the sialylation levels of the A form were 2–3.5 times lower compared to control, while their acidities (fractions with pI 4.25–5.1) increased, particularly with progression of secondary complications. Total serum NAG activities and percentages of A form were significantly higher (P < 0.001) in all diabetic groups and negatively correlated with the α-2,6-bound SA content of the A form. In addition, they decreased as secondary diabetic complications became more complex. The observed changes could be the consequence of structural changes in the A form due to significant increases in its acidity, i.e., negative charge, which originated from groups other than SA.

Keywords: N-acetyl-β-D-glucosaminidase; A isoenzyme isolation and characterisation; sialylation level, diabetes mellitus type 1; secondary complications.

INTRODUCTION

The serum N-acetyl-β-D-glucosaminidase (NAG, EC 3.2.1.52) activity and sialic acid (SA) are elevated in individuals diagnosed with diabetes mellitus1–3

*Corresponding author. E-mail: ljmandic@chem.bg.ac.rs
# Serbian Chemical Society member.
doi: 10.2298/JSC140430076J

1491
NAG is a lysosomal enzyme that is involved in the degradation of the oligosaccharide chains of glycoproteins, glycolipids and glycosaminoglycans. All serum NAG isoenzymes are glycoproteins that can be separated into two major isoenzymes, A and B, and several minor isoenzymes (I, P and S). They derive from different combinations of two polypeptide chains, \( \alpha \) and \( \beta \)-subunits, with additional heterogeneity conferred by posttranslational modifications. Among them carbohydrate part of NAG A contains the highest level of SA, which significantly contributes to the overall negative charge of its molecules. Therefore, NAG A has the lowest isoelectric point (its \( pI \) is close to 5).\(^7\) In addition, SA may contribute to the stability and survival of glycoproteins (e.g., NAG) in blood circulation.\(^8\) Many reasons for the increase in serum NAG activity in diabetics have been proposed, such as poor glycemic control;\(^9\) the glycomaterial deposits on blood vessels; long-term disruption of metabolic equilibrium in diabetics with an alleviated release of lysosomal enzymes, especially the A form,\(^10\) into the extracellular liquid, which interferes with the mechanisms controlling the half-life of enzymes.\(^11\) However, none of these explanations have been fully elucidated.

Increased NAG exocytosis, i.e., the redistribution of the percentage of secreted enzyme compared to the percentage transported to lysosome, as well as changes in the activity and half-life of serum NAG could result from posttranslational modifications of enzymes (changes in the SA content in their carbohydrate components) in patients with hyperglycaemia. To the best of our knowledge, there are no reports that examine the changes in the carbohydrate components of NAG isoenzymes in diabetic patients with various secondary complications. To gain insight into these changes, the NAG A form from the serum of patients with insulin-dependent diabetes mellitus (IDDM) with and without various secondary complications was isolated and characterized, and the SA in the carbohydrate components of the A forms was analyzed and their correlation with changes in the total NAG activity examined. In this study, for the first time, the reduced sialylation levels of the A form from patients with IDDM is reported. However, the acidity of the A form (negative charge) increased as the diabetic complications progressed. These findings provide a deeper understanding of the changes in NAG activity in diabetics compared to healthy individuals and between groups of patients with IDDM.

**EXPERIMENTAL**

**Subjects**

Eighty-one patients with IDDM were grouped according to the following secondary complications:\(^10\) without complications (W.Compl., \( n = 24 \)); with retinopathy (R, \( n = 19 \)); with retinopathy and polyneuropathy (R+P, \( n = 18 \)); and with retinopathy, polyneuropathy and nephropathy (R+P+N, \( n = 20 \)). The characteristics of these patients are listed in Table I. The
control group consisted of 25 healthy volunteers of appropriate ages and sex. This study was approved by the institutional ethics committee on human research.

TABLE I. Clinical and biochemical characteristics of the control group and groups of patients with IDDM; W.Compl. – without complications, R – with retinopathy, R+P – with retinopathy and polyneuropathy, R+P+N – with retinopathy, polyneuropathy and nephropathy. The values are expressed as the mean ± standard deviation (SD). \( aP < 0.001, bP < 0.01, cP < 0.05 \) compared with the control group. \( dP < 0.001, eP < 0.05 \) compared with W.Compl. \( fP < 0.001, gP < 0.01, hP < 0.05 \) compared with R. \( iP < 0.001, jP < 0.01, kP < 0.05 \) compared with R+P.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Control group</th>
<th>W.Compl.</th>
<th>R</th>
<th>R+P</th>
<th>R+P+N</th>
</tr>
</thead>
<tbody>
<tr>
<td>( n ) (M/F)</td>
<td>23 (11/14)</td>
<td>24 (12/12)</td>
<td>19 (7/12)</td>
<td>18 (11/7)</td>
<td>20 (9/11)</td>
</tr>
<tr>
<td>Age, y</td>
<td>30.2±8.7</td>
<td>24.6±7.4</td>
<td>26.2±2.2</td>
<td>33.8±14.8</td>
<td>31.2±10.1</td>
</tr>
<tr>
<td>Diabetes duration, y</td>
<td>6.6±5.5</td>
<td>14.8±5.5d</td>
<td>15.1±11d</td>
<td>18.3±9.4d</td>
<td></td>
</tr>
<tr>
<td>( SBP/\text{mmHg} )</td>
<td>109±9</td>
<td>115±8c</td>
<td>119±5e</td>
<td>122±7h,e</td>
<td>132±9a,d,g</td>
</tr>
<tr>
<td>( DBP/\text{mmHg} )</td>
<td>83±6</td>
<td>77±6</td>
<td>83±5</td>
<td>80±7</td>
<td>88±6</td>
</tr>
<tr>
<td>Glucose concentra- ( \text{tion, mmol L}^{-1} )</td>
<td>4.8±0.5</td>
<td>11.5±2.9a</td>
<td>14.3±2.6a,c,j</td>
<td>9.0±2.9a</td>
<td>12.9±3.1a,k</td>
</tr>
<tr>
<td>Albuminuria, mg per 24 h</td>
<td>4.9±0.5</td>
<td>9.3±0.8a</td>
<td>11.1±0.9a</td>
<td>10.1±1.4a</td>
<td>10.1±0.5a</td>
</tr>
<tr>
<td>NAG activity, IU L(^{-1} )</td>
<td>3.19±0.5a</td>
<td>4.54±1.15a</td>
<td>5.72±1.06e,e</td>
<td>5.44±0.92e</td>
<td>5.08±2.05a</td>
</tr>
<tr>
<td>A form, %</td>
<td>69.38±4.98</td>
<td>83.82±5.82a</td>
<td>84.48±5.2a</td>
<td>81.9±5.24a</td>
<td>76.22±7.42a,c,k</td>
</tr>
</tbody>
</table>

Chemicals and instrumentation

All chemicals that were used were of analytical grade and were purchased from Merck (Darmstadt, Germany) and Sigma–Aldrich (Steinheim, Germany). Con A-Sepharose 4B and Sephadex G-100 were obtained from GE Healthcare (Uppsala, Sweden). SNA-HRPO was purchased from MyBioSource (San Diego, CA, USA). Spectrophotometric measurements were performed using a Beckman DU-50 spectrophotometer (Fullerton, USA) and an LKB 5060-066 microplate reader (Uppsala, Sweden).

Serum samples

Blood samples were collected after the patients had fasted overnight between 07:00 pm and 07:30 am.

Biochemical methods

Using 2-methoxy-4-(2-nitrovinyl)phenyl-2-(acetylamine)-2-deoxy-\( \beta \)-D-glucopyranoside, the activity of NAG was determined as previously described. A unit of enzymatic activity (IU) was defined as the amount of enzyme used to hydrolyse 1 \( \mu \)mol of substrate in one minute at 37 °C (IU L\(^{-1} \) = \( \mu \)mol min L\(^{-1} \)). The intra- and inter-assay variations of the measurements of total NAG activity were 1.8 and 3.2 %, respectively.

The fasting serum glucose, glycated haemoglobin (HbA\(_{1c}\)) and microalbuminuria were determined with a Hitachi 912 autoanalyser using commercial kits (Randox, Crumlin, UK). The content of proteins was determined by the Bradford method.

Isoelectrofocusing and native polyacrylamide gel electrophoresis (PAGE) were performed according to the manufacturers’ recommendations using a Multiphor® II electropho-
resis system (Pharmacia BioTech Ltd., Little Chalfont, Buckinghamshire, UK) and a Hoefer® SE 260 electrophoretic unit (San Francisco, CA, USA), respectively. The enzyme activity was determined by dividing the polyacrylamide gels into sections (3 mm × 5 mm) and incubating each section with substrate at 37 °C for 24 h.

**Isolation and purification of the serum NAG A isoenzyme**

Ion-exchange chromatography on DEAE cellulose column was used to separate the NAG isoenzyme forms from fresh serum (4–6 mL) of each person from the control and the IDDM groups. Prior to the chromatography, the serum was dialysed overnight against 0.01 mol L⁻¹ of phosphate buffer, pH 7.0, at 4 °C. The B form was eluted with 0.01 mol L⁻¹ phosphate buffer, pH 7.0. The A form was isolated using a linear concentration gradient (0–0.3 mol L⁻¹) of sodium chloride in the same buffer. Fractions containing 90 % of the total A isoenzyme activity were pooled according to the groups, concentrated by ultrafiltration (PM-30; Amicon) and stored at −20°C.

**Gel chromatography.** All pooled solutions of the isolated A isoenzyme were dialysed overnight at 4 °C against 0.05 mol L⁻¹ phosphate buffer containing 0.15 mol L⁻¹ sodium chloride, pH 7.0 and applied to a Sephadex G-100 column. The enzyme was eluted with the same buffer. Fractions containing 35–50 % of the total eluted A isoenzyme activity were pooled, concentrated by ultrafiltration and dialysed overnight at 4 °C against 20 mmol L⁻¹ of 2-amino-2-(hydroxymethyl)propane-1,3-diol (Tris) buffer, pH 7.0, containing 1 mmol L⁻¹ manganese(II) chloride, 1 mmol L⁻¹ calcium chloride and 0.5 mmol L⁻¹ sodium chloride (wash buffer).

**Affinity chromatography.** The dialysed solution was then applied to a Concanavalin A-Sepharose 4B column equilibrated with wash buffer. The enzyme was eluted with 0.5 mol L⁻¹ α-methyl-D-mannoside dissolved in wash buffer. The two fractions with the highest enzyme activity were pooled and stored at −20 °C for electrophoresis, isoelectrofocusing and enzyme-linked lectin binding assays.

**Enzyme-linked lectin binding assays (ELBAs)**

The ELBAs were performed in 96-well microtitre plates (Nunc, Roskilde, Denmark) coated overnight at 4 °C with 200 µL of fetuin (0.01 mg mL⁻¹) or BSA (0.01 mg mL⁻¹) solution in 0.025 mmol L⁻¹ phosphate buffer, pH 7.4, containing 0.15 mol L⁻¹ sodium chloride (PBS) per well. The plates were then washed once with 200 µL of PBS/0.05 % Tween 20 buffer (PBS-T) and blocked with 200 µL of 1 % BSA dissolved in PBS-T buffer at room temperature for 1 h. After blocking, the wells were washed twice with 200 µL of PBS-T buffer and then 75 µL of the purified A isoenzyme of serum NAG (starting protein concentration of 1 mg mL⁻¹ that was serially diluted in PBS-T) of the control and IDDM patients and 75 µL of SNA conjugated with HRPO (diluted 1:5000 in assay buffer) were added. After incubation in the dark at room temperature for 2 h, the wells were washed twice with 200 µL of PBS-T buffer.

The spectrophotometric determination of the binding HRPO activity per well was performed by mixing 100 µL of urea–hydrogen peroxide (1.8 mg mL⁻¹) and 100 µL of TMB (1.4 mg mL⁻¹). After 10 min at room temperature, 50 µL of sulphuric acid (2 mol L⁻¹) was added, and the absorbance at 450 nm was measured. All sample dilutions were analysed in triplicate in two repeated experiments.

The reactivity of the A form of serum NAG with SNA lectin (binding SA) in the ELBA system was expressed as the concentration of the A form of NAG (in µg mL⁻¹), which led to 50 % inhibition of SNA linking to immobilised fetuin.
The procedures and experiments were in accordance with the ethical standards of the responsible committee on human experimentation (institutional and national) and with the Helsinki Declaration of 1975, as revised in 2000.

Statistical analysis

The Student’s t-test, linear regression analysis and Pearson’s correlation coefficient were used to analyse the data. Only values in which \( P < 0.05 \) were considered to be significant.

RESULTS

In all IDDM groups (W.Compl., R, R+P and R+P+N), the mean values of total NAG activity and percentage of the dominant A form (Table I) in serum were significantly higher \( (P < 0.001) \) compared to those of the control group \( (3.19±0.5 \text{ IU L}^{-1} \text{ and } 69.38±4.98 \% \text{, respectively}) \). Significant positive correlations were obtained \( (0.945, 0.943, 0.940 \text{ and } 0.963, \text{ respectively}; \ P < 0.001) \) by comparing these two parameters as well as the activity of the A form of NAG and glycaemia \( (0.511, 0.537, 0.609 \text{ and } 0.568, \text{ respectively}; \ P < 0.05) \).

During the separation of the NAG isoenzymes from the serum of the control and IDDM groups by ion-exchange chromatography, a difference in the bonding strength between the A form and DEAE-cellulose was observed (Fig. 1). Additionally, the A isoenzyme of patients with R+P+N was eluted with a slightly higher concentration \( ((6.95±0.89)×10^{-2} \text{ mol L}^{-1}) \) of sodium chloride compared to those of the other three diabetic groups. However, the mean values of the sodium chloride concentration used to elute 50 % of the serum A isoenzyme activity of the control and diabetic groups did not differ statistically (Table II).

To determine the origin of the increased negative charge of NAG A in diabetics, the isoenzymes of NAG A were isolated and purified from the serum of the control and all diabetic groups. In all groups, the A form was purified 180- to...
TABLE II. Mean values of NaCl concentration (in 0.01 M of phosphate buffer, pH 7.0) used to elute 50% of the NAG A isoenzyme from the DEAE-cellulose column from the serum of the control and IDDM groups; W.Compl. – without complications; R – with retinopathy; R+P – with retinopathy and polyneuropathy; R+P+N – with retinopathy, polyneuropathy and nephropathy

<table>
<thead>
<tr>
<th>Group</th>
<th>$c_{\text{NaCl}} / 10^{-2}$ mol L$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6.37±0.37</td>
</tr>
<tr>
<td>W.Compl.</td>
<td>6.35±0.62</td>
</tr>
<tr>
<td>R</td>
<td>6.35±0.72</td>
</tr>
<tr>
<td>R+P</td>
<td>6.40±0.51</td>
</tr>
<tr>
<td>R+P+N</td>
<td>6.95±0.89</td>
</tr>
</tbody>
</table>

200-fold, yielding 10.8–16% of enzyme (Table III). The purity of the isolated A forms was analysed by native PAGE electrophoresis (Fig. 2), whereby one dominant band was observed. This band was derived from the A form of NAG (molecular mass of 120 kDa), which was confirmed by determination of enzyme activity in the gel slice. The relative electrophoretic mobility ($REM$) of the A form gradually increased with progression of diabetic complications (Table III), indicating a slight increase in the acidity of the A form.

TABLE III. Purification – fold, yield, % and relative electrophoretic mobilities ($REM$) obtained by native PAGE of the A form isolated and purified from the serum of the control and IDDM groups; W.Compl. – without complications; R – with retinopathy; R+P – with retinopathy and polyneuropathy; R+P+N – with retinopathy, polyneuropathy and nephropathy

<table>
<thead>
<tr>
<th>Line</th>
<th>Group</th>
<th>Purification – fold</th>
<th>Yield, %</th>
<th>$REM$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>200</td>
<td>11</td>
<td>0.560±0.018</td>
</tr>
<tr>
<td>2</td>
<td>W.Compl.</td>
<td>195</td>
<td>16</td>
<td>0.582±0.020</td>
</tr>
<tr>
<td>3</td>
<td>R</td>
<td>180</td>
<td>10.8</td>
<td>0.619±0.024</td>
</tr>
<tr>
<td>4</td>
<td>R+P</td>
<td>190</td>
<td>12</td>
<td>0.629±0.022</td>
</tr>
<tr>
<td>5</td>
<td>R+P+N</td>
<td>185</td>
<td>13</td>
<td>0.639±0.027</td>
</tr>
</tbody>
</table>

Fig. 2. Native PAGE of purified serum NAG A form from control (lane 1) and IDDM groups (lane 2, W.Compl.; lane 3, R; lane 4, R+P; lane 5, R+P+N) showing the changes in mobility of the A form. The electrophoresis was performed on 9% polyacrylamide gels and the protein bands were visualised by Coomassie Brilliant Blue (CBB).
Significant differences in the distribution of the A form fraction between the control group and IDDM patients with secondary complications were observed by isoelectrofocusing (Fig. 3A). In the IDDM group, apart from the dominant A isoenzyme fraction (pI value approximately 5.6; pI range 5.1–6.0), more acidic fractions were observed (pI values in the range 4.25–5.10, Fig. 3B). Using densitometry, the percentage of these fractions (pI 4.25–5.10) was found to increase with the progression of secondary complications, so that they were 2–4 times higher compared to that of the control group (Table IV). In the R+P+N group, the percentage of this fraction was reached 51.5±1.8 %.

![Fig. 3. A) IEF (linear gel of pH 3–10) of purified A form from the serum of the control (lane 1) and IDDM groups (lane 2, W.Compl; lane 3, R; lane 4, R+P; lane 5, R+P+N). B) Densitometry of NAG A forms from the serum of the control a) and IDDM groups: b), c), d) and e). B.L., base line in the determination of the abundance of the more acidic fractions of the A form.]

**TABLE IV.** Percentage of the more acidic fractions (pI in the range 4.25–5.1) of the A form in the control group and the groups of patients with IDDM; The data presented are mean values from three experiments ± SD; W.Compl. – without complications; R – with retinopathy; R+P – with retinopathy and polyneuropathy; R+P+N – with retinopathy, polyneuropathy and nephropathy

<table>
<thead>
<tr>
<th>Group</th>
<th>More acidic fractions of A form, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>12.8±0.2</td>
</tr>
<tr>
<td>W.Compl</td>
<td>10.1±0.7</td>
</tr>
<tr>
<td>R</td>
<td>35.1±2.2</td>
</tr>
<tr>
<td>R+P</td>
<td>28.8±1.8</td>
</tr>
<tr>
<td>R+P+N</td>
<td>51.5±1.8</td>
</tr>
</tbody>
</table>
The SA content in the carbohydrate parts of isolated A isoenzymes from the serum of control and IDDM groups (Table V) was analysed using ELBA, which is based on the competitive binding of NAG and fetuin (an immobilised glycoprotein ligand) to SNA lectin (specific for terminally α-2,6-bound SA) conjugated with HRPO. The A form from the serum of the control group exhibited the highest inhibitory effect on SNA binding to fetuin (i.e., the highest α-2,6-bound SA content). In the case of the A isoenzyme from the serum of all IDDM groups, to achieve 50 % inhibition of SNA binding to fetuin, 2–3.5 times higher concentrations of the A form were required compared to that of the control group. Thus, the A forms in diabetic groups were less sialylated compared to those of the control group. Furthermore, comparisons between diabetic groups showed that the lowest and highest sialylation levels of the A form were observed in the R and R+P+N groups, respectively.

**TABLE V.** Content of α-2,6-bound SA in the isolated and purified A isoenzymes from the serum of the control group and patients with IDDM, which was estimated based on the concentration, μg mL⁻¹, of the serum A form leading to 50 % inhibition of SNA binding to fetuin. All measurements were performed in triplicate, in two repeated experiments, and are presented as mean ± SD; W.Compl. – without complications; R – with retinopathy; R+P – with retinopathy and polyneuropathy; R+P+N – with retinopathy, polyneuropathy and nephropathy

<table>
<thead>
<tr>
<th>Group</th>
<th>(c(A\text{ form})/\mu\text{g mL}^{-1}), leading to 50 % inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>18.99±0.84</td>
</tr>
<tr>
<td>W.Compl.</td>
<td>54.54±1.52</td>
</tr>
<tr>
<td>R</td>
<td>62.52±1.70</td>
</tr>
<tr>
<td>R+P</td>
<td>42.20±1.84</td>
</tr>
<tr>
<td>R+P+N</td>
<td>32.13±0.91</td>
</tr>
</tbody>
</table>

An overview of the changes in the total serum NAG activity, the contribution of the A isoenzyme activity in the total NAG activity and the NAG A isoenzyme α-2,6-bound SA content is presented in Fig. 4.

**DISCUSSION**

In all IDDM groups, the total NAG activity and percentage of the A form were significantly higher compared to those of the control group, which is in agreement with previously reported data. Changes in total NAG activity correlated with changes in the activity of the A form as diabetic complications become more complex (from W.Compl. to R+P+N), which was confirmed by the high correlation coefficients (from 0.940 to 0.963) determined between these two parameters. Due to the significant positive correlation observed between the activity of the serum NAG A form and glycaemia, it was concluded that the total NAG activity increased in IDDM patients mainly as a result of the increased exocytosis (release into circulation) of the A form in hyperglycaemic pat-
ACTIVITY OF N-ACETYL-β-D-GLUCOSAMINIDASE A FORM IN TYPE 1 DIABETES MELLITUS

Fig. 4. Summary of the changes in the total serum NAG activity (○), contribution of the A isoenzyme activity to the total NAG activity (●), and NAG A isoenzyme α-2,6-bound SA content (●) in the control and IDDM groups, which correspond to the concentration, μg mL⁻¹, of the A form leading to 50% inhibition of SNA binding to fetuin. W.Compl. – without complications; R – with retinopathy; R+P – with retinopathy and polyneuropathy; R+P+N – with retinopathy, polyneuropathy and nephropathy.

In hyperglycaemics, extracellular glucose passes through the metabolic ways independent of insulin, leading to an increase in the flux of N-acetylglucosamine (GlcNAc). Disturbed glycosaminoglycane metabolism (substance deposition in the blood vessels of diabetics with complications) and excess posttranslational O-glycosylation of proteins with GlcNAc (via O-GlcNAc transferase) could be involved in the pathogenesis of diabetes and the development of secondary complications. Thus, the increase in O-GlcNAc could result in the activation and release of NAG for its removal. However, because no statistically significant differences were found between the NAG activities from the serum of diabetic groups with various complications, it was concluded that the deposition of glycomaterial was not the main reason for the changes in NAG activity.

Increases in the flux of GlcNAc in diabetics could also cause increased intracellular posttranslational protein modifications, leading to changes in NAG carbohydrate components and NAG distribution inside and outside cells. In assays of NAG posttranslational modifications in fibroblast cultures, structural differences between the NAG carbohydrate components from lysosomes and NAG secreted into the medium were established.

During ion-exchange chromatography and native PAGE of the serum NAG isoenzymes, it was observed that the negative charge of the A form was higher in the IDDM groups than in the control group. In the IDDM groups, apart from the dominant A isoenzyme fraction (pI value approximately 5.6), more acidic fractions were observed (pI values in the range 4.25–5.1). The percentage of these fractions increased 2–4 times with the progression of secondary complications compared to that of the control group. In the R+P+N group, the percentage of fractions attained 51.5±1.8%. Considering that NAG α-subunits (only present in
the A form) contain oligosaccharide chains with SA as terminal residues\textsuperscript{21} and that changes in the SA content could affect an increase in the acidity of the A form, experiments were performed to determine whether there were increases in the sialylation levels of NAG in the IDDM groups.

The obtained results demonstrated that the degree of $\alpha$-2,6 sialylation of the A form in all diabetic groups was lower compared to that in the control group. Comparing the diabetic groups, the lowest sialylation level of the A form was observed in the R group, whereas the highest was observed in the R+P+N group. The reduced intensity of NAG sialylation in diabetics could be a consequence of a reduced activity of sialyltransferases or an increased activity of sialidases. In the endothelial cells of diabetic rats with hyperglycaemia, the sialidase activity was reported to be increased by 78\%, whereas the sialyltransferase activity was decreased by 15\%.\textsuperscript{22,23} On the other hand, an increase in serum neuraminidase\textsuperscript{6} and unchanged sialyltransferase activity\textsuperscript{24} were observed in diabetics. Thus, it is assumed that the activation/inhibition ratio of these enzymes in diabetics determine the sialylation levels of glycoproteins, resulting in the occurrence of various sialoforms as well as NAG. The molecular mechanisms of these effects, the physiological consequences of the structural and functional changes in molecules with modified glycosylation and how these changes are reflected in the development of diabetic complications are unknown. Only the effects of modified glycosylation patterns could be observed. The present results showed a reduced content of $\alpha$-2,6 bound SA in the IDDM groups. These results also indicated that the increased acidity of the A form in the IDDM groups compared to control group had another cause.

In addition to SA, the total negative charge of NAG could also be attributed to other negatively charged groups (e.g., phosphate or sulphate). Newly synthesised lysosomal enzymes lack or exhibit reduced levels of mannose-6-phosphate residues in their oligosaccharide chains, leading to hypersecretion and intracellular deficiency of multiple lysosomal enzymes.\textsuperscript{25} Thus, NAG, which is secreted in higher levels in diabetes, may have a reduced number of phosphate groups. A sulphate cap could be added to the end of complex-type carbohydrate chains\textsuperscript{26} or Tyr residues of secretory proteins.\textsuperscript{27} The sequence within the $\beta$-subunit of the A form of serum NAG could be a site for Tyr sulphation.\textsuperscript{27} Negative charges derived from sulphation may contribute to the changes in the structure and activity of the A form. Additional studies are required to understand better these changes in NAG A.

How do the changes in the acidity of the NAG A isoenzyme correlate with the increased activity of serum NAG in diabetics? Increased serum NAG activity in patients with hyperglycaemia could result from an increased rate of exocytosis and/or reduced clearance of enzymes. Hyperglycaemia could lead to an increase in the exocytosis of lysosomal enzymes through several mechanisms\textsuperscript{28–36} with
NAG packed in secretory vesicles with other secretory products. The clearance of lysosomal enzymes from the serum proceeds through the asialoglycoprotein (ASGR), mannose and cation-independent mannose 6-phosphate receptors (CI-MPR).\textsuperscript{31–33} The sialylation of the carbohydrate chains of protein masks the residues of D-galactose and N-acetyl-D-galactosamine, which are specifically recognized by ASGR. The asialo A form of NAG was reported to be rapidly eliminated after injection from the circulation of rats.\textsuperscript{34} The decreased sialylation of the A form in all IDDM groups compared to that of the control group should lead to the rapid removal of the isoenzyme from the circulation. However, increases in the A form and total NAG activities were observed in all diabetic groups (Fig. 4). Thus, it could be concluded that the rate of NAG exocytosis in patients with hyperglycaemia is higher than that of its clearance. The results obtained in the present study could be explained by the number of ASGR in diabetics, which drastically decreases on the cell surface.\textsuperscript{35} The expression of ASGR requires a balance between the intracellular concentrations of cGMP and c-AMP. In diabetics, an intracellular increase in c-AMP leads to a decrease in the number of ASGRs on the cell surface.\textsuperscript{36} The decreased number of ASGRs leads to slower NAG removal from circulation (i.e., to prolongation of the enzyme half-life), increasing the NAG activity in serum if the asialo derivative is active. In accord with the present data, Miller et al.\textsuperscript{20} showed that sialidase action (removal of SA) does not alter the activity of the NAG A form.

Lastly, the changes in total serum NAG activity and A isoenzyme profiles in IDDM patients with various secondary complications negatively correlated with changes in the SA content (Fig. 4) and negative charges (acidity, in general) of the most abundant NAG A form. Due to the presence of a larger number of negatively charged groups (compared to those of SA) on the surface of more than 50% of the A form isolated from the R+P+N group, the activity of the A form is reduced, leading to a significantly lower contribution to the total NAG activity compared to those of the W.Compl ($P < 0.01$) and R ($P < 0.05$) groups. Thus, in the diabetic groups, the decrease in total serum NAG activity with the progression of secondary complications could result from structural changes in the A form due to significant increases in the negative charge (acidity, in general). Based on the presented results, additional insight into these changes could be obtained by investigating the contribution of sulphate and phosphate residues to NAG, as well as changes in the activity of other lysosomal enzymes.

CONCLUSIONS

The total serum NAG and A isoenzyme activities in IDDM patients were negatively correlated with the $\alpha$-2,6-bound SA content of the NAG A form. In this study, for the first time, it was found that patients with IDDM have reduced sialylation levels of the A form. However, the acidity of the A form increased in IDDM compared to control group. These results indicate that the increase in aci-
dity of the A form in the IDDM groups has another cause (e.g., the negative charge could originate from groups other than SA).

**Abbreviations.** NAG, N-acetyl-β-D-glucosaminidase; IDDM, insulin-dependent diabetes mellitus; R, retinopathy; P, polyneuropathy; N, nephropathy; BSA, bovine serum albumin; TMB, 3,3′,5,5′-tetramethylbenzidine; SNA, *Sambucus nigra* lectin; HRPO, horseradish peroxidase; ELBA, enzyme-linked lectin binding assay; SD, standard deviation.

**Acknowledgement.** This work was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 172049).

**ИЗВОД**

КАКО САДРЖАЈ СИЈАЛИНСКЕ КИСЕЛИНЕ У А-ОБЛИКУ СЕРУМСКЕ N-АЦЕТИЛ-β-D-ГЛУКОЗАМИНИДАЗЕ УТИЧЕ НА ЊЕГОВУ АКТИВНОСТ У ДИЈАБЕТЕС МЕЛИТУСУ ТИПА 1?

ВЕСНА Б. ЈОВАНОВИЋ1, ЈЕЛЕНА М. АКИМОВИЋ1, ВЕСНА С. ДИМИТРИЈЕВИЋ2 и ЉУЂА М. МАНДИЋ3

1Хемијски факултет Универзитета у Београду, Катедра за биохемију, Сијалезиски центар 12–16, 11158 Београд и 2Институт за ендокринологију, дијабетес и болности мешаболизма, Клинички центар, Др Суботића 13, Београд

До сада је показано да је активност серумске N-ациетил-β-D-глукозаминидазе (NAG) повећана у дијабетесу, али промена садржаја сијалинске киселине (SA) у глико-компоненти A облика NAG и њен утицај на промене у укупној активности NAG код пацијената оболелих од дијабетеса типа 1 (IDDM, са секундарним компликацијама и без њих), нису разматрани. Стога је још економски облик NAG изолован и пречишћен из серума 81 IDDM пацијената, са секундарним компликацијама (глико- и нефипатија) и без њих, и из серума 25 здравих особа, а потом окрајерисан. Одређена је укупна активност NAG и активност A облика, садржај α-2,6 везане SA у пречишћеном A облику и профил овог изоензима. Заступљеност α-2,6 везане SA у А облику свих група дијабетичара била је 2–3,5 пута мања у поређењу са контролом, док је његова киселост (фракције са pI од 4,25 до 5,1) раста, посебно са напредовањем секундарних компликација. Укупне активности серумског NAG и процентни удел A облика у укупној активности биле су значајно повише (P < 0,001) код свих група дијабетичара, и у негативној корелацији са садржајем α-2,6 везане SA у А облику. Поред тога, оне су опадале са уско- живањем секундарних компликација. Утврђене промене могу бити последица промене структуре A облика услед значајног повећања његове киселости, односно негативне шарже која потиче од група које нису остаци SA.

(Примљено 30. априла, прихватио 9. јула, прихватио 17. јула 2014)

**REFERENCES**

24. G. Ronquist, O. Walinder, Diabetes Metab. 9 (1983) 212
33. M. Spiess, Biochemistry 29 (1990) 10009