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Biosensor BIAcore Application in Analysis of a Carbohydrate Moiety of Glycoproteins*

Zastosowanie biosensora BIAcore w analizie komponenty cukrowej glikoprotein

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Abstract

Background. Glycoproteins interact with the lectins, their specificities give information regarding structural features of the carbohydrate moiety of the studied proteins.

Objectives. The investigation of an interaction between human glycophorin A and human IgG with the selected lectins, using a biosensor BIAcore.

Material and Methods. Human glycophorin A (GPA) was isolated from the membranes of A, B, and O blood group erythrocytes; IgG was isolated from the sera of healthy individuals and the patients with rheumatoid arthritis (RA). The following lectins*: Ricinus communis* (RCA-I), *Griffonia simplicifolia* (GSL-II)*, Triticum vulgaris* (wheat germ agglutinin, WGA) and *Sambucus nigra* (SNA-I) were immobilized on a biosensor chip and interaction of these lectins with two studied glycoproteins was measured in a biosensor BIAcore, equipped with a surface plasmon resonance detector.

Results. It was shown that analyses performed in a biosensor can give information on different structural features of the glycoproteins, e.g. status of desialylation (in the case of GPA), status of reduction of disulphide bonds or degree of galactosylation (in the case of IgG).

Conclusions. SPR method may be used to analyze glycotopes, present in the proteins. The method may have some drawbacks, because for one of the immobilized lectins (WGA) a distinct decrease in analyte binding efficiency was observed during prolonged experiments (**Adv Clin Exp Med 2005, 14, 5, 897–903**).

Key words: glycophorin A, IgG, lectin, surface plasmon resonance, biosensor.

Streszczenie

Wprowadzenie. Glikoproteiny oddziałują z lektynami, których specyficzności dostarczają informacji dotyczących elementów struktury komponenty cukrowej badanych białek.

Cel pracy. Badanie interakcji dwóch glikoprotein: ludzkiej glikoforyny A oraz ludzkiej IgG z wybranymi lektynami, z zastosowaniem biosensora BIAcore.

Materiał i metody. Ludzką glikoforynę A (GPA) izolowano z membran erytrocytów grupy A, B i O; IgG izolowano z surowic osób zdrowych oraz surowic pacjentów chorych na reumatoidalne zapalenie stawów (r.z.s.). Następujące lektyny: *Ricinus communis* (RCA-I), *Griffonia simplicifolia* (GSL-II), *Triticum vulgaris* (WGA) oraz *Sambucus nigra* (SNA-I) immobilizowano na płytce biosensora; oddziaływanie tych lektyn z dwiema badanymi glikoproteinami analizowano za pomocą biosensora BIAcore, wyposażonego w detektor SPR (powierzchniowy rezonans plazmonowy).

Wyniki. Wykazano, że analizy wykonane za pomocą biosensora BIAcore umożliwiają rozróżnienie elementów strukturalnych glikoprotein, np. stanu odsialowania (w przypadku GPA), redukcji mostków dwusiarczkowych lub stopnia galaktozylacji (w przypadku IgG).

Wnioski. Metoda SPR może być stosowana do analizy glikotopów, obecnych w białkach. Możliwe jest ograniczenie metody – dla jednej z immobilizowanych lektyn (WGA) wykazano wyraźny spadek wydajności wiązania analitu wraz z upływem czasu (**Adv Clin Exp Med 2005, 14, 5, 897–903**).

Słowa kluczowe: glikoforyna A, IgG, lektyna, powierzchniowy rezonans plazmonowy, biosensor.

Most of the proteins, isolated and described so far, are glycosylated. To characterize these compounds, named glycoproteins, a structure of the polypeptide chain, together with a structure of the covalently attached carbohydrate chains should be described. Regarding the carbohydrate moiety of glycoproteins, several methods of analysis are available; some of them need previous release of oligosaccharide chains (paper chromatography, paper electrophoresis, column chromatography, ^{1}H-NMR), some of them are suitable for analysis of the glycoprotein molecules in a native, nonmodified form (ELISA, lectino- and immunoblotting, ^{1}H-NMR). One of the latter methods is the surface plasmon resonance (SPR), applied as a detection method in a biosensor BIAcore. The experimental procedure, used in the biosensor, needs that one of the substances investigated is immobilized in the working channel of a sensor chip (it is called a ligand) and the other substance (an analyte) is in a solution, which under precise flow control is introduced to the working channel; the analysis is performed in a real time. The practical methodology of ligand immobilization and analysis using the biosensor BIAcore has been described in detail [1].

Biosensor BIAcore can be used to measure interactions of two substances, exhibiting affinity to each other, for example an antigen and antibody or a glycoprotein and a lectin. To get insight into the problem regarding some basic experimental parameters and procedures of the biosensor methodology in the case of interaction between a glycoprotein and a lectin, the authors have chosen two glycoproteins, i.e. human glycophorin A (GPA) and human serum immunoglobulin G, and four plant lectins. Glycophorin A (GPA) is the major sialoglycoprotein of the human erythrocyte membrane [2]; it contains two types of oligosaccharide chains in the molecule, i.e. one N-glycan and about 12 O-glycans. The second glycoprotein under investigation was human IgG, originating from the sera of healthy individuals and the patients with rheumatoid arthritis (RA). This disease is known to considerably influence the galactosylation status of the conservative N-glycans, present in Fc fragments of IgG molecule and this effect is proportionally pronounced together with severity of the disease [3]. To perform the experiments the following four lectins were chosen: *Ricinus communis* (RCA-I), *Griffonia simplicifolia* (GSL-II), *Triticum vulgaris* (wheat germ agglutinin, WGA) and *Sambucus nigra*

(SNA-I). RCA-I recognizes terminal galactoses, GSL-II reacts with terminal GlcNAc residues, WGA recognizes GlcNAc and sialic acid residues and SNA-I reacts with NeuAc residues bound α2-6 to Gal (found in N-glycans) and α2-6 to GalNAc (found in O-glycans) [4–6]. The lectins were used throughout the biosensor experiments as the ligands, i.e. they were immobilized in the working channels of the sensor chips; two glycoproteins under investigation were used as the analytes.

Material and Methods

BIAcore sensor chips CM5 were purchased from Biacore AB (Uppsala, Sweden). *Sambucus nigra* lectin (SNA-I), *Ricinus communis* lectin (RCA-I) and two reagents used for the sensor chip activation procedure: EDC (N-ethyl-N'-(3-dimethylaminopropyl) carbodiimide hydrochloride) and NHS (N-hydroxysuccinimide) were from Sigma (USA); *Triticum vulgaris* lectin (WGA) was from Pharmacia (Sweden) and *Griffonia simplicifolia* lectin (GSL-II) was from VAKO Laboratories (USA). The working buffer HBS (10 mM Hepes, 0.15 M NaCl, 3.4 mM EDTA, 0.05% Tween 20) was used in this composition or was supplemented, when necessary, with 1 mM cations Ca^{2+}, Mg^{2+}, Mn^{2+} (in that case EDTA was omitted); pH of HBS was adjusted to 5.5 (for binding experiments with GSL-II) or 7.4 (for binding experiments with RCA-I, SNA-I and WGA).

Protein Determination

Protein was determined on a micro scale in 96-well polystyrene plates, using a colorimetric method with bicinchoninic acid (BCA), as described [7]. As a standard protein the bovine serum albumin (BSA) was used.

GPA Isolation

Human glycophorin A (GPA) was isolated from the membranes of outdated erythrocytes, obtained from the Regional Blood Transfusion Center in Wrocław, originated from healthy individuals with blood group A, B or O [8]. Briefly, after phenol-water extraction of the membranes the crude preparations were obtained, which were further purified by gel filtration on a Sephadex G-200 co-

lumn, in a buffer containing 1% SDS. Before final lyophilization SDS was removed from the purified samples as described [8].

GPA Desialylation

GPA samples were desialylated in 0.05 M sulfuric acid at 60°C for 4 h. After neutralization with NaOH solution the samples were dialyzed against distilled water and lyophilized. The content of residual sialic acid was determined by a colorimetric method [9].

IgG Isolation

IgG was isolated from the serum samples of healthy individuals and the patients with rheumatoid arthritis (RA), obtained from the Department of Rheumatology, Medical University in Wrocław, by an affinity chromatography on Protein A-Sepharose column (5 ml, Sigma, USA). Before chromatography each serum sample was diluted 1 : 1 with 50 mM TBS containing 0.02% sodium azide, pH 8.0 and centrifuged at 1400 rpm for 5 min. The supernatant was applied on the column and elution proceeded using TBS. Retained IgG, eluted from the column with 0.1 M glycine/HCl, containing 0.15 M NaCl, 0.02% sodium azide, pH 3.4, was immediately neutralized with 1 M TRIS. The content of IgG in the fractions was monitored by measuring the absorbance at 280 nm.

Reduction of IgG Disulfide Bbonds

Reduction was performed according to the previously described procedure [10]. Purified IgG was diluted with 50 mM Tris/HCl, pH 8.0 to a concentration 1–2 mg/ml. Nine volumes of IgG solution were mixed with one volume of 1 M 2-mercaptoethanol in 50 mM Tris/HCl, pH 8.0 and were incubated at 37°C for 2 h. Generated sulfhydryl groups were blocked with iodoacetamide, added directly to the solution to a final concentration 0.1 M; the incubation was performed at room temperature overnight. Finally, reduced IgG was dialyzed against relevant solution.

Ligand Immobilization

Immobilization of the lectins in the working channel of a sensor chip was performed according to the manufacturer's description, using EDC/NHS activating system. All the steps of the procedure, i.e. preconcentration, activation, ligand immobilization, deactivation with 1 M ethanolamine and washing with 0.5 M NaCl were performed as described [1].

Binding Studies

For the binding experiments the following time intervals, solutions and flow rates were used:

a) *Sambucus nigra* lectin (SNA-I) – (working buffer – HBS, pH 7.4):
- association – 2 μl/min for 10 min,
- dissociation – 2 μl/min for 7 min,
- regeneration – 60 μl/min for 10 min (0.5 M lactose/0.1 M HCl), for 3 min (HBS), for 4 min (0.0001 M NaOH);

b) *Triticum vulgaris* (WGA) – (working buffer – HBS, pH 7.4):
- association – 2 μl/min for 10 min,
- dissociation – 2 μl/min for 3 min,
- regeneration – 20 μl/min for 10 min (0.2 M GlcNAc in HBS);

c) *Ricinus communis* lectin (RCA-I) – (working buffer – HBS without EDTA, pH 7.4, containing 1 mM cations Ca^{2+}, Mg^{2+}, Mn^{2+}):
- association – 5 μl/min for 5 min,
- dissociation – 5 μl/min for 6 min,
- regeneration – 20 μl/min for 1.5 min (2 M guanidine hydrochloride in the working buffer);

d) *Griffonia simplicifolia* lectin (GSL-II) – (working buffer – HBS without EDTA, pH 5.5, containing 1 mM cations Ca^{2+}, Mg^{2+}, Mn^{2+}):
- association – 5 μl/min for 5 min,
- dissociation – 5 μl/min for 6 min,
- regeneration – 20 μl/min for 1.5 min (2 M guanidine hydrochloride in the working buffer).

To perform the above mentioned individual experiments the IgG and glycophorin samples were taken on the basis of the same protein content, respectively, determined by BCA method. In the case of immunoglobulin G analysis a reference sample (R) was prepared from a pool of six sera derived from healthy people. In all binding experiments, performed in biosensor BIAcore, the signal from SPR detector was expressed in the relative resonance units (RU); the signal is proportional to the mass increase as a result of a complex formation on the surface of a working channel [1].

Results

Reproducibility Test

This test was done using immobilized *Sambucus nigra* lectin (SNA-I) as a ligand and glycophorin as an analyte. During the preconcentration pro-

cedure performed for this lectin, the optimum pH, at which the lectin gave the highest SPR response, was 4.0. After immobilization of this lectin onto the working channel the signal from the SPR detector rose by 2552 RU. Glycophorin A, used in these experiments, was isolated from erythrocytes of the blood group A (GPA-A). Glycophorin has two types of the carbohydrate receptors, recognizable by *Sambucus nigra* lectin, i.e. NeuAcα2-6Galβ1-, present in N-glycan and NeuAcα2-6GalNAcα1-, present in most O-glycans; this makes glycophorin a very proper reagent for SNA-I. During 7 consecutive days the binding experiments were repeated 11 times under the same experimental conditions; the GPA samples were taken from the same batch solution and the data are shown in Fig. 1. The binding results, expressed as a detector response, are included in the range of 230–265 RU, which gives mean value of 242 RU. The standard error was ≤ 10%, which is well acceptable for this type of measurements. In the case of the experimental system applied: SNA-I as a ligand and glycophorin as an analyte, in the course of experiments no decrease of the detector response was observed.

Analysis of Native and Desialylated Glycophorin

Glycophorin samples, derived from the blood group A, B and O erythrocytes, were analyzed in a native form and after desialylation, using a sensor chip with immobilized WGA lectin. During the pre-
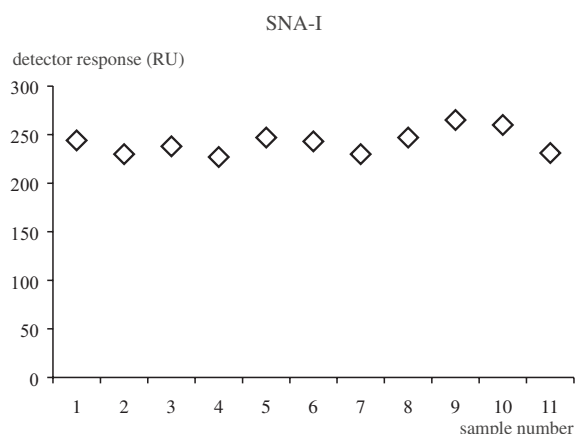

Fig. 1. SPR analysis of GPA-A. Eleven aliquots of GPA-A sample, each containing, 7 µg protein in 100 µl HBS solution, pH 7.4, were analyzed in a working channel with immobilized SNA-I during seven days in eleven consecutive experiments

Ryc. 1. Analiza glikoforyny GPA-A metodą SPR. 11 próbek glikoforyny, przygotowanych z jednego roztworu, zawierających każda po 7 µg białka w 100 µl HBS, pH 7,4; analizowano w kanale pomiarowym z immobilizowaną lektyną SNA-I przez 7 dni

concentration procedure the optimum pH at which WGA gave the highest SPR response, was 4.5. After immobilization of the lectin a signal from the SPR detector rose by 4180 RU. The native Gpa samples exhibit sialic acid (Neu5Ac) content CA 20% (w/w), whereas after desialylation procedure the sialic acid content decreased to 1.4–3.7% (w/w), as determined colorimetrically (data not shown). The experiments of GPA binding to WGA lectin were performed during 7 days and the results are presented in Fig. 2. As can be seen, two distinct experimental features were observed. First, there was approximately a constant difference in binding, for all three GPA preparations, between native and desialylated samples; in accordance with WGA specificity the native samples exhibited the higher binding, due to the presence of sialic acid residues. Second, in the course of experiments using the same working channel, there was a slow, proportional in time, decrease of the detector response. As can be seen, there were no distinct differences in binding to WGA between glycophorin samples from blood group A, B or O erythrocytes.

Analysis of IgG Samples

The samples of human serum IgG, in a native form and after disulfide bridges reduction, were analyzed using two lectins: RCA-I and GSL-II. During the preconcentration procedure for the first lectin the optimum pH value was 5.0; after immobilization of this lectin the SPR baseline increased by 3615 RU. Accordingly, during the preconcentration for GSL-II the optimum pH was 4.0 and after immobilization the signal from SPR detector increased by 2877 RU. The results of binding experiments are shown in Fig. 3. Both lectins reacted better with the reduced IgG samples than with the native ones, with the difference being more pronounced in the case of RCA-I. This indicates that in the native IgG samples RCA-I has a worse access to its recognizable carbohydrate structures, as compared with GSL-II. Reduction of IgG molecules is known to considerably improve access of the conservative N-glycans to the outside environment of the molecule, which enables a better reaction with the lectins, which the authors observed in the case of RCA-I and GSL-II, Fig. 3.

From the binding studies with GSL-II it can be seen that IgG reference sample (R), which originated from the pool of six sera from healthy people, reacted relatively weak (detector signal ca 100 RU). Most of the studied IgG samples of the RA patients showed a bigger reaction with GSL-II, which is in accordance with a known fact that in RA the conservative N-glycans of IgG significantly lack galactoses, which uncovers the next glyco-
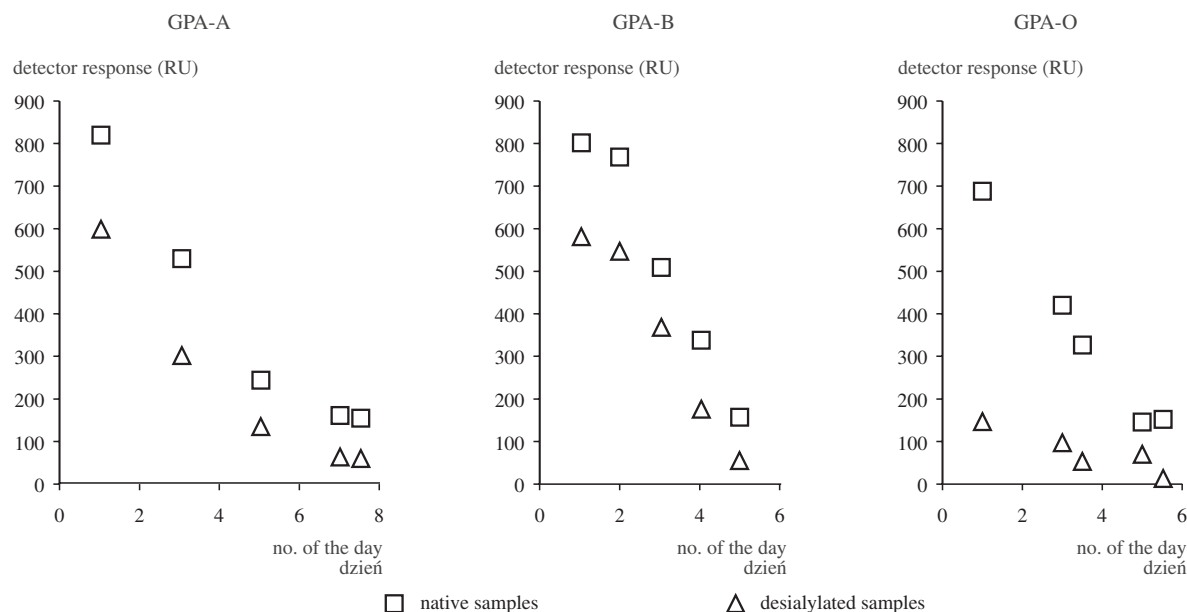
Fig. 2. SPR analysis of the native and desialylated GPA, derived from the group A (GPA-A), B (GPA-B) and O (GPA-O) erythrocytes, respectively. Each analyzed sample contained 2.5 µg protein in 100 µl HBS solution, pH 7.4; the working channel contained immobilized WGA

Ryc. 2. Analiza metodą SPR natywnej i odsialowanej glikoforyny, izolowanej z krwinek grupy A (GPA-A), grupy B (GPA-B) oraz O (GPA-O). Każda analizowana próbka zawierała 2,5 µg białka w 100 µl HBS, pH 7,4; w kanale pomiarowym była immobilizowana lektyna WGA

tope in a sequence, i.e. GlcNAc residues and makes them accessible for GSL-II [3]. Some of the IgG samples, like 45A, 67 and 1A exhibit approximately the same reaction as the reference sample (R), which suggests that severity of the disease in these patients, based on biochemical parameter of IgG, was low. On the contrary, a high reaction with GSL-II of the samples 21A, 58, 52, 49 and 61 indicates a high degree of agalactosylation which suggests substantially more advanced development of the disease in these patients.

Discussion

Presented results of the binding experiments of two glycoproteins: human glycophorin A and human serum IgG with four lectins, in biosensor BIAcore, regarded various experimental conditions and brought interesting remarks. It was shown that depending on the immobilized lectin the response of the detector to the same amount of the same analyte could be approximately unchanged (Fig. 1, immobilized SNA-I) or could simultaneously decrease in the course of experiments (Fig. 2, immobilized WGA). In the latter case two explanations are probable: one – that WGA is generally less stable than SNA-I under experimental conditions applied, or second – that WGA was exposed to a more drastic environment during regeneration of the working channel and, therefore, was continuously loosing

its binding properties during each regeneration cycle. Regeneration seems to be a crucial step in biosensor analysis because it enables to maintain the constant properties of the working channel, which is very important for comparison of the consecutive binding experiments with one another.

Reaction of the reduced IgG samples with RCA-I and GSL-II lectins, Fig. 3, according to the known specificities of these two lectins, should be reciprocal to each other, because RCA-I reacts with terminal Gal residues in oligosaccharides and GSL-II reacts with terminal GlcNAc residues, which are exposed in protein oligosaccharides after galactose removal. As can be seen, for some samples these reactions are not exactly reciprocal, and two probable explanations can be considered. First, which seems to be a general remark, that – what is measured using the lectins is not in practice the total amount of a specific glycotope present on a protein but rather the amount of this glycotope which is currently accessible for the lectin. Unfolding of a polypeptide backbone after IgG reduction strongly influences this feature. The second explanation may be that oligosaccharide chains, present statistically in Fab fragment of IgG [11], influence the total reaction of the reduced IgG with a given lectin, as measured using the surface plasmon resonance technique and may increase this reaction. The above mentioned remarks, resulting from the present investigation, are worth to be explained during future experiments with the biosensor BIAcore.
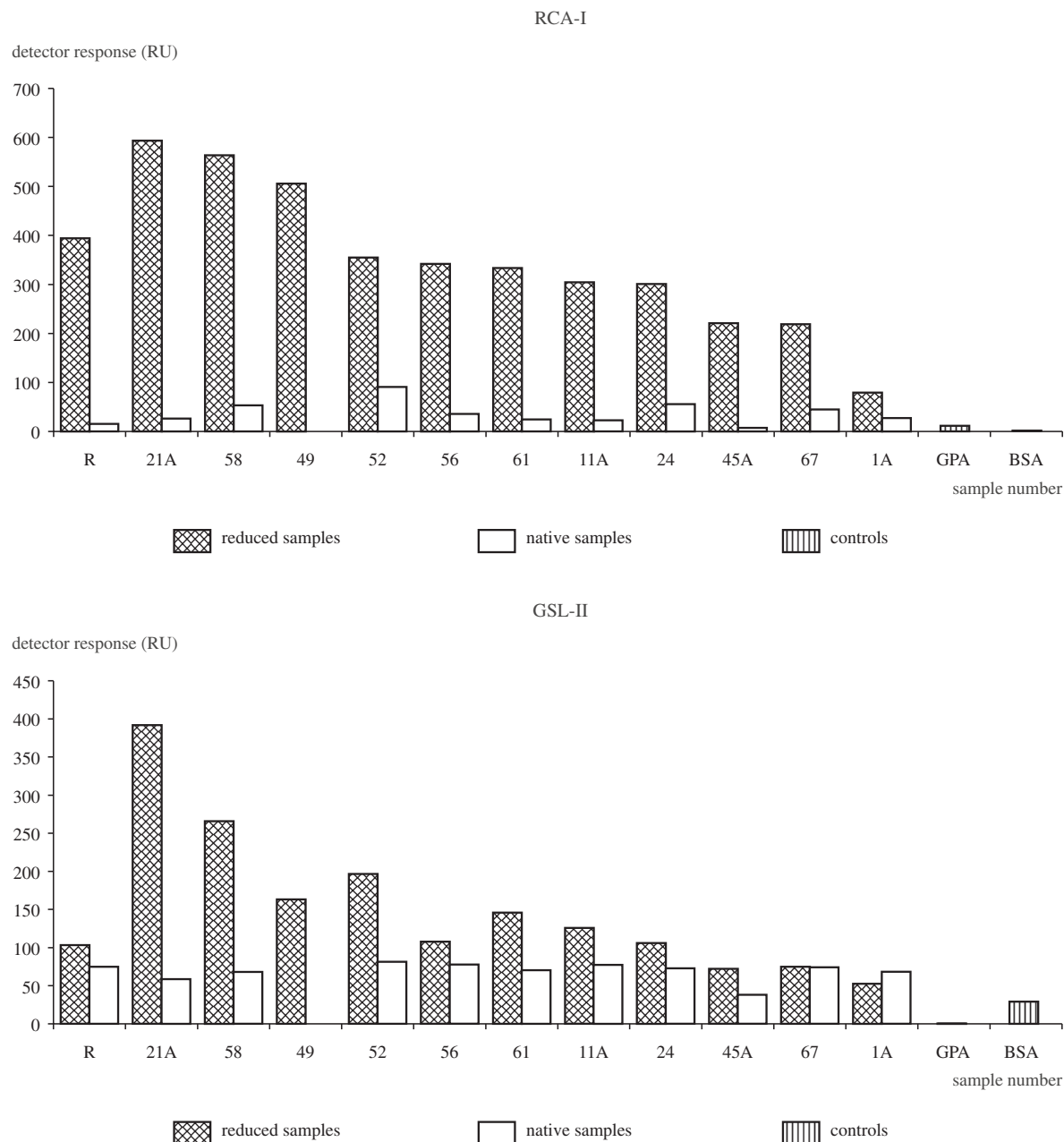
RCA-I

detector response (RU)

GSL-II

detector response (RU)

Fig. 3. SPR analysis of human IgG, derived from the sera of patients with rheumatoid arthritis. Each analyzed sample contained 5 µg protein in 300 µl HBS solution, supplemented with 1 mM cations (for details see Material and Methods)

Ryc. 3. Analiza metodą SPR ludzkiej IgG, izolowanej z surowicy pacjentów z r.z.s. Każda analizowana próbka zawierała 5 µg białka w 300 µl HBS, wzbogaconego w trzy kationy o stężeniu 1 mM każdy (szczegóły w rozdziale Materiał i metody)

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