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In situ Monitoring of *In vitro* Sialylation by Inclusion Bodies

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Abstract

Sialic acids usually terminate oligosaccharide chains expressed on the cell surfaces and glycoproteins of vertebrates, higher invertebrates and some microorganisms. They have various important biological roles. Two of these roles, determination of anti-inflammatory activity of IgG antibodies and increasing the circulating half-life of glycoproteins and blood cells, can find application in pharmaceutical industry and medicine. For this reason, there is a need for efficient *in vitro* protein sialylation processes. To evaluate the efficiency of *in vitro* sialylation, a method for *in situ* monitoring in high throughput screens has been developed. It is represented by hemagglutination caused by inclusion bodies of SabA lectin. SabA lectins are proteins expressed on the cell surface of bacteria *Helicobacter pylori* and bind to sialic acids on the host cell surfaces. They are responsible for *H. pylori* mediated agglutination of erythrocytes *in vitro*. This presented method is fast, simple and one can avoid time-consuming purification of analyzed glycoprotein. In addition, it could provide a basic diagnostic method for determination of sialylation level of erythrocytes.

Keywords: Protein sialylation; Inclusion bodies; Lectin SabA

Introduction

Sialic acids (Sias) are acidic sugars that belong to a large family of neuraminic acid derivatives. They are often found in terminal position of oligosaccharide chains decorating cell surfaces and glycoproteins of vertebrates, higher invertebrates and some microorganisms [1]. Located there, Sias are implicated in various important processes including prevention of clearance of glycoproteins and blood cells from circulation [2] and determination of IgG antibody activity [3].

Sias increase the circulating half-life of glycoproteins by many different mechanisms [2,4,5]. In modern medicine, many diseases are treated by therapeutical proteins. However, rapid degradation and/or elimination from circulation limit their efficiency [5]. Several authors demonstrated that sialylation improve pharmacokinetic properties of therapeutical proteins, e.g. erythropoietin, human growth hormone and interferon alpha [4,6,7]. In a similar way, Sias determine also life span of blood cells. Decreased level of erythrocyte sialylation affects their aggregation, what may raise the risk of some cardiovascular diseases (e.g. acute myocardial infarction, atherosclerosis) [8].

As it was already mentioned, Sias determine also the activity of IgG antibodies. Fc region of IgG antibody contains two oligosaccharide chains that are crucial for antibody activity. IgG S2 glycoform carrying two terminal Sias has anti-inflammatory properties [3]. In sera of patients suffering for inflammatory autoimmune diseases (e. g. rheumatoid arthritis) was found increased concentration of IgG G0 glycoform that is responsible for activation of inflammatory processes. These diseases are treated by intravenous immunoglobulin (IVIg) that is capable to inhibit inflammation. However, this activity is result of sialylated IgG antibodies that are present in IVIg only in relatively small amount. *In vitro* sialylation of IVIg leads to remarkable increase of its anti-inflammatory activity [9].

Sialylation brings new perspectives into pharmaceutical industry and medicine. Using *in vivo* techniques, level of sialylation is variable and often incomplete [5]. For this reason, present research focuses on development of efficient *in vitro* sialylation method. Important step during development of such a method is selection of sialyltransferases that show the highest *in vitro* activity. There are analytical techniques (e.g. mass spectrometry, nuclear magnetic resonance spectroscopy) which can provide very exact data about sialylation level of final product of enzymatic reaction. The disadvantage is that the analyzed glycoprotein must be purified in clumsy and time-consuming process. Therefore, the aim of our work was to develop a fast and simple method for high-throughput analysis of protein sialylation.

Presented method is based on hemagglutination-inhibition test (HAI) widely used in microbiology. This test has been already used for various lectin characterizations [10-12], as well as for testing of influenza hemagglutinin inhibitors [13]. However, we come up with modification of this approach. Instead of regular lectin molecules, SabA lectin was applied in the form of inclusion bodies (IBs). Protein in the form of insoluble IBs remains active and specific. SabA lectin originates from bacteria *Helicobacter pylori* and serves for binding of host cell Sias. Interaction of SabA lectin with Sias on the surface of erythrocytes results in hemagglutination [14,15]. Sialylated glycoprotein acts as an inhibitor of this interaction, because it binds SabA lectin as well. According to the level of inhibition, it is possible to determine the sialylation level of analyzed protein.

Materials and Methods

Cloning, expression and isolation of inclusion bodies

The method described by Nahálka et al. [15] was used for production of SabA lectin in form of active IBs. IBs were isolated from *Escherichia coli* BL21 (DE3) transformed by plasmid vector pET-34b(+). Insert carried by vector was truncated gene HP0062 isolated from genomic DNA of *Helicobacter pylori* ATCC700824D. This gene was inserted in such a way that N-terminus of resulted protein is fused with the cellulose-binding domain of *Clostridium cellulovorans*. Described fusion initiates physiological aggregation of protein into the active IBs. Transformed *E. coli* was cultivated in LB medium (10 g/l trypton, 5 g/l yeast extract, 10 g/l NaCl) with added kanamycine (30 μg/ml) as described by Nahálka et al. [15]. After cultivation, cells were lyophilised. IBs were isolated from 10 mg of lyophilised cells using 500 μl of non-ionic lytic detergent. Lysate was subsequently centrifuged (13000 g, 10 min., 4°C) and washed three times in 750 μl Tris-HCl (50 mM, pH 7,5). Finally, pellet was suspended in 1 ml of PBS (0.8% NaCl, 0.02% KCl, 0.115% $Na_2HPO_4.7H_2O$, 0.02% KH_2PO_4, pH 7.2).

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Comparison of three samples of IBs

Individual samples of IBs were 2-fold serial diluted in PBS in 96-well microtiter plate. Human erythrocytes in PBS (55 µl of human blood in 55 ml of PBS) in volume 50 µl were added to 25 µl of diluted IBs. Wells containing 25 µl of PBS with 50 µl suspension of blood (no IBs) were considered as negative control. Microtiter plate was incubated for 1 hour at room temperature and subsequently 3 hours at 4°C.

Comparison of sialylation level of erythrocytes

Suspension of IBs was diluted in ratio 1:8 and then 1,3-fold serial diluted. Erythrocytes were prepared in concentration of 42.5×10^6 erythrocytes/ml in PBS. Negative control and incubation conditions were as for previous experiment.

Interaction of IBs with sialylated protein fetuin

Fetuin (Sigma-Aldrich, USA) dissolved in PBS (100 mg/ml) was 1,5-fold diluted in 25 µl PBS. Fetuin was then titrated with non-diluted IBs suspension in volume of 50 µl. Wells with no fetuin represented negative control. Microtiter plate was incubated for 4 hours at 30°C and subsequently 16 hours at 4°C. After incubation, electronic record was used to measure the diameter of sediment at the well bottom.

Hemagglutination-inhibition test

Suspension of IBs was diluted in ratio 1:8 and resulted suspension was 1,3-fold serial diluted in PBS (final volume of 15 µl). Fetuin (Sigma-Aldrich, USA) solution in PBS (100 mg/ml) was 1,5-fold serial diluted and added to IBs suspension in volume of 10 µl. Finally, blood suspension was added in volume of 50 µl. Wells containing only blood in PBS and blood with IBs suspension (no fetuin) were considered as negative controls. Microtiter plate was then incubated for 1 hour at room temperature and subsequently 3 hours at 4°C. After incubation, electronic record was used to measure the diameter of inner circle of agglutinated erythrocytes.

Inhibition of hemagglutination by composition of fetuin and asialofetuin

Suspension of IBs was diluted in ratio 1:25 and pipetted into wells of microtitter plate in volume of 15 µl. At the same time, fetuin (Sigma-Aldrich, USA) solution (100 mg/ml) as well as asialofetuin (Sigma-Aldrich, USA) solution in PBS (100 mg/ml) were 1,5-fold serial diluted. Corresponding concentrations of fetuin and asialofetuin were added into wells and variably mixed to obtain final volume of 10 µl. IBs and proteins were then titrated with 50 µl erythrocytes in PBS. Wells containing only blood in PBS and blood with IBs suspension (no fetuin, no asialofetuin) were considered as a negative control. Microtiter plate was then incubated for 1 hour at room temperature and subsequently 3 hours at 4°C. After incubation, electronic record was used to measure the diameter of inner circle of agglutinated erythrocytes.

Results

SabA lectin of bacteria *H. pylori* is responsible for Sia-dependent *in vitro* hemagglutination [14]. We had three batches of *E. coli* transformants producing IBs of SabA lectin. In the first step, the activity of these three batches of IBs was examined. They were all active, although slight differences in their activity were observed. It was possible to recognize four different zones depending on IBs concentration. The first one, zone of haemolysis, is the result of presence of large IBs quantities that cause disintegration of erythrocyte membrane. Following is area of prozone effect. The amount of IBs is too low to haemolyse and too high to induce positive hemagglutination, because they occupy all oligosaccharide chains on erythrocyte surface. Balanced amount of IBs and terminal Sias on erythrocyte surface leads to positive hemagglutination that represents third zone. The last area appears when the concentration of IBs is too low and thus not capable to induce hemagglutination (Figure 1). Although in all samples, we isolated IBs from 10 mg of lyophilized cells, IBs were not propagated in the same amount (concentration of IBs in Sample 1 and Sample 2 was 1 mg/ml, in Sample 3 was 0.5 mg/ml). Also activity of compared IBs was moderately different. Mentioned differences are visible as variability of borders of described zones among individual IBs samples.

We also demonstrated that by using of single type of IBs, agglutination process of erythrocytes of three volunteers was slightly variable. As shown on Figure 2, various concentrations of IBs were needed to induce positive and/or negative hemagglutination what was caused by diverse Sia content on the erythrocyte surface. It provides evidence, that this method is sensitive enough to show variability in erythrocyte sialylation of individual persons.

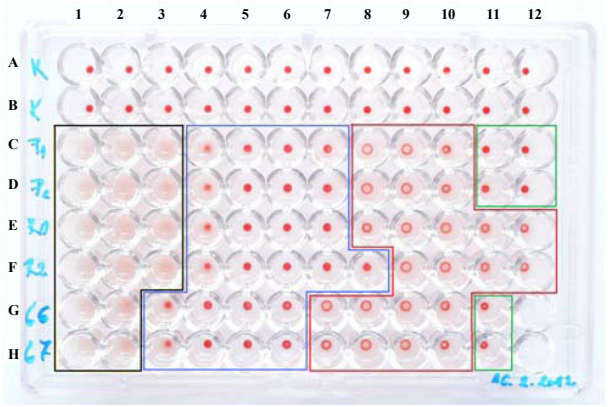

Figure 1: Comparison of three samples of IBs via hemagglutination. Lines A, B – negative control (no IBs); lines C, D – Sample 1; lines E, F – Sample 2; lines G, H – Sample 3 of IBs 2-fold diluted from column 1 to column 12 in 25 µl PBS and titrated with 50 µl washed human erythrocytes. Black frame –heamolysis; blue frame – prozone effect; red frame – positive hemagglutination; green frame – negative hemagglutination.

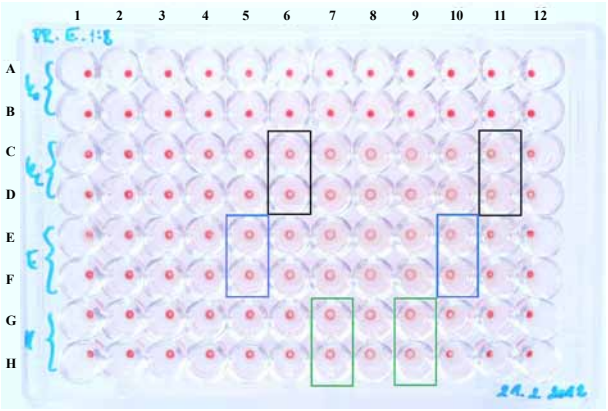

Figure 2: Comparison of sialylation levels of erythrocytes. Lines A, B – negative control (no IBs); lines C, D – erythrocytes of Volunteer 1; lines E, F – erythrocytes of Volunteer 2; lines G, H – erythrocytes of Volunteer 3. Erythrocytes interact with 1,33-fold diluted IBs suspension (diluted in ratio 1:8) from column 1 to 12. Border wells of zones of positive hemagglutination are in frames, black frames – Volunteer 1; blue frame – Volunteer 2, green frame – Volunteer 3.

IBs also react with sialylated proteins, e.g. fetuin. Fetuin was bound by hydrophobic interactions to the conic surface of well bottom. Added IBs interacted with fetuin what resulted in creation of grey sediment. Diameter of this sediment was variable, depending on concentration of fetuin in individual wells. In wells containing low concentration of fetuin and/or in control wells without fetuin, IBs settled to the bottom forming light grey point. According to our results, there is a logarithmic regression between sediment diameter and fetuin concentration (R^2=0,979) (Figure 3).

However, more desirable results were obtained using hemagglutination-inhibition test. In this proposed test, reaction of IBs with erythrocytes is inhibited by sialylated protein fetuin. Proportionally to increasing fetuin concentration, we observed shortening of prozone effect area and, in opposite, zone of positive hemagglutination was extending. We also found linear regression of hemagglutination level on fetuin concentration in presence of identical concentration of IBs (Figure 4). Hemagglutination level corresponds to the diameter of inner circle in reaction.

The last step represents generation of a calibration curve enabling to evaluate concentration of sialylated protein in the mixture with nonsialylated one. At constant IBs concentration, inhibition of hemagglutination by composition of fetuin and asialofetuin mixed in different proportions confirmed linear regression (R^2=0,911) of hemagglutination level on fetuin concentration (Figure 5). These results also confirmed specificity of interaction between IBs and Sias.

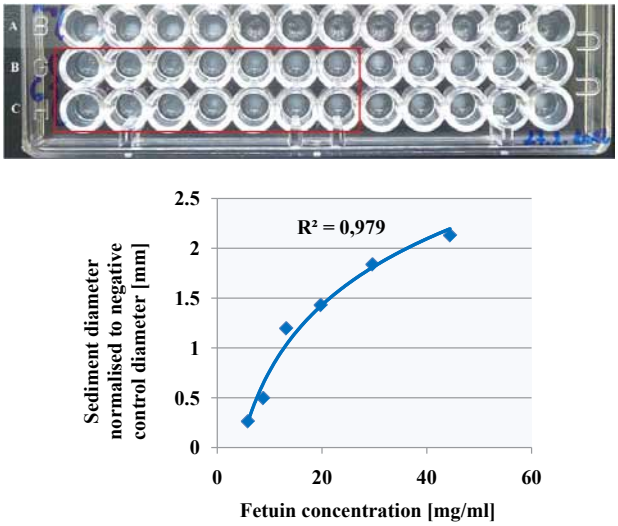

Figure 3: Interaction of IBs with sialylated protein fetuin. Line A – negative control (no fetuin); lines B, C – interaction of 50 µl IBs with 1,5-fold diluted fetuin (66,67 mg/ml) in 25 µl PBS (final concentration 22,22 mg/ml in well) from column 1 to 12; red frame – positive interaction. Diagram represents logarithmic regression between sediment diameter and fetuin concentration.

Discussion

High efficiency is very important criteria for biotechnologically used enzymes. Because sialyltransferases have huge potential for application in medicine and pharmaceutical industry, our work is focused on development of simple and fast method to analyze products of enzymatic reaction and thus to determine the most efficient enzymes.

In the present, primary detection of Sias is often done by antibodies and/or lectins. However, lectins are usually preferred due to various advantages, including useful specificities, lower costs, better characterization and higher stability [16]. Widely used are lectins from *Sambucus nigra* (SNA) and *Maackia amurensis* (MAA) that specifically binds α2,6-sialylated and α2,3-sialylated compounds, respectively [17,18] as well as lectin from *Limax flavus* nonspecifically recognizing Sias in any linkage [19].

Our method provides an analogy to hemagglutination-inhibition test (HAI) that represents the 'gold standard' for diagnosis of infectious diseases, e.g. influenza virus [20,21]. It has been already applied for characterization of selected lectins or their inhibitors [10-13]. Used Sia-binding lectins are mimicking viral particles or pathogen cells that are capable to induce hemagglutination. Because resulted hemagglutination is dependent on Sias, sialylated compounds are competitive inhibitors of this reaction and thus, they act similarly to antibodies in diagnostic HAI.

Our proposed approach represents a novel alternative to these used methods. In presented experiments, lectin SabA from *H. pylori* was applied in the form of IBs and used for determination of sialylated protein concentration. As we already published before, proteins fused with CBD_{clos} domain aggregate into insoluble IBs that remain active [15].

At first, we investigated and compared activity of three samples of IBs propagated in three different batches of transformed *E. coli*. Our results revealed that amount as well as activity of IBs in different

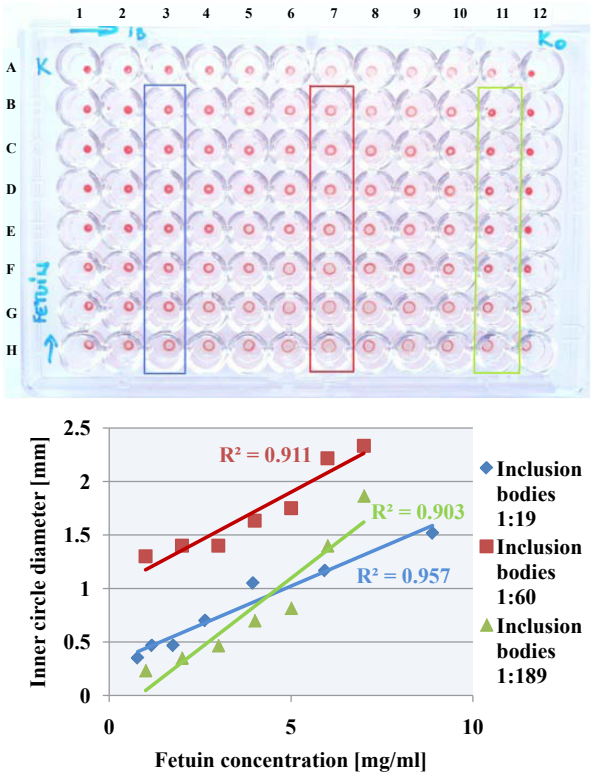

Figure 4: Hemagglutination-inhibition test. Well A12-negative control (no IBs, no fetuin); line A (1-11) - negative control (no fetuin); lines B-H-inhibition of hemagglutination by fetuin. Suspension of IBs was diluted in ratio 1:8 and resulted suspension was 1,3-fold serial diluted in 15 µl PBS from column 1 to column 12. Fetuin (66,67 mg/ml) was 1,5-fold diluted in 10 µl PBS (final concentration 8,89 mg/ml in well) from line H to line B. Described mixture was then titrated with 50 µl of washed erythrocytes. Wells in frames correspond to linear regressions depicted on diagram.

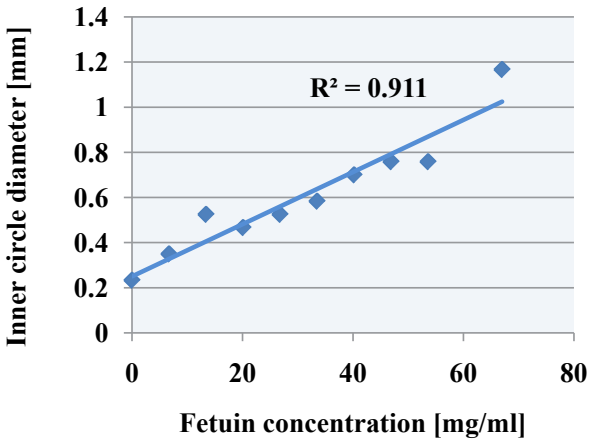

Figure 5: Calibration curve to determine concentration of fetuin in mixture with asialofetuin. In each point, the final mixture of fetuin and asialofetuin contained 66,67 mg/ml of protein. Zero point contained 66,67 mg/ml of asialofetuin.

batches may vary. However, it is possible to recognize typical four zones – haemolysis, prozone effect, positive hemagglutination and negative hemagglutination.

Hemagglutination caused by our IBs also could serve as a simple diagnostic method for primary determination of decreased sialylation level of erythrocytes. As it was already mentioned, reduced content of Sias is probably associated with some cardiovascular diseases [8]. Our analysis proved the applicability of this method in diagnostic, as it was possible to recognize individual variability in Sia content. However, one would need standardized sample as a reference. Positive correlation between Sia content and age was not proved. For better understanding of these results, knowing of health conditions of individual volunteers would be necessary.

In the next step we tested interaction of our IBs with fetuin. Although we identified logarithmic regression between reaction level and fetuin concentration, this method is not proper for high-throughput screening for several reasons. The reaction is hardly visible and requires high concentrations of fetuin and IBs.

Hemagglutination-inhibition test can eliminate drawbacks of former approach. Hemagglutination inhibited by fetuin is clearly visible and lower concentrations of sialylated protein as well as IBs are needed. At constant concentration of IBs, linear regression represents relationship between hemagglutination level and fetuin concentration. Additional experiment using mixture of fetuin and asialofetuin also showed linear regression between hemagglutination level and fetuin concentration. This regression line can serve as a calibration curve to determine concentration of sialylated protein in the mixture with non-sialylated one after enzymatic reaction and thus, establish the efficiency of this reaction. At the same time, these results confirm that observed interactions are specifically dependent on Sias.

These results suggest that our proposed approach fulfils all requirements for high-throughput method and has a high potential to become a suitable alternative to already known methods for Sia detection. In contrary to various analytical methods, it is less precise but does not require time-consuming purification step and thus it is more beneficial for primary screening of efficiency of *in vitro* enzymatic reactions. The drawback of proposed method is necessity of standardization with respect to used blood, to isolated IBs as well as to specific protein. However, once the standardization is done, it is possible to examine a lot of samples in relatively short time, which means it is applicable in evaluation of efficiency of biotechnological processes.

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