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Short communication

Improvement of an enzyme linked lectin assay to determine recombinant mistletoe lectin I

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Abstract

Extracts from Viscum album leaves, with mistletoe lectin I (ML I) as the main therapeutic agent, are commonly used as an immunomodulating adjuvat in tumour therapy. Because of its popularity against cancer and the possibility for a better standardisation a recombinant ML I (rML I) was developed by Eck et al. To improve the sensitivity of an already established enzyme linked lectin assay (ELLA) for rML I human haptoglobins with different phenotypes (1.1, 2.1 and 2.2) are used to replace asialofetuin as matrix. To determine the carbohydrate binding specificity of the tested glycoproteins the ELLA was realised in the presence of the competitive carbohydrate β-D-lactose. It could be shown that using haptoglobin phenotype 1.1 instead of asialofetuin improved the test results markedly. Both, the limit of detection and the limit of quantitation were decreased by an order of magnitude. However, this positive result was obviously accompanied by a loss in specificity of the test. The specificity of asialofetuin for rML I is almost six-fold higher than for the tested haptoglobins. Thus, in cases where high specificity and less sensitivity values for rML I is required the ELLA should still be run with asialofetuin as binding partner.

Keywords: Enzyme linked lectin assay (ELLA); Carbohydrate binding; Recombinant mistletoe lectin I; Asialofetuin; Haptoglobin

1. Introduction

Extracts from mistletoe have been used for many years as an alternative complementary in cancer treatment [1,2]. Since the glycoprotein mistletoe lectin I (ML I) has been discovered as the main therapeutic agent, a standardisation of extracts is possible.

ML I is a highly potent, cytotoxic and immunomodulatory drug, that causes apoptosis in cancer cells [2] and a considerable increase and activation of natural killer cells [3–5], initiating an immune cascade.

ML I is a heterodimeric disulfide-linked molecule, consisting of two functional subunits, the A- and B-chain (Fig. 1). Due to the rRNA cleaving enzymatic activity of the A-subunit ML I belongs to the family of type II ribosome-inactivating proteins (RIP). The second domain (B-chain), the sugar-binding region, is responsible for the clinical effect which is based on the specific binding on carbohydrates of target cell surfaces [6]. Fig. 2 shows the three-dimensional structure of mistletoe lectin I [7].

The A domain has *N*-glycosidase activity and inactivates the 28S ribosomal subunit, resulting in cell death, because of protein synthesis inhibition and induction of apoptosis [2].

The B-chain of ML I is specific for sialic acid (*N*-acetylneuraminic acid) and has also a less pronounced carbohydrate binding specificity for D-galactose [8–11]. This specificities enable ML I to bind to the surface of many cell types leading to agglutination of these cells.

Because of the popularity to use from plants derived ML I against cancer and the possibility of a better standardisation, a new recombinant version of the extract's main active agent [12] was developed by Eck et al. [13,14]. This recombinant mistletoe lectin I (rML I) was obtained by cloning and a separate expression of the individual A- and B-chains in *Escherichia coli*, gaining the active disulfide-linked heterodimeric protein [14] with structural similarity to the original mistletoe lectin I. Bacterially expressed proteins are not glycosylated and the rML I differs in this respect from its natural model but demonstrates the same properties [9,10,14]. This difference does not seem to influence the effectiveness of the drug as cytotoxic agent [14,15]. The recombinant holoprotein also shows an enzymatic activity with its A-domain and a carbohydrate binding activity resulting

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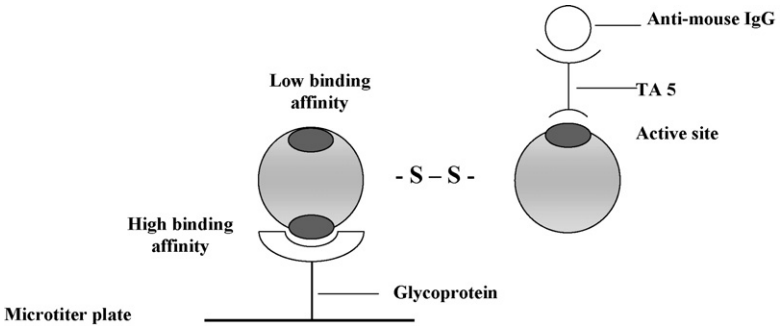

Fig. 1. Illustration of mistletoe lectin I (ML I) adapted from [2]. The A- and B-chain are linked by a disulfide bridge. The A-chain exhibits *N*-glycosidase activity and inactivates the 28S ribosomal subunit. The B-chain is the carbohydrate binding domain.

from the B-domain [16], in particular a sialic acid specificity [9,10,15]. Both, the A- and B-domain, are substantial for the cytotoxic apoptose effect and the use as an anticancer drug, too.

A common analytical method to determine the natural and also recombinant ML I activity, respectively the B-chain binding properties, is the enzyme linked lectin assay (ELLA). In this assay, the active binding of mistletoe lectin to a glycoprotein, here asialofetuin, is measured (Fig. 1) [17]. But recent studies demonstrate that asialofetuin, a desialylated glycoprotein, shows a reduced affinity to both recombinant and ML I compared to the sialofetuin [9,15,18,19].

Human haptoglobin is a plasma glycoprotein, which is able to bind haemoglobin and thereby prevent oxidative tissue damage caused by free haemoglobin [20,21]. Haptoglobin consists of two polypeptide chains, α and β, connected by disulfide bonds. In humans three major phenotypes exist, named haptoglobin 1.1, 2.1 and 2.2 [22]. These glycoproteins carry α-2,6-sialylated structures [9]. Binding assays performed with these sialohaptoglobins indicate a strong affinity for recombinant and from plant derived ML I [9,15,19]. By contrast, measurements with deglycosylated haptoglobin as binding partner show no affinity to ML I [19]. These results confirm the α-2,6-sialic acid specificity of rML I and ML I. Because of this α-2,6-sialic acid specificity different human haptoglobins are used to replace asialofetuin as matrix, in order to improve the sensitivity of the ELLA to recombinant ML I.

To give detailed information about the carbohydrate binding specificity of recombinant ML I on the different glycoproteins,

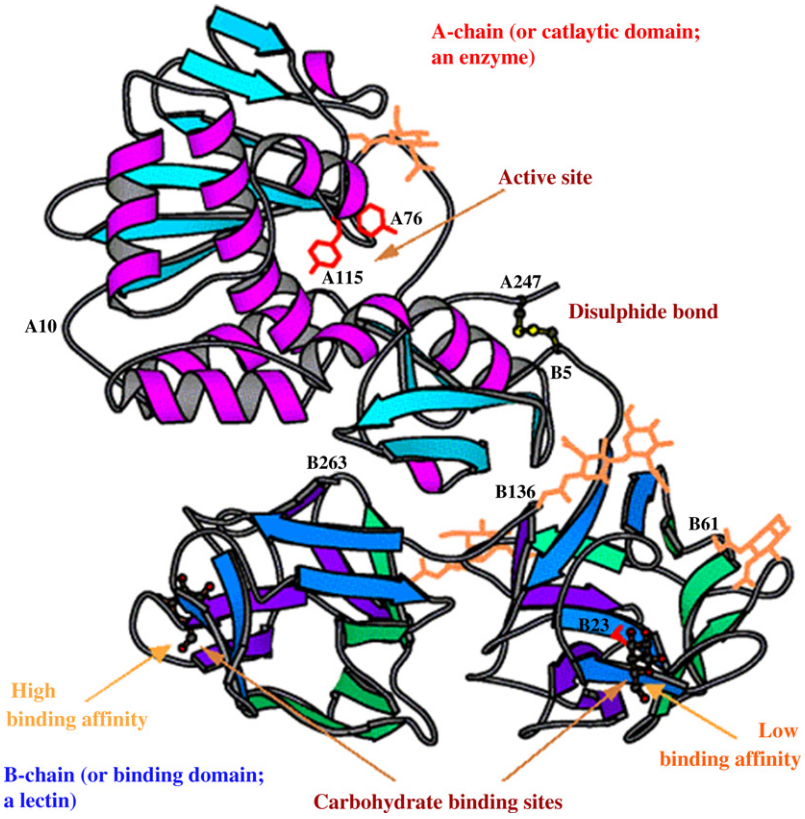

Fig. 2. Cartoon plot from mistletoe lectin I showing the A- and B-chains. The high and low carbohydrate binding sites in the B-chain are marked. Adapted from URL: http://people.cryst.bbk.ac.uk/~ubcgx1d/project.html#structure.

the ELLA was performed in the presence of the competitive carbohydrate β-D-lactose.

2. Experimental

2.1. Chemicals and reagents

Haptoglobin phenotype 1.1, 2.2 and pooled, asialofetuin, goat anti mouse IgG peroxidase conjugate, bovine serum albumin (BSA) and β-D-lactose were purchased from Sigma–Aldrich Chemie (Steinhafen, Germany). Disodium hydrogen phosphate dihydrate, sodium dihydrogen phosphate monohydrate, sodium chloride extra pure, Triton X100 and Polysorbate 20 (Tween 20) from Merck (Darmstadt, Germany), were used to prepare isotonic phosphate buffer pH 7.4 (PBS) and PBS-containing solutions.

Recombinant mistletoe lectin (rML I) was provided from the Gesellschaft für Biotechnologische Forschung (GBF) (Braunschweig, Germany). Anti-mistletoe lectin serum from goat was supplied from Biotechnology Research and Information Network (BRAIN) GmbH (Zwingenberg, Germany).

3,3′,5,5′-Tetramethylbenzidine (TMB) was obtained from Biotrend Chemikalien GmbH (Cologne, Germany).

Double-distilled water was used for all preparations. All used chemicals were of pharmacopoeial or reagent grade.

2.2. Instruments

The used equipment was a Molecular Devices Spectra Max 190 microtiter plate reader (Union City, U.S.A.), a Vortex Genie 2^{TM} from Scientific Industries Inc. (New York, U.S.A.), a Columbus microtiter plate washer from Tecan (Crailsheim, Germany) and an incubator from Heraeus instruments (Hanau, Germany).

2.3. Methods

2.3.1. Determination of rML I activity for LOD and LOQ

The recombinant mistletoe lectin I activity was determined by an enzyme linked lectin assay (ELLA) according to Eck et al. [14]. Here, the specific binding of rML I to the usually applied asialofetuin matrix is compared with other possible glycoprotein binding partners. Glycoproteins tested were haptoglobin phenotype 1.1, phenotype 2.2 and haptoglobin pooled.

To fix the glycoprotein matrix, 100 μl of 100 μg/ml glycoprotein in PBS/NaCl (13 mM sodium phosphate buffer, 128 mM NaCl, pH 7.4) was given in 96-well microtiter plates and incubated for 24 h at room temperature. After washing with PBS/NaCl/0.1% Tween 20 the plates were incubated subsequently for 1 h at 37 °C with 200 μl PBS/NaCl/0.1% Tween 20 + 1% (w/v) bovine serum albumin (BSA) per well to saturate unspecific binding sites. The used test concentration range for rML I was between 0.2 and 250 ng/ml in PBS/NaCl + 0.05% (w/v) BSA. Each concentration was performed eight-fold. After incubating for 24 h at 4 °C, the microtiter plates were washed three times with PBS/NaCl/0.1% Tween 20. Afterwards, each well was loaded with 100 μl anti-mistletoe lectin serum (1:2500 dilution of serum pool) in PBS/NaCl + 0.1% (w/v) BSA + 1% Triton X100 and incubated for 2 h at 37 °C. This complex was added to 100 μl goat anti mouse IgG peroxidase conjugate (1:3500 dilution) in PBS/NaCl/0.1% Tween 20 and stored for 1 h at 37 °C. The plates were subsequently washed three times. After adding the detection substrate TMB and stopping this reaction with 20% sulphuric acid, the immune complex absorption was measured photometrically at 450 nm.

2.4. Limits of detection (LOD) and quantitation (LOQ)

LOD and LOQ were calculated by measuring eight samples for each test concentration (0.2–200 ng/ml). The parameter used to determine the limit of detection was based on a signal-to-noise ratio (SNR) ≥5. The relative standard deviation (R.S.D.) for the limit of quantitation is usually defined ≤10% [23,24]. The LOD and LOQ were evaluated by accomplishing the ELLA test and measuring the absorbance at 450 nm using a microtiter plate reader.

2.5. Inhibition assay and test selectivity

Carbohydrate binding specificity was examined by the same competitive ELLA described in Section 2.3.1. The selectivity of this analytical method refers to the extend at which certain analytes can be determined in the sample under given conditions without interactions of other components.

This assay performance showed the specific binding of a dedicated amount of rML I on different matrices in the presence of β-D-lactose.

Differing from the described test procedure, 100 μl of a fixed rML I concentration (100 ng/ml) was examined. To determine the binding specificity, the samples were additionally incubated with the carbohydrate ligand β-D-lactose (0–140 mM). Differing from the sample incubation described above, after loading the microtiter plates with rML I and β-D-lactose, the incubation time was 2 h at 37 °C.

The rML I residual binding to the different matrices is plotted versus the log competitor concentration of β-D-lactose. The concentration of the inhibitor which caused 50% reduction in binding was obtained graphically for each glycoprotein and was represented in the IC_{50} value for rML I. The IC_{50} value was chosen to compare the tested glycoproteins.

3. Results and discussion

3.1. Limits of detection (LOD) and quantitation (LOQ)

To obtain information about the binding specificities of rML I, an ELLA was performed using microwells coated with different glycoproteins.

Curves resulting from this assay show a strong binding interaction of rML I towards all tested glycoproteins (data not shown here). Solely, asialofetuin-coated microwells reveal an apparently weaker binding of rML I. Human haptoglobin phenotype 1.1, 2.2 and pooled exhibit (nearly similar) comparable

and linear binding profiles in the microwell assay. As already reported, on microwell adsorption assays of human glycoproteins with recombinant ML I [9] human haptoglobin shows strong positive reactions, too. This behaviour was observed for glycoproteins predominantly carrying α-2,6-sialylated structures, like human haptoglobin for example [10]. However, asialofetuin, a desialylated fetuin, which shows also positive reactions with ML I, indicates an only marginal binding [9].

To give detailed predications for the tested glycoproteins a determination of the limits of detection and quantitation was performed. By means of these results, the human glycoprotein haptoglobin phenotype 1.1 as binding partner shows the lowest values for LOD and LOQ with 1 ng/ml. This glycoprotein is clearly more sensitive than the generally applied asialofetuin (both LOD and LOQ 5 ng/ml). The LOD and LOQ for asialofetuin are equal to human haptoglobin phenotype 2.2. Only human haptoglobin pooled represent a limit of quantitation above 10 ng/ml and a limit of detection above 5 ng/ml.

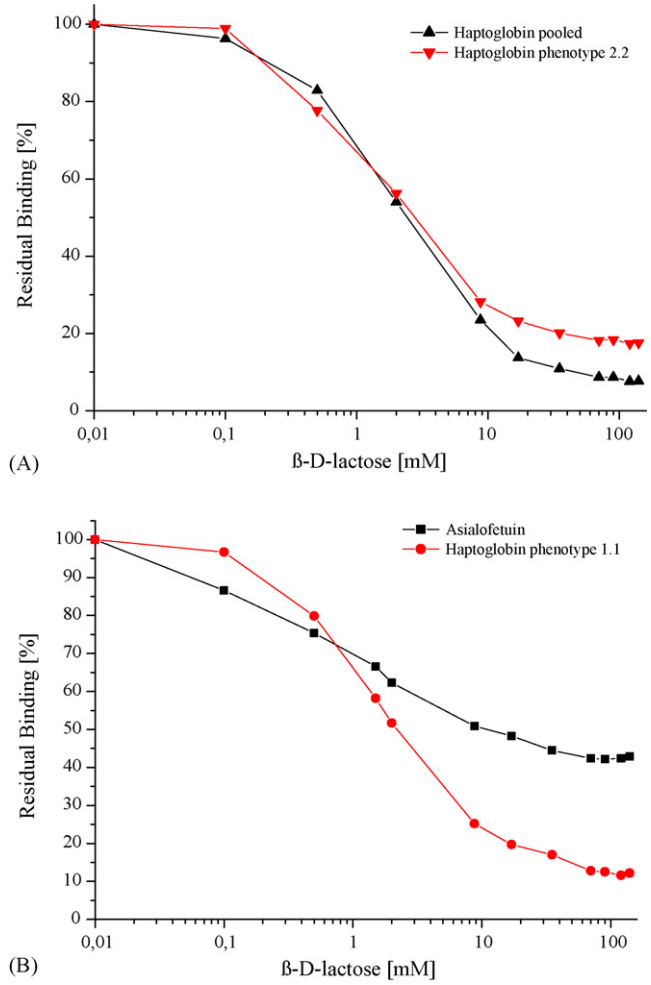

Fig. 3. Determination of carbohydrate binding specificity of rML I with different glycoproteins using a competitive ELLA. The residual binding of rML I to different matrices is plotted against the specific β-D-lactose concentration: (A) haptoglobin pooled (▲) and haptoglobin phenotype 2.2 (▼) and (B) asialofetuin (■) and haptoglobin phenotype 1.1 (●).

3.2. Inhibition assay and test selectivity

The inhibition assay was used to evaluate the binding affinity of rML I molecules on different glycoproteins in the presence of the competitive carbohydrate β-D-lactose. According to numerous studies [10,11,25] it was found that ML I, in addition to an α-2,6-sialylated structure affinity, has a noticeable liability to Galα- and Galβ-linked units. On this account β-D-lactose was chosen to compete with the carbohydrate specific binding of rML I B-chain [14]. The results of this study are presented in Fig. 3.

The comparison of the four binding partners reveal significant differences of IC_{50} data. The amount of β-D-lactose, which is needed to compete 50% of rML I with asialofetuin as matrix, is 11.78 mM. Human haptoglobin phenotype 1.1, 2.2 and pooled yield similar lower signal intensities for 50% competition, approximately 2 mM. The specificity of asialofetuin for rML I is almost six-fold higher than for the tested haptoglobins.

This inhibition assay with β-D-lactose as a competitive carbohydrate suggests that asialofetuin as matrix for the used ELLA is more specific than the tested haptoglobins, at least in the concentration range tested. Asialofetuin seems to show a higher binding capacity to rML I than the other tested glycoproteins.

The sigmoidal inhibition curves shown in Fig. 3 all settled down at the highest inhibitor concentration at approximately 10–18% level. Only the matrix asialofetuin levelled off at higher concentrations (40%). Apparently, the competing carbohydrate β-D-lactose is not able to displace the whole amount of rML I bound to the matrix.

4. Conclusion

Considering the limit of detection (LOD) and the limit of quantitation (LOQ), haptoglobin phenotype 1.1 seems to be favorable. This human glycoprotein appears to be more qualified than asialofetuin which is so far frequently used as a binding partner.

However, the carbohydrate binding of rML I seems to show a higher specificity to asialofetuin than to the tested human haptoglobins. Already low β-D-lactose concentrations for the human haptoglobins indicate a 50% competition.

Thus, the results presented in this paper suggest that it is possible to select the ELLA method for the quantification of rML I either with the focus set on specificity or sensitivity by choosing an appropriate coating molecule for the microtiter plates.

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