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Research paper

Recognition intensities of submolecular structures, mammalian glyco-structural units, ligand cluster and polyvalency in abrin-a-carbohydrate interactions☆

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ABSTRACT

Abrin-a is the most toxic fraction of lectins isolated from *Abrus precatorius* seeds and belongs to the family of type 2 ribosome inactivating proteins (RIP). This toxin may act as a defense molecule in plants against viruses, fungi and insects, where attachment of abrin-a to the exposed glycans on the surface of target cells is the crucial and initial step of its cytotoxicity. Although it has been studied for over four decades, the recognition factors involved in abrin-a-carbohydrate interaction remains to be clarified. In this study, roles of mammalian glyco-structural units, ligand clusters and polyvalency in abrin-a recognition were comprehensively analyzed by enzyme-linked lectinosorbent binding and inhibition assays. The results indicate that: (i) this toxin prefers oligosaccharides having α-anomer of galactose (Gal) at the non-reducing terminal than the corresponding β-anomer; (ii) Galα1-3Galα1- (**B**$_\alpha$), Galα1-4Gal (**E**), Galβ1-3GalNAc (**T**) and Galβ1-3/4GlcNAc (**I**/**II**) related oligosaccharides were the active glyco-structural units; (iii) tri-antennary **II**$_\beta$, prepared from *N*-glycan of asialo fetuin, played a dominant role in recognition; (iv) many high-density polyvalent **I**$_\beta$/**II**$_\beta$ and **E**$_\beta$ glycotopes enhanced the reactivity; (v) the carbohydrate recognition domain of abrin-a is proposed to be a combination of a small cavity type of Gal as major site and a groove type of additional one to tetrasaccharides as subsites with a preference of α1-3/4/6Gal, β1-3GalNAc, β1-3/4/6GlcNAc, β1-4/6Glc, β1-3DAra and β1-4Man as subterminal sugars; (vi) size of the carbohydrate recognition domain may be as large enough to accommodate a linear pentasaccharide and complementary to Galα1-3Galβ1-4GlcNAc β1-3Galβ1-4Glc (gailipenta) sequence. A comparison of the recognition factors and combining sites of abrin-a with ricin, another highly toxic lectin, was also performed to further understand the differences in recognition factors between these two type 2 RIPs.

1. Introduction

Abrin-a, the most toxic fraction of four abrin isoforms, was isolated from the seeds of the subtropical leguminous climber *Abrus precatorius* (Jequirity bean) by a combination of Sepharose 4B and DEAE-cellulose column chromatography [1]. This toxic lectin is one of the ribosome-inactivating proteins (RIPs) that catalytically damage ribosomes and inhibit protein synthesis, causing apoptotic cell death [2]. It is a galactose specific heterodimeric lectin, consisting of a toxoforic A chain subunit of 30 kDa and a lectin active B chain subunit of 31 kDa, covalently linked by a single disulphide bond [3]. The toxoforic A chain is responsible for cytotoxicity and subsequent cell death whereas lectin active B chain is very crucial for the differentiation of cellular targets [4,5], lectin mediated attachment and further endocytosis of toxoforic A chain in the cytosol [6]. Despite numerous studies highlighting the importance of B chain as a determining factor in the cytotoxicity of abrin-a, the complete carbohydrate recognition profile

Abbreviations: Con A, Concanavalin A lectin; Lentil, *Lens culinaris* lectin; PSA, *Pisum sativum* lectin; gp, glycoprotein; ps, polysaccharide; poly-, polyvalent; BNG, bird nest glycoprotein; BSM, bovine submandibular gp; OSM, ovine submandibular gp; PSM, porcine salivary mucin; PTG, porcine thyroglobulin; THGP, Tamm–Horsfall gp; CRD, carbohydrate recognition domain; **A**, GalNAcα1-3Gal, human blood group A disaccharide structure; **A**$_h$, GalNAcα1-3[LFucα1-2]Gal, human blood group A-specific trisaccharide containing crypto **H** determinant; **S**, GalNAcβ1-4Gal; **B**, Galα1-3Gal, human blood group B disaccharide structure; **B**$_h$, Galα1-3[LFucα1-2] Gal, human blood group B-specific trisaccharide containing crypto **H** determinant; **E**, Galα1-4Gal, blood group P^k active and P$_1$ disaccharide structure; **T**, Thomsen–Friedenreich disaccharide, Galβ1-3GalNAc; **I**/**II**, Galβ1-3/4GlcNAc, human blood group type I/II precursor sequence; RIP, ribosome inactivating protein; TBS, Tris-buffered saline; TBS-T, TBS with Tween 20; ELLSA, enzyme-linked lectinosorbent assay; RP, relative potency.

☆ Codes and abbreviations of the blood group antigens and lectin determinants used to express recognition profile of lectins are in bold.

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has not yet been well portrayed [7,8]. Furthermore, the concept of the combining sites of many lectins has now been extended to five or more sugars [9]; sub specificities of broad affinity and crucial role of ligand polyvalency in the recognition of many lectins have also been reported [10–13]. Therefore, to elucidate the functional role of lectins and for a wider scope of immuno-glycobiological use, the glycan recognition profile of abrin-a was systematically analyzed using the enzyme-linked lectinosorbent assay (ELLSA) method that is one of the most accurate and convenient methods to explore ligand clustering and polyvalency [14]. These analyses included: (i) the specificity towards monosaccharides (Gal, GalNAc, and/or Man), their anomers and derivatives; (ii) the reactivity toward mammalian glyco-structural units; (iii) the most active ligand; (iv) the size and shape of combining site, carbohydrate recognition domain (CRD); and (v) the effect of ligand clustering and polyvalency exerted by macromolecules of known glycotopes in the lectin-glycoprotein binding. A comparison of the recognition factors, including the size and shape of combining site (CRD), of abrin-a and ricin [11] was also performed to gain a closer insight into their differential roles in recognition and/or clinical use.

2. Materials and methods

2.1. Abrin-a purification and biotin labeling

Abrin-a was purified and separated from other isotoxic lectins from the seeds of *A. precatorius* by a combination of Sepharose 4B and DEAE-cellulose column chromatography following the procedure of Lin et al. [1]. The lectin was labeled with biotinamidocaproate-*N*-hydroxy-succinimide ester (biotin ester; Sigma–Aldrich, USA) in a 2:1 (w/w) ratio according to the procedure of Duk et al. [14].

2.2. Polyvalent glycotopes from natural glycoproteins

The mucus type bird nest gp (BNG) from the salivary gland of Chinese swiftlets (genus *Collocalia*) was extracted with distilled water at 60 °C for 20 min from commercial birds nest substances (Kim Hing Co., Singapore). The water-soluble fraction was concentrated to 30 mg/ml and dialyzed against 25 volumes of water for 3 days with 3 changes. Contaminated *N*-glycans were removed by immobilized mannose specific lectins such as ConA, PSA and Lentil etc. It primarily consists of sialic acid rich *O*-glycans of mammalian structural units $\mathbf{E}_\beta$ (Galα1-4Galβ1-), $\mathbf{T}_\alpha$ (Galβ1-3GalNAcα1-) and $\mathbf{F}_\alpha$ (GalNAcα1-3GalNAcα1-) [15].

Human and bovine α_1 acid gps (Sigma–Aldrich, USA) contain complex type sialyl *N*-linked glycans of multi-antennary $\mathbf{II}_\beta$ (Galβ1-4GlcNAcβ1-) structural units [16,17]. Porcine thyroglobulin (Sigma–Aldrich, USA) has complex type sialyl *N*-linked glycans of multi-antennary $\mathbf{II}_\beta$ structure (both di- and tri-antennary) [18]. Laminin from Engelbreth-Holm-Swarm murine sarcoma cells (Sigma–Aldrich, USA) is a sialylated poly-$\mathbf{II}_\beta$ and repeating $\mathbf{II}_\beta$ containing a highly glycosylated large multidomain glycoprotein [19].

Fetuin (Gibco Laboratories, Grand Island, USA) is the major gp in fetal calf serum, containing six glycan chains per molecule, three mucin type sialyl *O*-glycans of $\mathbf{T}_\alpha$ structural units and three complex type sialyl *N*-glycans, contain tri- and di-antennary $\mathbf{II}_\beta$ (and very little $\mathbf{I}_\beta$) in a ratio of 2:1 [20]. Bovine, porcine, and ovine submandibular mucins (BSM, PSM and OSM) were purified according to the method of Tettamanti and Pigman [21–23]. Asialo PSM contains *O*-glycans mostly $\mathbf{T}_\alpha$ together with **Tn** (GalNAcα1-Ser/Thr) and **A** (GalNAcα1-3 Gal, human blood group A-specific disaccharide) sequences, as most of the outer fucosyl residues are cleaved by the mild acid hydrolysis. Asialo BSM is a rich source of clustered **Tn** and GlcNAcβ1-3**Tn** and asialo OSM has about 75% **Tn** among the carbohydrate side chains.

Hog gastric mucin#4, a blood group **A**- and **H**- antigen exposed substance over *O*-linked repeating blood group $\mathbf{I}_\beta/\mathbf{II}_\beta$ structural units [13], was derived from crude hog gastric mucin as described previously [24]. Treatment of mucin#4 with HCl (pH 1.5, 100 °C, 2 and 5 h) results in hog gastric mucin#14 and #21. Extended hydrolysis leads to impairment of blood group activities.

Human blood group **ABH** and **Lewis** active substances (e.g., cyst Beach, Mcdon, Tighe and MSS) were purified from human ovarian cyst fluid by phenol/ethanol extraction as described previously [25–28]. They are polydispersed macromolecules (molecular mass > 200 kDa) of similar composition (75–85% carbohydrate and rest are amino acids), presenting multiple mucin type *O*-linked branched glycans [29]. Regardless of their blood group activity, the above purified water-soluble substances have similar core and backbone structure Galβ1-3GlcNAcβ1-3Galβ1-3GalNAcα1-Ser/Thr with four branches of mostly $\mathbf{II}_\beta$ (minor $\mathbf{I}_\beta$).

Tamm-Horsfall gps (THGPs) with Sd(a^+) blood group activity ($\mathbf{II}_\beta$ and **S**) purified from the urine of two individual donors (W.M. and A.Y.) by the method of Tamm and Horsfall [30], were kindly provided by late Dr. W.M. Watkins (Royal Postgraduate Medical School, Hammersmith Hospital, University of London, UK). *Pneumococcus* type 14 polysaccharide of repeating $\mathbf{II}_\beta$ units [31] was a generous gift from the late Prof. E.A. Kabat (Department of Microbiology, Columbia Medical Center, NY, USA). Antifreeze gps from the Antarctic fish (*Trematomus borchgrevinki*) containing only $\mathbf{T}_\alpha$ as carbohydrate chains [32], were provided by Dr. R.E. Feeney (Department of Food Science and Technology, University of California, Davis, CA, USA) through the late Dr. E.A. Kabat (Columbia Medical Center, NY, USA). Poly-2,8-*N*-acetylneuraminic acid capsular polysaccharide (colominic acid) from *E. coli* and pectins from apple and citrus fruits were purchased from Sigma–Aldrich (USA).

Removal of sialic acids from sialylated glycoproteins was performed by mild acid hydrolysis in 0.01 N HCl at 80 °C for 90 min, followed by extensive dialysis to remove small fragments [21].

2.3. Glycotope cluster, glyco-structural units and sugars

Mono-, di- and oligosaccharides were either purchased from Sigma–Aldrich (USA) or prepared by Dextra (Reading, Berkshire, UK). **Tn**-glycopeptides (GalNAcα1-Ser/Thr, molecular mass < 3000 Da) were prepared from asialo OSM [33]. Tri-antennary *N*-glycopeptides with most $\mathbf{II}_\beta$ termini and 2,4,2-branching pattern were prepared from asialo fetuin [34].

2.4. Microtiter plate lectin-enzyme binding assay

The assay was performed according to the procedure described by Duk et al. [14]. The volume of each reagent solution was 50 μl/well, and all incubations, except for immobilization, were performed at 20 °C. The reagents, if not indicated otherwise, were diluted in TBS containing 0.05% Tween 20 (TBS-T) that was also used for washing plates, in between incubations. Ninety-six well microtiter plates (Maxisorp, NUNC, Denmark) were coated with gps and pss in 0.05 M sodium carbonate buffer (0.05 M $NaHCO_3$/0.05 M Na_2CO_3), pH 9.6, and kept overnight at 4 °C. After washing the plates, 10.0 ng biotin labeled abrin-a in TBS-T was added to each well, and incubated for 30 min. The plates were carefully washed to remove free lectin, and ExtrAvidin-alkaline phosphatase solution (diluted 1:10,000; Sigma) was added to detect the specifically bound probes. After 1 h, the plates were washed to remove the conjugate and then incubated with *p*-nitrophenylphosphate (phosphatase substrate, 5 mg tablet; Sigma–Aldrich) in 0.05 M carbonate buffer, pH 9.6, containing 1 mM $MgCl_2$ (1 tablet/5 ml). The resulting absorbance was monitored at 405 nm after 4 h incubation at 20 °C in the dark with substrate-containing solution.

For inhibition studies, serially diluted inhibitor samples were mixed with fixed amount of biotin labeled abrin-a (2.5 ng/well).