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Yeast expressed ArtinM shares structure, carbohydrate recognition, and biological effects with native ArtinM

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ABSTRACT

Recent advances in glycobiology have revealed the essential role of lectins in deciphering the glycocodes at the cell surface to generate important biological signaling responses. ArtinM, a D-mannose-binding lectin isolated from the seeds of jackfruit (*Artocarpus heterophyllus*), is composed of 16 kDa subunits that are associated to form a homotetramer. Native ArtinM (n-ArtinM) exerts immunomodulatory and regenerative effects, but the potential pharmaceutical applicability of the lectin is highly limited by the fact that its production is expensive, laborious, and impossible to be scaled up. This led us to characterize a recombinant form of the lectin obtained by expression in *Saccharomyces cerevisiae* (y-ArtinM). In the present study, we demonstrated that y-ArtinM have a similar subunit formation, oligomerization degree, and carbohydrate recognition specificities that n-ArtinM. We showed that y-ArtinM can exert n-ArtinM biological activities such as erythrocyte agglutination, neutrophil migration and degranulation, mast cell degranulation, and induction of interleukin-12 and interleukin-10 production by macrophages. In summary, the expression of ArtinM in yeast resulted in successful production of an active, recombinant form of ArtinM that is potentially useful for pharmaceutical application.

1. Introduction

Lectins are a heterogeneous group of proteins containing at least one domain that selectively and reversibly binds to free carbohydrates or glycans attached to proteins or lipids [1]. Lectins are ubiquitous in nature, as they are found in a variety of organisms ranging from viruses to humans. The binding of glycans to the cell surface modifies the plasma membrane and/or promotes signaling that triggers diverse phenomena such as proliferation, migration, and cell death [2]. Due to their important biological activities, lectins are increasingly being studied, and the growing understanding of lectin–carbohydrate interactions at the molecular level provides new and important insights that can be applied in biomedical research.

ArtinM (also known as KM+ or Artocarpin) is a D-mannose-binding lectin that was isolated from the seeds of the *Artocarpus heterophyllus* (jackfruit). It consists of a 64 kDa homotetramer formed by the association of 16 kDa non-glycosylated subunits [3,4]. This association, although non-covalent, is resistant to detergents such as sodium dodecyl sulfate (SDS), and thermal denaturation is required to obtain electrophoretically detectable monomers from these homotetramers. ArtinM has a high specificity for Manα1–3(Manα1–6)Man, the trimannoside core of N-glycans [5]. By interacting with N-glycans of cell surface receptors, ArtinM triggers cellular responses such as neutrophil

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migration and activation by binding to the CXCR2 receptor [6,7], mast cell degranulation by binding to immunoglobulin (Ig) E or its receptor FcεR [8,9], and interleukin12 (IL-12) production by inducing activation of macrophages and dendritic cells triggered by interaction with the Toll-like receptor 2 (TLR2) ectodomain [10,11].

Because of its pleiotropic action, ArtinM is an interesting immunomodulatory agent. Systemic administration of ArtinM induces Th1 immunity that confers protection against various intracellular pathogens [10,12–15]. Topical administration of ArtinM promotes regeneration of injured corneal epithelium and oral mucosa [16,17]. Because of its potential pharmaceutical applications, there is considerable interest in large-scale production of ArtinM, and obtaining active, recombinant forms of the protein is a first step toward attaining this goal.

By cloning and expressing the ArtinM gene in *Saccharomyces cerevisiae* and *Escherichia coli*, we have produced the recombinant forms y-ArtinM and b-ArtinM, respectively [18]. In this study, we analyzed the carbohydrate binding specificity of y-ArtinM and examined its ability to mimic the activities exerted by the native ArtinM (n-ArtinM). The results showed that y-ArtinM has biological properties that are similar to those previously described for n-ArtinM, indicating that expressing the protein in yeast cells is a promising method for large-scale production of ArtinM for the development of new immunomodulatory drugs.

2. Materials and methods

2.1. Ethics statement

The animal studies were approved by the Committee on Ethics in Animal Research (CETEA) of the College of Medicine of Ribeirão Preto of the University of São Paulo, and were conducted in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation (COBEA) Protocol no. 025/2010. Erythrocytes and neutrophils were isolated from blood samples obtained from healthy donors who provided written informed consent, and with approval of the Ethics Committee and the Institutional Review Board of the Clinical Hospital of Ribeirão Preto, University of São Paulo, Brazil (approval number 10012/2009).

2.2. Lectins

n-ArtinM was purified, as previously described [3], from a saline extract of *A. heterophyllus* (jackfruit) seeds *via* affinity chromatography on sugar columns. y-ArtinM was expressed in *S. cerevisiae* and purified as previously reported [18]. Briefly, the pYES-DEST52 (Invitrogen, Carlsbad, CA, EUA) expression vector containing the ArtinM coding sequence was used to transform *S. cerevisiae* strain BJ3501 (MATalpha pep4: HIS3 prb1-delta1.6R his3-delta200 ura3-52 gal2 can1). Transformants were selected on uracil-devoid minimal induction medium (SC-U) agar plates. Selected transformants were cultured in SC-U in which glucose was replaced by 2% galactose and 1% raffinose and collected by centrifugation. The cell pellets were suspended in sodium phosphate buffer supplemented with protease inhibitors and the cells were lysed by passage through a French pressure cell press (SLM-AMINCO, Urbana, IL, USA). y-ArtinM was purified by affinity chromatography on D-mannose columns (Sigma–Aldrich, St Louis, MO, EUA). Before use for biological assays, n-ArtinM and y-ArtinM preparations were incubated with a polymyxin solution (Sigma–Aldrich) for 1 h. The purity of the preparations was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE). The protein concentration in the samples was estimated by densitometric scanning of the SDS gels.

2.3. Analytical ultracentrifugation

Sedimentation velocity measurements were performed using a Beckman XL-A analytical centrifuge equipped with both absorbance and interference optics. All data were acquired at a rotor-speed of 50,000 rpm at 20 °C using a Beckman An60Ti rotor. For each sample, 100 scans were acquired at 120 s intervals. The data were analyzed with the program SEDFIT [19] using a c(S) distribution. Buffer density and viscosity as well as the partial specific volume of the protein were calculated using SEDNTERP (Alliance Protein Laboratories, Thousand Oaks, CA, USA).

2.4. Glycan array analysis

The native and recombinant ArtinM forms were biotinylated as previously described [20] and quantified by determining their absorbance at 280 nm (OD_{280}). Microarrays were composed of lipid-linked oligosaccharide probes robotically printed in duplicate on nitrocellulose-coated glass slides at 2 and 7 fmol per spot (in-house designation sets 18–21bis) using a non-contact instrument, as previously described [21]. The microarray binding assays of biotinylated ArtinM proteins were performed at 19–20 °C, as previously described [22]. In brief, the arrayed slides were blocked with 1% w/v bovine serum albumin (BSA; Sigma–Aldrich) in casein blocker solution (Pierce Chemical Co, USA) for 1 h. The biotinylated ArtinM preparation was overlaid at 50 μg/mL, and binding was detected with Alexa Fluor 647-labeled streptavidin (Molecular Probes-Life Technologies, CA, USA) at 1 μg/mL in blocker solution. Glycoarray data analysis was performed with dedicated software [23]. The binding signals were probe-dose dependent.

2.5. Animals

Male BALB/c mice were acquired from the animal house of the Campus of Ribeirão Preto, University of São Paulo and housed in the Animal Facility of the Department of Cell and Molecular Biology of the Faculty of Medicine of Ribeirão Preto, University of São Paulo, under optimized hygienic conditions. All experiments were conducted in accordance with the ethical guidelines of the Committee on Ethics in Animal Research. The mice were used for experiments at 6–8 weeks of age.

2.6. Human neutrophil purification

Neutrophil purification was performed as previously described [7]. The cell preparation had a purity of 98% as determined by flow cytometry, and over 95% of the neutrophils were viable as determined by trypan blue exclusion.

2.7. Hemagglutination assay

Human group O blood was collected in a heparinized tube, the erythrocytes were separated, and the hemagglutination assay was performed, as previously described [24]. Both ArtinM forms (800 μg/mL) were serially diluted (2-fold) in PBS and added to suspensions of human erythrocytes (3% in PBS) in each well. The lectin titer is represented as the reciprocal of the highest dilution of ArtinM protein able to cause detectable agglutination of erythrocytes. One hemagglutination unit (HU) is defined as the minimum lectin concentration required for complete agglutination. Specific hemagglutination activity corresponds to HU per microgram lectin (HU/μg).

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