Carbohydrate Polymers 103 (2014) 392–397

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Inulin isoforms differ by repeated additions of one crystal unit cell

Peter D. Cooper[a,b,c,*], Thomas G. Barclay[d], Milena Ginic-Markovic[d], Andrea R. Gerson[d], Nikolai Petrovsky[a,e]

[a] Vaxine Pty Ltd, Flinders Medical Centre, Bedford Park, SA 5042, Australia
[b] Cancer Research Laboratory, Australian National University Medical School at The Canberra Hospital, Garran, ACT 2605, Australia
[c] John Curtin School of Medical Research, The Australian National University, Acton, ACT 2601, Australia
[d] The Mawson Institute, University of South Australia, Mawson Lakes, SA 5095, Australia
[e] Department of Endocrinology, Flinders Medical Centre/Flinders University, Bedford Park, SA 5042, Australia

ARTICLE INFO

Article history:
Received 1 November 2013
Received in revised form
19 December 2013
Accepted 20 December 2013
Available online 31 December 2013

Keywords:
Adjuvant
Carbohydrate
Inulin
Isoform
Polymorph
Vaccine

ABSTRACT

Inulin isoforms, especially delta inulin, are important biologically as immune activators and clinically as vaccine adjuvants. In exploring action mechanisms, we previously found regular increments in thermal properties of the seven-member inulin isoform series that suggested regular additions of some energetic structural unit. Because the previous isolates carried additional longer chains that masked defining ranges, these were contrasted with new isoform isolates comprising only inulin chain lengths defining that isoform. The new series began with 19 fructose units per chain (alpha-1 inulin), increasing regularly by 6 fructose units per isoform. Thus the 'energetic unit' equates to 6 fructose residues per chain. All isoforms showed indistinguishable X-ray diffraction patterns that were also identical with known inulin crystals. We conclude that an 'energetic unit' equates to one helix turn of 6 fructose units per chain as found in one unit cell of the inulin crystal. Each isoform chain comprised progressively more helix turns plus one additional fructose and glucose residues per chain.

1. Introduction

Inulin is a versatile polysaccharide with many applications (Barclay, Ginic-Markovic, Cooper, & Petrovsky, 2010). Our interest lies in micro-particulate inulin (MPI) isoforms with demonstrated utility as potent vaccine adjuvants for a wide range of antigens in animals and humans (Cooper, Barclay, Ginic-Markovic, & Petrovsky, 2013a; Cooper & Petrovsky, 2011; Gordon et al., 2012; Honda-Okubo, Saade, & Petrovsky, 2012; Larena, Prow, Hall, Petrovsky, & Lobigs, 2013; Petrovsky et al., 2013; Saade, Honda-Okubo, Trec, & Petrovsky, 2013) and also having anticancer activity (Cooper, 1993; Korbelik & Cooper, 2007). The biological importance of these isoforms accordingly lies in both in vitro immune activation and clinical application. A major advantage of inulin-based adjuvants for human vaccines is that they are non-inflammatory, with low reactogenicity and high human and animal safety, in contrast to more traditional adjuvants that activate inflammatory pathways (Petrovsky, 2013; Petrovsky & Aguilar, 2004). This paper further explores the structural basis of these useful activities.

The chemical make-up of inulin is well known (Franck & De Leenheer, 2002, chapter 14). Chicory inulin comprises a family of linear (branching < 2%: De Leenheer & Hoebregs, 1994) chains of β-D-[2 → 1] poly(fructo-furanosyl) α-D-glucose with a range of degrees of polymerization (DP) up to 100 or more hexose moieties. Inulin solutions readily deposit particles visualized as layers of crystalline lamellae (André, Mazeau, et al., 1996; André, Putaux, et al., 1996; Cooper & Petrovsky, 2011; Hébette, Delcour, Koch, Booten, & Reynaers, 2011), each comprising inulin chains helically folded into rigid rods in parallel arrays. The arrays form broad sheets with the rods perpendicular to the lamellar plane, isoforms presumably reflecting variations in the rods' makeup.

MPI polymorphic forms/isoforms have long been recorded (Cooper & Carter, 1986; Cooper & Petrovsky, 2011; Katz & Weidinger, 1931; McDonald, 1946; Phelps, 1965). We recently described a total of seven polymorphic forms/isoforms of inulin (Cooper, Barclay, Ginic-Markovic, & Petrovsky, 2013b),

Abbreviations: AI-1, alpha-1 inulin; AI-2, alpha-2 inulin; BP, British Pharmacopoeia; DI, delta inulin; EI, epsilon inulin; FRM, filtered raw material (methods); GI, gamma inulin; MPI, microparticulate inulin; NA, not applicable; ND, not determined; OI, omega inulin; PBS, phosphate buffered saline; RI, refractive index; RT, room temperature (20–21 °C); Tc, critical temperature; USP, United States Pharmacopoeia; WFI/bic, 1 mM Na bicarbonate solution in water for injection; ZI, zeta inulin.

* Corresponding author at: Vaxine Pty Ltd, Flinders Medical Centre, Bedford Park, SA 5042, Australia. Tel.: +61 2 62319926; fax: +61 8 82045987.
E-mail addresses: peter.cooper@anu.edu.au, doddcoop@ozemail.com.au (P.D. Cooper), nikolai.petrovsky@flinders.edu.au (N. Petrovsky).

0144-8617/$ – see front matter © 2014 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.carbpol.2013.12.066

comprising a fixed incremental series starting with amorphous inulin developing with increasing temperatures of treatment into forms designated alpha-1 (AI-1) → alpha-2 (AI-2) → gamma (GI) → delta (DI) → zeta (ZI) → epsilon (EI) → omega (OI); each has higher aggregate H-bonding strength than its precursor. Their biological activities also varied. Isoforms are the usual presentation of inulin polymorphic forms but differ in including increasingly longer inulin chains while the true polymorphs all have identical chain compositions; isoforms and polymorphs may share the same phenotype (thermal properties). Each was characterized either by its aqueous dissolution point or critical temperature (Tc) as identified as the point of abrupt phase shift, or by its dry melting point (MP) as measured by modulated differential scanning calorimetry (MDSC). The Tc or MP of these seven inulin isoforms/polymorphs increased in a strikingly periodic and step-wise manner, with a regular increment of 6–10 °C or ca 2.9 °C per step, respectively (Cooper et al., 2013b). This suggested that each isoform step simply reflected a regular addition of some energetic unit to a defining structure. These samples contained the entire upper range of inulin chain lengths (plus-format preparations) that obscured the minimal structure defining each isoform.

We wished to understand inulin polymorph/isoform assembly in terms of the lamellar organization of MPI. Our objectives here were to obtain a new series of isoform isolates expected to provide only that minimal definition ('monoformat' preparations), to determine the number average degrees of polymerization (DPn) of these defining structures, and to compare their X-ray diffraction characteristics with those of André, Putaux, et al. (1996).

2. Materials and methods

2.1. Materials

Chicory inulin (Raftiline HP) was supplied as a single large batch in powder form by BENEO-Orafti, Tienen, Belgium. Inulin enzymically synthesized in vitro (DPn ~20) was a gift from Fuji Nihoh Seito Corporation, Tokyo, Japan. The filtered raw material (FRM) quoted here was a 100 mg mL^{-1} solution of the raw chicory inulin as supplied, ion-exchange- and 200 nm-filtered to British/United States Pharmacopoeias compliance, prepared and monitored as described by Cooper et al. (2013b). Close temperature control is critical for this work and methods are also described in that paper. Materials were sterile and handled aseptically. All solutions and suspensions were in WFI/bic (pH ~8: 1 mM Na bicarbonate in water for injection; Baxter, Sydney NSW) unless otherwise stated. Inulin concentrations were measured in the dissolved state by RI using a Brix-Mettler Toledo "Quick-Brix" 90 hand refractometer calibrated by the supplied standard sucrose solution, restricting sample concentrations to <200 mg mL^{-1}.

2.2. Preparation of isoforms in monoformat

We aimed for samples stable to heating at about the Tc of their plus-format analogues with similar physical properties but with evidence of little or no ability to form higher or lower isoforms. Final yields were usually rather small (<2 g). Letters in bold face type in this section indicate the final monoformat preparation. The precipitate after thawing (7 days, 5 °C) a frozen FRM solution includes all crystallizable inulin chains, presented in a mixed alpha plus-format. Usually, this material was first heat-converted to the GI plus-format (80–100 mg mL^{-1}, 45 °C, 90 min), annealed at room temperature (RT, 20–21 °C, 1–2 weeks), adjusted to 40–50 mg mL^{-1} and heated at a temperature (39 °C) midway between the Tc of AI-2 and GI (Table 1) to extract a maximum of AI-2 chains with a minimum of GI chains.

Table 1
Significant temperatures (°C) for monoformat inulin isoforms.

	AI-1	AI-2	GI	DI	ZI	EI	OI
Tc/Conversion[a]	17	32	45	54	60	64	71
Anneal[b]	5	20	20	37	37	45	45
Extract[c]	25	39	49	57	62	68	N/A
1st wash	0[d]	29	43	52	58	62	68
Assay (OD_{700}) [e]	10	21	39	49	55	60	68

[a] 80-100 mg mL^{-1}, 90 min.
[b] 7-14 days.
[c] 40-50 mg mL^{-1}.
[d] All washes are at 0 °C.
[e] T (°C) to distinguish that isoform from the next lower isoform.

However, the series may be entered at any point to obtain a particular isoform. The centrifuged (10–30 min, 650 g, to obtain a clear supernatant) GI precipitate resuspended to 40–50 mg mL^{-1} was first washed once at 43 °C (2 °C lower than the Tc of GI) and then at RT to supernatant RI < 2 mg mL^{-1} to yield GI in plus-format. The 39 °C extract of mixed AI chains was processed as in the next paragraph. This convert/anneal/extract/wash procedure was repeated using the known Tc values for each of the isoforms (Table 1) in turn to extract solutions expected to contain the chains of each of the isoforms in crude monoformat. All second and subsequent washes were at RT except for AI-1, which was always handled at 0 °C. Re-crystallizing becomes mandatory when extracted particles are too light to centrifuge easily. Crude monoformat extracts containing GI and higher chains (DI, ZI, EI and OI) were concentrated either by freeze–thaw crystallization (5 °C, 1–2 weeks) or 80%-ethanol precipitation, followed by re-crystallization and re-conversion up the series. Optimal conversion occurs at the Tc, and other working temperatures are given in Table 1.

Except for OI, the suspensions were finally converted as if to the *next higher* isoform (Table 1), which ensures that assay will reveal any higher isoform chain type contamination. The convert/anneal/extract and/or wash cycles were repeated on each isolate until the acceptance criteria (Section 3.1) were met. The incidence of sample faults described in Section 3.1 was minimized by use of (a) adequate annealing times and temperatures, (b) the difference between optimal extraction and washing temperatures, (c) extraction concentrations (40–50 mg mL^{-1}) low enough for efficient leaching despite finite saturation concentrations, and conversion concentrations high enough (80–100 mg mL^{-1}) to lessen losses during conversion. The intent was to obtain monoformat material with the thermal characteristics of the typical plus-format isoform (Cooper et al., 2013b) in order to determine the DPn of its defining chain type.

Both alpha monoformats may be sourced either from supernatants (5–10 mg mL^{-1}) of FRM crystallized at 5 °C or from the 39 °C extracts described in the previous paragraph. Both sources were ethanol-precipitated or freeze-dried then recrystallized (100 mg mL^{-1}, 5 °C, 7–10 days) and the whole suspension converted as if to GI (90 min, 45 °C) then annealed (7–14 days, RT). AI-1 chain type was extracted (40–50 mg mL^{-1}, 25 °C, 45 min) and the centrifuged (10 min, 650 g) AI-2 precipitate washed once at 29 °C then at RT to supernatant <2 mg mL^{-1}. The clear AI-1 extract was dried, dissolved to 100 mg mL^{-1} and recrystallized (5 °C, 7–10 days), the whole suspension then converted as if to AI-2 (90 min, 32 °C) and annealed (7–14 days, 5 °C), then washed at 0 °C to supernatant <2 mg mL^{-1}. A single exception was a solution of synthetic inulin (DPn 20), which spontaneously precipitated material with the properties of AI-1 monoformat (100 mg mL^{-1}, 7 days and 5 °C). This was purified as for other AI-1 precipitates.