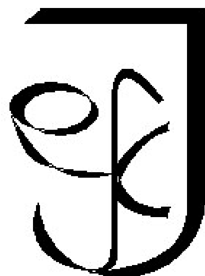
Review Paper



Inulin - a versatile polysaccharide with multiple pharmaceutical and food chemical uses.

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ABSTRACT

α-D-glucopyranosyl-[β-D-fructofuranosyl](n-1)-D-fructofuranoside, commonly referred to as inulin, is a natural plant-derived polysaccharide with a diverse range of food and pharmaceutical applications. It is used by the food industry as a soluble dietary fibre and fat or sugar replacement, and in the pharmaceutical industry as a stabiliser and excipient. It can also be used as a precursor in the synthesis of a wide range of compounds. New uses for inulin are constantly being discovered, with recent research into its use for slow-release drug delivery. Inulin, when in a particulate form, possesses anti-cancer and immune enhancing properties. Given its increasing importance to industry, this review explains how inulin's unique physico-chemical properties bestow it with many useful pharmaceutical applications.

KEY WORDS: Inulin, polysaccharide, fructose, excipient, vaccine, adjuvant

INTRODUCTION

α-D-glucopyranosyl-[β-D-fructofuranosyl](n-1)-D-fructofuranoside (inulin, shown in Figure 1) is a natural renewable polysaccharide resource with a significant number of diverse pharmaceutical and food applications. In the food industry it is used as a fat or sugar replacement and soluble dietary fibre (1-5), but it also has important pharmaceutical applications, as an excipient or stabiliser, and as an injectable for clinical measurement of kidney function (5-8). Inulin may also have utility as a slow release drug delivery medium (2, 6, 9) and as a stabiliser for protein and peptide-based drugs and vaccines (10). Additionally inulin has interesting biological effects, being a potent complement pathway activator when in a particulate form and having anti-cancer (11, 12) and immuno-modulatory properties (13-16).

These pharmaceutical applications rely upon a number of unique chemical and physical properties. Firstly, soluble inulin is largely biochemically inert and non-toxic. Specifically, its β(2-1) glycosidic bonds make it indigestible by humans and other higher animals that do not possess inulinase enzymes. Inulin is,

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however, digestible by certain microorganisms living in the gut that have inulinase activity including lactobacilli (2, 17-23). This means inulin can pass though the human digestive system relatively intact, until it reaches the large intestine, where it is digested by bifidobacteria (20, 23). This encourages growth of a healthy intestinal micro flora that in turn produces important metabolic by-products including butyric and propionic acid, which suppress colon cancer development. Inulin also induces production of a glucagon-like peptide (GLP)-1 hormone, an important endogenous stimulator of insulin secretion and appetite suppressant (24, 25). This makes inulin a valuable probiotic dietary fibre (2, 4, 5, 17-23). The indigestibility of inulin also means it has relatively low food energy for humans and this, combined with its bland to sweet taste, means that it can be used as a low calorie bulking agent in food, replacing sugar, flour and fat (2, 13, 18, 23). Inulin's use as a bulking agent, in particular as a fat replacement, is aided by its particular properties of water solubility. Parts of the molecular structure, specifically the hydroxyl groups, are more able to interact with water than other parts. This provides inulin with some surfactant character and it is able to form stable gels with water at concentrations of 13-50% (22, 23, 26, 27). These gels provide similar textual characteristics to fat, allowing it to be used to replace fat, resulting in low fat foods that are palatable and have good mouth feel (2, 18, 22, 23, 28).

Further advantage can be taken of the solubility of different molecular weights of inulins in water. Inulin's solubility is closely related to the chain length of the polymer, and thus shorter oligomers are much more soluble than long chain polymers (22, 28, 29). This means applications can utilize either inulin's solubility or its insolubility, depending on which polymer length is utilised. For example, in the food industry it is generally required that the inulin is dissolved in processing to make the gels that are important for texture and bulk (22). In contrast, crystalline forms of inulin with low solubility in water activate the complement system, relevant to its use as a vaccine adjuvant (13-16) or cancer treatment (11, 12). These medicinal applications benefit from inulin's biochemical inertness and lack of toxicity in the human body (8). In kidney function testing, intravenously administered inulin is rapidly excreted by the kidney without being metabolised or reabsorbed in the renal tubules enabling it to be used as a measure of glomerular filtration (6, 7, 30). Inulin's rapid excretion from the body in the urine means it could potentially be used in drug delivery to the urinary tract (6). Further, inulin's ability to form gels, particularly when modified with cross-linking groups (9), also provides potential utility as a drug delivery vehicle for water-soluble drugs to other parts of the body (9, 26, 31).

The biochemical properties of inulin are also utilised in the chemical industry, with microbial fermentation of inulin used to produce alcohols, including ethanol (5, 6). More usually, though, it it is the basic chemical structure that is more frequently taken advantage of, being used itself, or as a source of the constituent sugars, as precursors in the production of a number of chemicals such as glycerol, as a component in detergents, and for many less prosaic species (6, 32).

In the optimisation of inulin applications, a good understanding of the physio-chemical properties of inulin polymers is required. Much work has been conducted into inulin over the last century, but, given the diversity of applications, the literature is highly fragmented. It is the aim of this review to consolidate the knowledge of inulin's physio-chemical properties to assist future research of this interesting polymer.

POTENTIAL INULIN USES

Inulin's various uses have been addressed by previous reviews of its chemical (2, 6), industrial (2, 6), food (2, 6, 18), and pharmaceutical applications (2, 5, 6, 18). This review will largely focus on the potential of new pharmaceutical applications of inulin.

Therapeutic effects of dietary inulin

Dietary inulin inhibits development of colon cancers in animal models. Similar tumour-inhibitory effects are seen with fermentation products of inulin, particularly the short chain fatty acids butyric and propionic acids, both of which inhibit growth of colon cancer cells. Butyrate has multiple actions that may contribute to its anti-cancer effects including inhibition of histone deacetylases (33). The effect of dietary inulin is not just seen locally on colon tumours, as dietary inulin also suppressed methylnitrosourea-induced mammary carcinogenesis in Sprague-Dawley female rats and, in addition, the growth of muscle-implanted tumour cells (34). This indicates a systemic anti-tumour effect of dietary inulin, presumably mediated by one or more soluble mediators induced by inulin. Several ongoing studies are seeking to confirm these findings in humans. Treatment of subjects with colon polyps with inulin plus probiotics resulted in reductions in DNA damage, colonocyte cell proliferation and faecal water genotoxicity (35, 36). Part of the beneficial effect of inulin in suppressing tumours may be mediated by its enhancement of gut immune function (37). Notably, inulin has been shown to exert immuno-modulatory effects and induces differentiation in several intestinal cell types independently to its effects on the gut flora (38). Dietary inulin has been shown to have an immunomodulatory effect on the gut, increasing secretory immunoglobulin (Ig) A and interleukin-10 production and decreasing the oxidative burst activity of blood neutrophils (37). It also increases the capacity of peripheral blood mononuclear cells to produce interferon gamma (39). Inulin-fed rats had a higher number and proportion of dendritic cells in gut Peyer's patches, and greater *ex vivo* splenocyte secretion of IL-2, IL-10 and interferon-gamma (40). The mechanisms of the immuno-regulatory effects of dietary inulin may include indirect effects such as changes in the composition of the intestinal flora, and the enhanced production of short chain fatty acids with immuno-regulatory actions (41). In mice dietary inulin enhanced the response to an oral salmonella vaccine consistent with an enhancing effect on mucosal immunity (42). Similarly, mixtures containing fructo-oligosaccharides enhanced influenza-specific delayed-type hyper-sensitivity responses in mice receiving influenza vaccination (43, 44).

Dietary inulin may reduce risk of cardiovascular disease. A recently reported human trial showed that dietary inulin reduced serum concentrations of the proatherogenic molecule, p-cresyl sulphate, in haemodialysis patients (45). Inulin may also reduce cardiovascular risk by its favourable effect on plasma cholesterol and glucose levels. A recent study to investigate the effects of dietary inulin in young healthy men found a significant increase in HDL-cholesterol and reductions in total cholesterol/HDL-cholesterol ratio, serum triglycerides, fasting glucose, fructosamine, HbA1c and insulin resistance as measured by homeostatic model assessment (HOMA-IR) (46). Gastric emptying was also significantly delayed in the group receiving inulin-enriched pasta. At least part of this favourable metabolic effect may be mediated by an effect of inulin on production of a glucagon-like peptide (GLP)-1 mentioned above.

Dietary inulin has also been shown to increase calcium and magnesium absorption and bone mineralisation in young adolescents, primarily through an effect of increasing calcium absorption in the large intestine (47-49).

Use of inulin for drug delivery

Drug delivery systems aim to maximise the exposure of a drug to the tissue requiring treatment and can also be used to stabilise labile drugs such as proteins and peptides. This helps to minimise dosage, and reduce side effects and cost, while maximising efficacy and enabling patient preferred mechanisms of administration, such as oral, rather than injected routes. Inulin is generally biochemically inert, non-toxic and can form hydrogels. These

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properties provide the opportunity for its use as a drug delivery vehicle (9). These factors are assisted by inulin's pharmacological inertia and ready availability (31). Although inulin is able to form aqueous gels without any chemical modification, its use as a hydrogel in drug delivery applications may require it to be cross-linked to improve gel stability, allowing more controlled drug release. An example is its use as an orally-delivered drug delivery system targeting the colon, to allow delayed absorption of drugs that have adverse effects in the stomach (26) or to provide treatment of diseases that show a peak in symptoms in the early morning (9, 50, 51). Inulin is an ideal vehicle to deliver drugs to the colon as the β(2-1) glycosidic linkages are stable to the endogenous enzymatic action of the human digestive system, but it is still broken down by colonic bacteria, releasing the drug payload. While inulin hydrogels might be ideal for drug transport to the colon, they must be stable to the range of pH and ionic strength observed in the human gastro-intestinal tract and the gel swelling characteristics must be such that the bulk of the drug is released during colon transit (50, 51).

Hydrogel stability can be increased by cross-linking and for inulin polymers this has been achieved via functionalisation with vinyl groups. The vinyl groups are subsequently cross-linked by free radical polymerisation (9, 50, 51). Hydration of these dry materials occurs relatively quickly in a manner independent of the degree of cross-linking, until a certain value (~15% of fructosyl units substituted) is reached, whereupon further cross-linking leads to increased swelling time (50). Exposure to varying pH, matching those expected within the gastro-intestinal tract (GIT), shows that hydrogels are stable to the mildly acidic and basic pHs expected within the human GIT (50). For the range of ionic strengths in the GIT the equilibrium degree of swelling was relatively small (50). Similarly, exposure to esterases showed no significant destructive effect upon the inulin hydrogel, despite the ester bonds introduced with the cross-linking groups. In contrast, exposure to inulinases, like those used by the bifidobacteria in the colon, leads to enzymatic degradation of the inulin hydrogel at a rate inversely related to the degree of cross-linking (50).

Cross-linking of inulin hydrogels has also been achieved through functionalisation with both methacrylic anhydride and succinic anhydride, cross-linking triggered by subsequent irradiation with ultra violet light (26). This cross-linked inulin hydrogel, which was designed to deliver the anti-inflammatory agent diflunisal to the gut, exhibited pH-sensitive swelling, with reduced swelling in acidic environments and increased swelling in more neutral to alkaline environments of the intestine (26). The cross-linking did not prevent degradation of the hydrogel by inulinases (26).

Inulin, inulin acetate and modified inulin acetate microspheres have been used for the transport of water soluble model drugs (31) with the modified inulin acetate microspheres being supramolecularly associated with 1,2-dodecanedicarboxylic acid. Encapsulation was similar for all three species, being maximised at 65% (31). Drug release was initially quick as the drug was solubilised when absorbed onto the outside of the microspheres. For inulin and inulin acetate 58-62% of the drug was released within the first 5 minutes whereas for modified inulin acetate only 32% was released within the first 15 minutes (31). After this time the drug was released slowly by diffusion for approximately 1 day, after which the erosion of the microspheres led to quicker release until complete after 3.5 - 4 days (31).

Use of inulin as a stabiliser

Protein and peptide drugs, including monoclonal antibodies, are increasingly important as human therapeutics. A drawback to these drugs is their lability, particularly in aqueous solution, which may limit their shelf life. Drying is a way to avoid this, often requiring use of a protective agent to prevent

loss of protein activity. Polysaccharides can act as protective agents, as they provide multiple hydroxyl groups, that can replace the hydrogen bond interactions as the water is removed during drying (10). This helps to maintain the protein's native conformation and prevent denaturation. For the protective effect to work, the protein or peptide must set the molecular organisation, meaning that the polysaccharide must not crystallise in the drying process (52). Furthermore, the molecular mobility must be low after drying to prevent degeneration of the protein structure in the dried product. For this reason a glassy polymeric structure is best able to protect the protein or peptide and retain the native active conformation over time (53). Requirements for polysaccharides to act as a protective agent in the freeze-drying of protein and peptide based drugs include a high glass transition temperature, low hygroscopicity, low crystallisation rate and a lack of reducing groups (10). Inulin compared well to a control polysaccharide, trehalose, for these properties and was found to successfully protect alkaline phosphatase during drying, with longer inulin polymer lengths being the most effective (10).

Vaccine adjuvant activity of specific inulin isoforms

Insoluble inulin particles, but not soluble inulin, activate complement via the alternative pathway (APC) (13, 15, 16, 54, 55). The APC forms a key part of the innate immune system, and particulate forms of inulin have been shown to have vaccine adjuvant activity (13, 16, 55). and δ-inulin isoforms, together referred to as microparticulate inulin (MPI), activate complement whereas earlier described more soluble α- and β-inulin isoforms do not (13, 55, 56), explaining why these are the only - and -inulin that have shown vaccine adjuvant activity.

A problem with modern vaccines based on recombinant proteins or peptides, is their relative lack of immunogenicity, compared to older vaccines based on living or inactivated whole organisms. This poor immunogenicity can be alleviated though the use of adjuvants (13, 30, 55, 57-62). Adjuvants are agents that act non-specifically to increase the specific immune response or responses to a co-administered antigen (55, 63). MPI is a good candidate to replace traditional aluminium-derived adjuvants because it stimulates both humoral (antibody-mediated) and cellular (T cell-mediated) immunity to co-administered antigens. Inulin's safety and lack of toxicity are major attributes favouring its use as a vaccine adjuvant (13, 15, 30, 54, 55).

CHEMICAL PROPERTIES OF INULIN

All the above pharmaceutical applications of inulin require a thorough understanding of its chemistry and physico-chemical behaviours which will be discussed in detail below. Inulin is a natural storage polysaccharide of various plants that are mostly, but not exclusively, part of the *Compositae* family including chicory, dahlia, and Jerusalem artichoke (2, 19, 20, 22, 23, 55, 64-68). Inulin is also produced by microorganisms including a single known natural bacterial species, *Streptococcus mutans* (69). Other microorganisms that have been shown to be able to produce inulin in the lab are fungal species, specifically members of the *Aspergillus* family (70, 71), although whether inulin is produced natively by these fungi is debatable as they have no significant natural source of sucrose (23).

Chemically described as α-D-glucopyranosyl-[β-D-fructofuranosyl](n-1)-D-fructofuranoside, inulin is a polymer of fructans consisting of linear chains of fructosyl groups linked by β(2-1) glycosidic bonds terminated at the reducing end by an α-D-(1-2)-glucopyranoside ring group (Figure 1) (5, 30, 54, 55, 64-66, 72-77). Generally, plant inulins are found to have chains incorporating 2-100 or more fructose units, chain length and polydispersity depending on plant species and the point in its life cycle (1, 2, 6, 17, 19, 21-23, 28, 66, 74, 78-80). Microbial

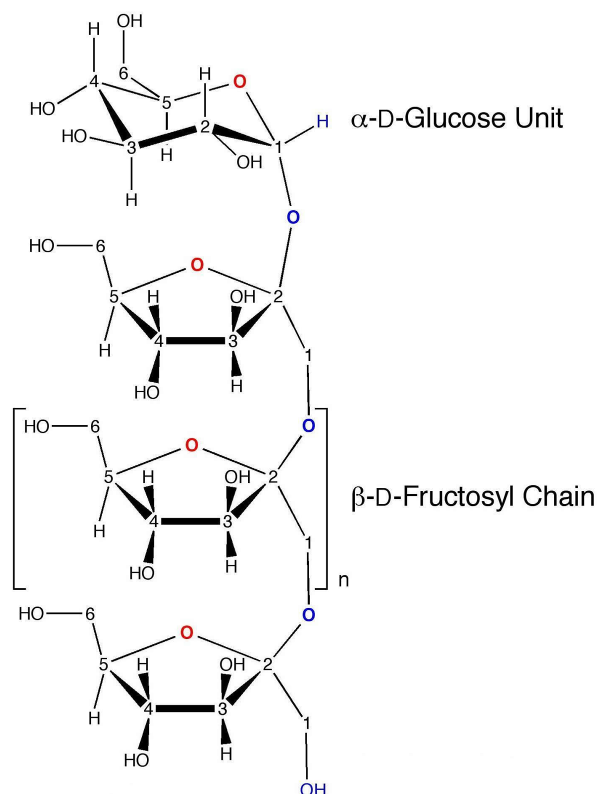

Figure 1 Inulin Polymer

inulin has much larger degree of polymerisation ranging from 10,000 to 100,000 (23).

The chemistry of fructose and glucose

To understand the chemistry of the polymeric and oligomeric forms of inulin some understanding of the basic chemistry of the constituent sugars is required. This is because, while the behaviour of polysaccharides is often quite different from that of its constituent monosaccharides (81), much of the chemistry the polymer can undergo is the chemistry of the constituent sugar, even if some avenues of reactivity are lost in polymerisation. Also, an understanding of the chemical decomposition of the polymeric forms, which requires an understanding of the how synthesis might occur from monomers, helps manage the desired or undesired decomposition of inulin during processing (29).

Inulin comprises a chain of fructose molecules

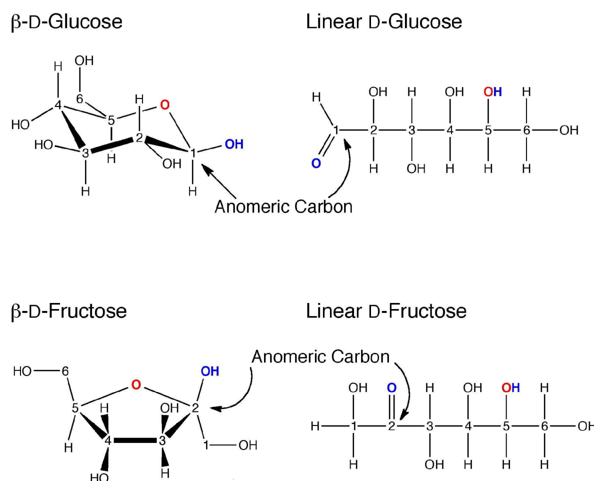

Figure 2 Cyclic and acyclic forms of glucose and fructose

terminated at the reducing end with glucose. While fructose and glucose both crystallise as cyclic forms, in solution the free sugars have a very small equilibrium amount of an acyclic form, the formation of which creates a carbonyl group (Figure 2) (81, 82). This carbonyl group is reactive to hydroxyl groups and it is the intramolecular reaction between the carbonyl group and a hydroxyl group present within the sugar that closes the ring, reforming the hemiacetal cyclic structure (81). For six carbon carbohydrates, like glucose and fructose, the ring closing reaction can occur with more than one of the hydroxyl groups, leading to isomerisation and multiple cyclic forms (81). Most often this ring structure has five or six members. Seven-membered rings are also possible for glucose, though the six membered rings are most highly favoured (81). Fructose is more complex as it is crystallised as a 5-membered ring or furanose, but mostly exists as a 6-membered ring or pyranose in solution (2, 83). Further complexity is provided as each of these rings has multiple conformations, as is also the case for glucose. Additionally, the ring opening and closing reactions about the chiral anomeric carbon means that two stereoisomers or anomeric forms for each conformation of the sugar exist (77, 81, 82).

The reactivity of the acyclic carbonyl group is not just limited to intramolecular cyclisations and provides a range of reactivity for the sugar

that is, to some extent, dependant on the carbonyl form. Which form the carbonyl group takes depends on the way biosynthesis of the sugar occurs, initially with carbon dioxide and water combining to make oxygen and formaldehyde (81). The manner in which the formaldehyde intermediates combine dictates whether the carbonyl chemistry of the acyclic sugar is terminal to the linear chain, making an aldehyde, or incorporated within the chain, making a ketone. Sugars that have an aldehyde acyclic form are known as an aldose, of which glucose is an example. Fructose is a ketone in the acyclic form and these types of sugars are known as ketoses (81). The reactivity of the carbonyl group is generally lower for ketones compared to aldehydes as the acidic carbonyl attached hydrogen in aldehydes makes them much more susceptible to oxidation reactions (82). Nonetheless, both carbonyl chemistries are susceptible to reduction and addition-elimination reactions with nucleophiles. Another variance between ketoses and aldoses, which affects reactivity oppositely, is that ketoses generally form more constrained, less stable ring structures. Consequently ketoses have higher concentrations of the acyclic form in solution than aldoses, the higher concentration increasing relative reactivity (82).

The complexity of structure and carbonyl chemical reactivity of glucose and fructose can be prevented by substitution through the anomeric hydroxyl group in the formation of glycosidic bonds (77, 81, 82). This bond prevents the chemistry involved in the ring opening reaction, fixing the cyclic structure, including its stereochemistry (77), and not allowing the acyclic carbonyl chemistry like reduction reactions. As such, sugars substituted here are described as non-reducing (77, 82). An example of a non-reducing sugar is sucrose. Sucrose is a disaccharide that bridges the anomeric carbons of α-D-glucose and β-D-fructose (77) creating a non-reducing sugar that is most notable for its use as table sugar, but which also provides an inert starting group in the biosynthesis of inulin (Figure 3).

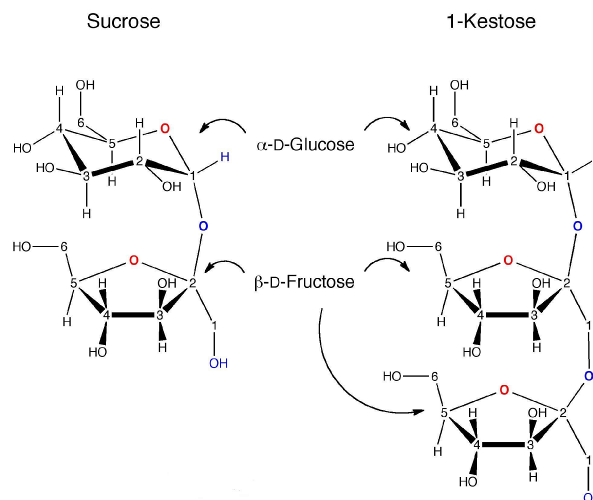

Figure 3 Starting materials of the biosynthesis of inulin

Polymerisation through the anomeric hydroxyl group, as is the case for inulin, similarly protects the component sugars with glycosidic bonds (77) reducing the types of chemical reactivity available to the polymer (though hydrolysis of the glycosidic bond is a new one that is discussed in detail in the next section). The basic monomeric reactivity that is available to the polymer revolves around the reactivity of the hydroxyl groups attached to each ring. These alcohol groups are generally relatively inert, but can undergo a number of organic reactions in the presence of appropriate reagents. Both deprotonation and protonation can occur in the presence of strong bases and acids respectively. These create much more reactive intermediates with deprotonated derivatives becoming very strong nucleophiles and protonated intermediates readily undergoing nucleophilic substitution and dehydration reactions. Other reactions of alcoholic groups include esterification reactions with carboxylic acids and oxidation reactions to form carbonyl chemistry, aldehydes and carboxylic acids for primary alcohols and ketones for the secondary alcohols. In general, the primary hydroxyl groups attached to C6 of both fructose and glucose components and attached to C1 of the terminal fructose moiety are more reactive than the other secondary hydroxyl groups making selective substitution possible (9).

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Another form of chemistry that is brought to inulin by the chemical structure of the precursor molecules is supramolecular interaction. That is, the polarity of the oxygen to hydrogen bond in the hydroxyl groups of glucose and fructose, and by extension inulin, also makes supramolecular chemistry significant, with non-covalent interactions with other similarly polar molecules easily achieved.

The chemistry of inulin polymers and oligomers

Inulin is biosynthesized from a starting molecule of sucrose (Figure 3), explaining the presence of the single glucose residue in the polymer (84). Enzymes then progressively transfer fructose from another sucrose molecule to begin the inulin polymer chain. The attachment of the incoming fructose is to the relatively reactive primary hydroxyl group (77) linked to the anomeric carbon through the methylene group at C1 of the fructose group in the sucrose substrate. This reacts with the anomeric hydroxyl group of the fructose, cleaved from the incoming sucrose, forming the glycosidic bond. This initially forms 1-kestose, essentially a sucrose molecule with an additional fructosyl group attached (Figure 3). In the case of bacterial and fungal synthesis of inulin additional fructose is then added to the terminal fructose in 1-kestose from sucrose, with the longest chain polymers being the best acceptors leading to the quick synthesis of long chains (23). In plants, 1-kestose is also used as a substrate for chain elongation with new fructose being added, not from sucrose, but from 1-kestose or other nascent polymers using a separate enzyme to the one that forms 1-kestose (23). As with bacterial or fungal synthesis, the longest chains are the most efficient fructose acceptors, along with the starting sucrose, which favours the production of longer chains (23).

The biosynthesis of inulin builds a polymer with β(2-1) linking glycosidic bonds. Polymerisation in this way means each incoming monomer becomes inert to carbonyl chemistry upon the formation of the new glycosidic bond, locking the fructose units in the furanose form (77). The growing end of the nascent polymer is described as the terminal end, the other end of the chain being generally known as the reducing end. Of course, in the case of inulin, what would be a reducible end of polymer chain made purely from fructose is ostensibly non-reducing due to the presence of the glucose end group. This is the reason that inulin is chemically relatively inert, although cleavage of the polymer chain at any of the glycosidic bonds will produce a reducing end that then is susceptible to the reactions of carbonyl groups (2, 8, 84). Interestingly, while the fructosyl units in the chain and the terminal fructose unit have furanose form, when free of glucose the reducible end fructosyl group is in the pyranose, or 6-membered ring form (85).

Inulin is enzymatically synthesised for energy storage by various plants, and this inertness of the glucose protected chain is valuable because the storage medium does not break down spontaneously. To access this energy when required these plants have enzymes (inulinase) able to cleave fructose from the terminal end (23). Under appropriate conditions this can lead to high concentrations of fructose, and under such conditions the enzyme that usually elongates the inulin chains can begin to add fructose to fructose monomers creating fructose only chains that have a reducing end (23). This is why significant quantities of inulin are found that have no glucose protecting groups in samples isolated from their natural source and why there is such a large polydispersity in chain length, despite the fidelity common to enzymatic synthetic systems (64).

Branching through reactions of the primary hydroxyl group at C6, rather than through the typical C1 group, has been reported for inulin, but it is usually at low levels in plants (1-4%) (19, 23, 28, 64, 86), and a little higher for microorganisms (5-15%) (23, 69). Carpita *et al.* (28, 64) suggest this is not actually branching, but that the detection of β(2 6) bonds is due

to the presence of a small proportion of β(2 6) 'inulin' synthesized by a different enzyme (64). The basis for this claim is that otherwise there must be two enzymes working to make a branched polymer, as enzymatic systems usually achieve such high fidelity (64).

Inulin hydrolysis

Inulin undergoes cleavage of the glycosidic bond though the addition of water in a process known as hydrolysis (4, 23, 87). Eventually this reaction will lead to the decomposition of the polymer into the component monosaccharides (29). While inulin is relatively stable to hydrolysis at room temperature and neutral pH, the rate of this reaction can be increased by increasing temperature and extremes of pH (4, 23, 29, 88, 89).

The hydrolysis of inulin proceeds by rates that vary depending on the properties of the glycosidic bond. In particular, the glucosyl-fructosyl bond is 4-5 times more resistant to acid hydrolysis than the fructosyl-fructosyl bond (90). Also, terminal fructose units are cleaved more easily than internal ones (90), most likely due to the change of conformation required of the fructosyl group during hydrolysis (91). This change is more easily achieved by an end group than one internal to the polymer (92). Despite these effects, for short chain oligomers where solubility issues and viscosity effects are negligible, the rate of hydrolysis is directly proportional to concentration of inulin (up to 40%/w) (29). This suggests that the hydrolysis follows first order or pseudo first order kinetics (29, 89).

At extremes of temperature, pH or both, decomposition of the hydrolysed monosaccharides can occur during the hydrolysis reaction (87, 88). Understanding this is of importance when trying to establish definitive parameters for the hydrolysis reaction. Therefore, although neither glucose or fructose are particularly susceptible to this, and relatively gentle conditions can be used in the hydrolysis of inulin, factors for allowing the correction of this effect have been developed (87). A way to avoid this monomeric decomposition altogether is to use methanolysis instead of hydrolysis because the methylated monosaccharides are more stable (87). Excluding oxygen when hydrolysing with HCl or trifluoroacetic acid also helps prevent decomposition of the monosaccharides (87).

Thermal hydrolysis of inulin

In the presence of water an increase in temperature leads to an increase in hydrolysis of inulin that follows first- or pseudo-first-order kinetics at both neutral and acidic pHs (29, 89). The rate of hydrolysis of inulin at neutral pH can be considered insignificant for processing time frames up to ~ 60ºC, but can be more relevant at higher temperatures (88, 89). Perhaps more important is the effect that temperature has on hydrolysis of inulin in the acidic conditions often used in processing (4, 29).

Acid hydrolysis of inulin

Inulin is susceptible to acid hydrolysis due to its high energy content (77), the acid promoting hydrolysis by protonating the glycosidic oxygen, activating the leaving group (4, 89). Even in distilled water, where the dissolution of carbon dioxide has reduced the pH marginally below neutrality to less than 6.8, hydrolysis has been observed (7, 93). The hydrogen ion concentration is found to affect the kinetics of inulin hydrolysis in a first-order manner (29). The combination of the first- or pseudo-first-order kinetics of the hydrolysis of inulin with respect to both acid concentration and temperature (29, 89) have been used to show that the hydrolysis of inulin follows the Arrhenius rate law for temperatures, from 7 - 130ºC, and pHs, from 2.0 - 4.2. These conditions encompass those most commonly used in inulin processing (29).

Base hydrolysis of inulin

Base-induced hydrolysis of inulin occurs through a carbonyl group, meaning it can only

occur from the reducing end of an inulin chain that does not contain a protecting glucose moiety (2, 77, 94, 95). Given that commercially available inulin usually contains only a relatively small amount of reducing sugar such hydrolysis is generally slow. Nonetheless, during conditions of raised temperature, where the increase in thermally induced internal scission will form smaller chains with reducing ends (8), base-induced hydrolysis can become significant (94). To avoid base hydrolysis of inulin at lower temperatures, treatment with a reducing agent, such as sodium borohydride, can protect inulin as the terminal ketone is reduced to an alcohol (8).

Reducing chemistry of inulin

Inulin is ostensibly a non-reducing sugar, but the existence of chains free of glucose, whether created by natural enzymatic processes or by internal scission of the chains during hydrolysis, means that some small amount of reducible inulin is invariably present (95). Such inulin can undergo reactions not accessible to non-reducing inulin, including, but not limited to, the basic hydrolysis described in the previous section. Reduction of the acyclic reducing end ketone to a secondary alcohol is another example of this chemistry. Significantly this chemistry has been used to identify the amount of glucose-free chains, so that calculations of Degree of Polymerisation (DP) based on ratios of glucose to fructose become more accurate (95).

Supramolecular chemistry of inulin

Inulin is rich in hydroxyl groups that are able to take part in supramolecular interactions, in particular hydrogen bonding. These interactions can be both intermolecular and intramolecular, though modelling of the inulin structure based on X-ray diffraction analysis of the solid form suggests that crystalline inulin has only intermolecular hydrogen bonding between chains (72). The gelation of inulin is described as particle gel in which three dimensional networks form of aggregated colloidal particles of inulin (28, 96). In this instance hydrogen bonding between inulin particles is intrinsic to gel formation and stabilisation.

PHYSICAL PROPERTIES OF INULIN

The polymeric structure of linear chains of inulin resembles a polyethylene oxide backbone, being made up of the anomeric C2 carbon, the C1 carbon and its attached oxygen molecule for each of the fructose units (Figure 1). This means that only one atom of the polymer backbone is attached to the fructose ring making inulin unusually flexible in conformation for a polysaccharide. This confers its ability to assemble into a range of different structures (13, 28, 66, 76, 77, 82, 97, 98), which along with the high polydispersity of inulin and rotational ability of the primary hydroxyl groups, complicates the structural analysis of inulin (7, 76, 77). Nevertheless, many inulin structures have slowly been elucidated.

Determination of degree of polymerisation and polydispersity

Some of the most important physical properties of inulin are the molecular weight of the polymer and the polydispersity, because these factors have a large influence on its suitability for various applications. Several chromatographic methods used for inulin are described below. Preferably chromatography is done at temperatures and pH that will not increase hydrolysis (7, 19, 93), i.e. at neutral or slightly basic pH and room temperature. Often chromatographic techniques have been combined, utilising the optimal separation of each of the processes (90, 99). General weaknesses of chromatography for inulin analysis are that it is only useful for pure samples, DP can be unreliable as different fractions are known to run together, lack of resolution for higher molecular weights and the lack of standards for calibration (17).

High Performance Liquid Chromatography

Standard HPLC techniques have effectively separate low molecular weight oligomers of

inulin up to a maximum of about DP 16 (17, 19, 23, 68, 75, 86, 90, 100). Most usually this analysis used columns specifically designed for carbohydrate analysis (19, 21, 75, 86, 90), requiring elution at pH and temperatures likely to induce hydrolysis, thus affecting accuracy (19, 86). Further, these columns often use chemical functionalities, such as amino groups, and gel size that allow combinations of separation mechanisms such as ion exclusion, ion and ligand exchange, reversed phase, normal phase and even size exclusion. These characteristics are ideal for most carbohydrates, but the conformationally flexible backbone of inulin means that in chromatography it behaves unusually for a polysaccharide so that simple reversed phase columns have been shown to be similarly effective (68, 90, 100). Even with these more simple columns the conformational variation in inulin can cause problems. For example, elution from a C18 column gave fractions that clearly separate DP of 11-16, however, DP 8 and 9 eluted in the same fraction as did DP 6, 7 and 10 and DP from 2-5 required separation using another technique (68). Indeed, in general most forms of HPLC are only capable of separating oligomeric forms of inulin and longer polymer chains elute together (100). One form of HPLC, however, does provide improved separation for a larger range of DP, as follows.

High performance anion exchange chromatography

High performance anion exchange chromatography (HPEAC) provides improved sensitivity and resolution in the analysis of polysaccharides, compared to other types of HPLC, but also uses high salt concentrations, that can be difficult to remove from the sugar after analysis (100). Different molecular weights of inulin have been variously separated by HPEAC, most often using pulse amperometric detectors (PAD) (1, 19, 21, 65, 68, 86, 93), but at least in one case a refractive index (RI) detector was used (99). PADs are usually used because elution is mostly using a gradient of acetate concentration (65, 99), and the RI detector introduces the added complexity of maintaining a constant refractive index for the solvent as the concentration of acetate increases. The benefit is that the PAD and RI detector responses can be compared to allow better quantitative analysis (99). HPAEC PAD is able to provide improved separation of inulin oligomers and resolution of larger chains is better than for standard HPLC techniques (1). However, HPAEC PAD is still unable to resolve long chain polymers with much success, due to the lack of sensitivity for such species by the PAD detector (1, 23).

One of the major issues for using HPAEC PAD to determine inulin molecular weight has been the lack of relevant standards, that is, it provides qualitative, rather than quantitative results (1, 17, 23, 93). To this end Ronkart *et al.* (1) generated standards via hydrolysis of globe artichoke inulin by endo-inulinase into small oligosaccharides (mostly 3 and 4 fructose units). Due to the large starting DP relatively few glucose terminated oligomers are created, thus presenting minimal interference in the development of fructose oligomer standards (1). The fructose oligomers were separated by size exclusion chromatography and the DP determined by mass spectroscopy before use as standards in HPAEC PAD (1). Nevertheless longer chains have created problems and complete hydrolysis followed by end group analysis was required to quantify the longer chains.

Hydrolysis of inulin for determination by HPLC

One way to improve the performance of HPLC is to hydrolyse the inulin first and then make determinations of the DP based on the ratio of glucose and fructose, which can be separated easily using HPLC (1, 21, 93, 101, 102). This process, described as end group analysis, uses assumptions that can have their accuracy eroded by the presence of fructose, glucose and sucrose monomers in the initial material, as well as, glucose-free chains of inulin. The effect of these interferants can be minimised by

quantifying them before hydrolysis (21, 93, 101), but even if this is accurately carried out, there will no separation by DP. Consequently, only an average measure of molecular weight is determined with no indication of polydispersity (93).

Gas chromatography

Gas chromatography (GC) has also been used to determine the molecular weight of inulin, though only DP up to 9-10 can be volatilised by silylation of extracted sugars, and longer chains require the hydrolysis of the inulin followed by end group analysis (17, 19, 23). Even to achieve the analysis of short chain oligomers requires the use of apolar columns capable of heating to 440ºC (19, 23).

Linkage and branching of inulin has been determined by permethylation followed by reductive cleavage of the polymer and then acetylation. Subsequent GC, with detection by either flame ionisation (23) or mass spectroscopy (64, 100), is able to separate and identify the constituent sugars with varying methylation patterns, allowing branching to be determined. Similar techniques have also been used to determine the quantity of reducing sugar in polysaccharides, by labelling the reducing end group through redox chemistry prior to hydrolysis (95).

Gel permeation chromatography

Gel Permeation Chromatography (GPC) or Size Exclusion Chromatography (SEC) has also been used to determine the DP of inulin (54, 79, 90, 103). The same issues arise as with other chromatographic methods, with an inability to resolve larger polymers (maximum resolution being up to a DP of about 10) and also with calibration (79, 90, 98). Praznik *et al.* (79) tried to combat these problems by using polymaltotriose as a calibrant on a polyacrylamide column. For higher molecular weights two different columns in series were required, ostensibly providing separation in the range of 200-50,000 using the glucan series as a calibrant (79). Though individual polymers can still not be resolved by such analysis, at least the elution is progressive, allowing reasonable calculations of polydispersity (79, 103).

Microbial high molecular mass inulin

Very high molecular mass inulin, isolated from bacteria and fungi, has been analysed by GPC and found to have a small root-mean-square radii of gyration with respect to their molecular mass, suggesting a compact molecular conformation (69, 71). Theoretical prediction and experimental evidence both agree that a globular shape is likely (69). The globular shape is here predicted to be created by branching (5-7%) and indeed variation between the branching architectures of the bacterial and fungal inulins are predicted by this work, despite both forms having the same amount of branching (69). A separate theory suggests that if the inulin is essentially linear, due to limited branching, or if the branches are short then intramolecular supramolecular associations between distant residues can also cause the globular shape (71).

Molecular organisation of inulin

There is great variation in the molecular conformations for inulin for the shortest oligomers with DP of below about nine (75, 98). It seems likely that some significant organisation starts with oligomers of DP 4 and 5 that are predicted to favour a cyclic (75, 99), or single helical structure (98). Oligomers of DP 6-8 have organised though less well determined conformations (98), and this change in structure is responsible for the unusual sequence of chromatographic elution in this DP range (discussed previously in the section on High Performance Liquid Chromatography) (23). This means the increase in chain length through this range of DP provides some significant alterations to molecular packing such that the polarity exposed to solution and/or the shape of the oligomer changes sufficiently to affect chromatography (75). Inulin oligomers with a DP of 9 and above form a regular helical structure, argued both to be five- (75, 97) and

six-fold helices (72). The right-handed six-fold helical structure has generally become accepted now. The helical structures of each chain are able to pack together into a range of isoforms, the nature of which is dependant on the kinetics and thermodynamics of formation.

Crystal structure of inulin

The more organised, crystalline structures of inulin have been analysed by X-ray and electron diffraction studies. This data has been used in combination with theoretical considerations (65, 72, 74, 78, 97) to predict an isotactic arrangement of fructosyl units about the polymer backbone (97), as well as, the helical structure (72, 74, 97). Electron diffraction studies showed that helical crystalline inulin exists in two polymorphs, a hemihydrate and a monohydrate (72). The hemihydrate form has one water molecule for every two fructosyl subunits (72) and is created by vacuum drying of the hydrated form, removing all the labile water molecules with the remaining water being securely bound in the crystal structure (74). Due to the labile nature of the extra proportion of the water contained in the monohydrate the electron diffraction results were less reproducible, but suggest that it has one water molecule for each fructosyl unit (72). It is the monohydrate that is likely to be significant for medicinal aspects of inulin, and its crystal structure has been worked out as a unit cell containing two anti-parallel right-handed helices of six fructosyl residues per turn, made into a rigid packing structure by six intermolecular hydrogen bonds, in some cases via water molecules (72).

Crystalline isoforms of inulin

Perhaps the most useful property of inulin from a medicinal viewpoint is its occurrence in multiple, distinct molecular packing structures (polymorphs or isoforms). Precipitation from ethanol yields the β form while water yields inulin at room temperature or lower (7, 54, 66, 104, 105). Theoretical studies show that many forms are allowable (69, 70). In practice it was found that both α- and β-forms were unstable in water, soon progressing (54) to a novel third form ( ) that was largely insoluble at body temperature. This property allows a range of strong biological effects (12, 13, 15). A fourth isoform, (56) which is even more active has now been identified. These isoforms are all soluble at concentrations of 10-15% w/w at temperatures below 75°C, enabling their ready purification for clinical use with minimal hydrolysis. They are conveniently distinguished by their solubility temperatures in water (Figure 4), representing a phase-shift as abrupt as a melting point.

The isoforms comprise an increasing series in the sequence β-α- -δ, in which lower isoforms are converted to higher ones at specific temperatures, and all higher isoforms can be returned to lower by complete dissolution and re-crystallisation. Thus the structures are formed by reversible bonding rather than covalently, and higher isoforms have a higher mean DP (54). The isoforms spontaneously assume a characteristic molecular packing structure, presumably determined by hydrogen bonding, when crystallised at specific temperatures. This unusual behaviour is attributed to the flexible polyoxyethylene-like polymer backbone (55), allowing multiple

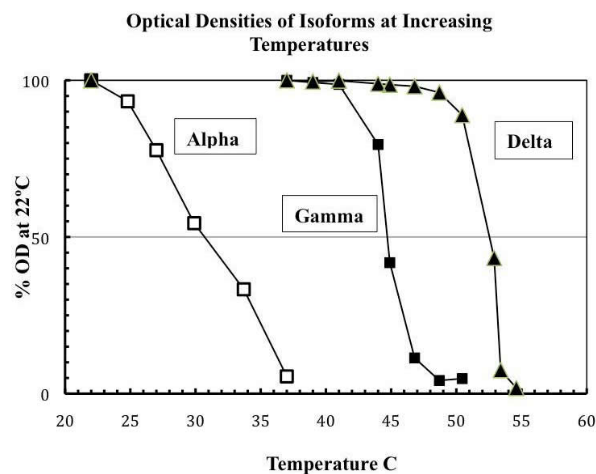

Figure 4 The temperature solubilities of inulin isoforms illustrated by the changes in turbidity of dilute suspensions (<0.5 mg/mL) at different temperatures. The isoforms are distinguished by several parameters that compare strengths of intermolecular non-covalent bonding

conformations both thermodynamically and kinetically favoured. The structural implications of the existence of such isoforms are of considerable interest.

Inulin particles of the various isoforms normally crystallise from water as ovoids of 1-10 μm diameter (14, 54) if the suspension is briskly stirred. Particle size is determined by a number of factors including temperature, inulin concentration, ionic strength and rate of stirring. The isoform of inulin has been exploited (56) to form a range of much smaller particle sizes. In this method the δ particles are partially dissolved, when they break into many very small fragments that in turn act as crystallisation micronuclei to grow a larger number of particles from the same weight of inulin. The new particles are accordingly much smaller. By further fragmenting the micro-nuclei while hot by shear stress (passing under pressure through a small orifice) or by ultrasonication, the particle size is further reduced to form a wide range of sizes down to 100 nm in a manner controlled by the degree of shear stress introduced. This is expected to expand the potential uses of inulin.

Solubility and gelation of inulin

The variation in molecular conformation, along with DP, affects solubility and short chain oligomers of inulin are relatively soluble in aqueous solution being soluble at up to 80% concentration (28). Longer chain inulin polymers are much less soluble and will precipitate in the crystalline forms discussed in the previous section. Due to a degree of surfactant character inulin can also gelate aqueous solutions from >25% inulin in water for shorter chains, and from >13% for longer chains by cooling a hot dissolved solution or by shearing inulin suspensions (22, 23, 27). These processes form a gel network of crystalline particles of about 100 nm diameter that aggregate to form larger clusters of 1-5 μm (23, 28), which trap a large amount of water in the network. It has been shown that the thermal methods used to produce inulin gels create stronger and smoother gels than the shearing techniques used more usually in industry due to the generation of smaller, more narrowly distributed particle sizes (27). A range of other variables also affect the character of the final gel including inulin concentration, pH, molecular weight and solvent (27, 28).

Inulin gels are important for several applications, currently the most commercially relevant being in food production, where it is used as a low calorie, bulking agent, replacing fat, sugar and flour, while providing valuable dietary fibre (1, 3-5). Inulin gels imitate fat extremely well in terms of texture, mouth-feel, glossy appearance and balance of flavour release, with longer chain inulin polymers providing the best results in terms of these characteristics (23). Importantly, inulin exhibits synergistic effects with most gelling agents, many being used in food production, together creating stronger gels than the sum of the components (22, 23).

The prospective use of inulin gels in drug delivery means that improvement to gel characteristics and to suitability of their chemical nature have been made through chemical modification (26, 31, 50, 51). Such modifications include functionalisation with vinyl groups for subsequent cross-linking reactions to stabilise the gel structure (50, 51). Inulin gels have also been cross-linked by derivatisation with methacrylic anhydride and succinic anhydride, followed by UV irradiation. In this case gel swelling is pH sensitive, ideal for drug delivery to the colon (26). Supramolecular associations between inulin acetate and 1,12-dodecanedicarboxylic acid have also been used to stabilise inulin gels for drug delivery (31).

Amorphous inulin

Industrial inulin raw material produced by the convenient and cost effective spray drying procedure is often amorphous (106, 107). Such a structure is not kinetically in equilibrium

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because of the rapid drying process (107), and when heated above the glass transition temperature the amorphous solid can reorganise into crystalline forms. The glass transition temperature can also be lowered by water absorption, the water acting as a plasticizer for inulin (3).

Analysis of relative crystallinity of inulin

The state of inulin has significant effect on the utility and stability of the product, amorphous and semi-crystalline materials have the highest industrial value (107, 108). Crystallisation of amorphous and semi-crystalline inulin by water absorption or heating results in a reorganisation of molecular structure, leading to caking (3, 26, 108). Caking of particulate inulin is due to extrusion of water which is no longer accommodated in the more organised structure and then absorbed onto the surface of neighbouring particles, creating a liquid interface between them. This sticks the particles together (108). Caked inulin is undesirable both as a raw material and in final products, as the crystalline/amorphous balance and processability are changed (108). As such, this transition between amorphous and crystalline inulins has been extensively investigated.

One of the most important properties in understanding the relative crystalline or amorphous qualities of a polymer is the glass transition temperature (T_g). The glass transition is an effect noticed in solid polymers with at least some amorphous character in which there is a reorganisation of polymer chains into more ordered crystalline forms at a temperature below the melting point. This transition is easily identifiable by various techniques and may be altered by a number of parameters such as the use of plasticisers and curing.

Plasticising effect of water on inulin

One of the most investigated effects on the T_g of inulin is the plasticising effect, or reduction in T_g, caused when water is absorbed by amorphous inulin (3, 65, 78, 106, 108). Water is known to act as a plasticizer in hydrophilic polymers such as gelatin (109). In such polymers, when reducing the temperature below 0ºC, there is often water that is able to crystallise (unbound water) and water that cannot be crystallised (bound water) (109, 110). It is the uncrystallised water that effectively mobilises the polymer chains, leading to the reorganisation and plasticising effect (65). For inulin this effect is significant with T_g going from 110ºC to -10ºC as water content increases from 0-15 g per 100 g of inulin in an essentially linear fashion (65). Crystallinity, as determined by Wide Angle X-ray Scattering (WAXS) analysis (see below), continues the trend observed in T_g showing that amorphous content disappears at 15.7 mg/100 mg and that the crystalline character of inulin continues to increase up to 18.8 g of water to every 100 g of inulin (106). This means that when sufficiently wet, the T_g of inulin can be depressed to the extent that crystallisation can occur at room temperature. Of course, given the multiple isoforms for crystalline inulin, this process is not a simple one.

The complexity of the crystallisation process due to the plasticising effect of water has been investigated using Differential Scanning Calorimetry (DSC). Semicrystalline and crystalline samples often exhibit multiple endotherms for melting transitions between 170ºC - 180ºC, due to the multiple crystalline isoforms of inulin. For humidity of 75% and above for semicrystalline samples a third melting endotherm develops at ~160ºC due to extra crystallinity induced by the reordering possible in a hydrated sample (3). Meanwhile an amorphous sample exposed to such humidity will only develop a single melting endotherm at ~160ºC (3, 108).

X-ray diffraction and scattering techniques have also been used to investigate the plasticising effect of water on amorphous inulin (65, 78, 106, 108). X-ray diffraction studies showed that crystallinity increases with increasing water content (65, 78, 106). Subsequent removal of water from samples crystallised by the

plasticising effect of water does not remove the crystalline character (65). More specific investigations made by WAXS showed crystallinity developing for amorphous inulin when stored in humidity of 75% or above. Indeed, at 94% humidity greater crystallinity is detected than that seen for a sample of precipitated crystals (108). The lower relative level of crystallinity observed for the precipitated samples is attributed to 'cross-linked' crystalline domains, constraining that portion of amorphous material caught between them from reorganising into crystalline structures. This constriction of the amorphous material prevents the glass transition from occurring before melting, superimposing the melting and glass transition peaks (106, 108).

Visual inspection of amorphous inulin particles by Environmental Scanning Electron Microscopy (ESEM) shows caking below 20ºC for humidity between 59% and 75% (108). This indicates that some molecular reordering involving the exclusion of water occurs at humidity lower than detectable using other techniques.

Effect of Curing on inulin crystallisation

Many investigations have been conducted trying to establish the effect of varying crystallisation regimes on the final make-up of the solid inulin. It has been found that when inulin is dissolved in water at 96ºC, then cooled slowly to room temperature, and held at this temperature for one hour, DSC shows two endotherms for the dispersed mixture between 40ºC and 90ºC that are indicative of crystalline melting (66, 107, 108). After 48 hours curing at room temperature two additional endotherms between 40ºC and 90ºC have reached maximum intensity (66). It seems such endotherms are intrinsically related to the four endotherms also found for the dry solid between 170ºC and 180ºC in DSC analysis (90, 91). These melting endotherms are attributable to different crystalline forms (107), possibly the four basic crystalline species previously described as α-, β-, γ- and δ-isoforms (13, 54, 56). In another process a solution was cooled from 96ºC to an intermediate curing temperature of 65ºC, where it was held for 12 hours, before slowly cooling to room temperature. Initially in this case, again, only two endotherms between 40 and 90ºC were observed using DSC, but only one additional endotherm formed in the same temperature range after storage at room temperature (66). This endotherm occurs at the same temperature of the highest temperature endotherm for inulin recrystallised without the hot curing stage and has enthalpy equivalent to the two highest endotherms found in the sample without hot curing (66). It seems likely that the hot curing procedure strongly favours crystallisation into only one of the high temperature isoforms of inulin.

The various melting transitions for crystalline inulin induced by curing and plasticisers are additionally affected by various parameters of the recrystallisation such as cooling rate, inulin concentration and average molecular weight of the analysed inulin. Indeed, to a significant extent the varied crystallisation forms fractionate inulin by molecular weight. Unfortunately there is co-crystallisation at both high and low temperatures with portions of low molecular weight material crystallising out with the high molecular weight material at high temperature and vice versa (66, 106). Despite co-crystallisation, the character of the various crystalline forms differs, with Small Angle X-ray Scattering (SAXS) revealing different long periods for structures crystallising at high and low temperatures (66). Overall, it is clear from these results that inulin has a complex character with multiple crystalline forms, the formation of which is determined by the kinetics and thermodynamics of the recrystallisation procedure (3, 66).

Effect on crystalline form of inulin by crystallisation method and DP

As already stated, crystalline inulin created by hydration has a melting point of ~ 160C and that created by precipitation melts between 170ºC and 180ºC (66, 67, 103, 107, 108),

depending on crystalline form and DP (103). Further investigation of this anomaly used samples crystallised by hydration and by precipitation with roughly equal crystalline content, both being established as the monohydrate (106). Heating the precipitated form in WAXS studies created a new peak attributed to the transition from the monohydrate to the hemihydrate. This same behaviour did not occur for the amorphous solid crystallised by hydration (3). TGA was used to follow the same phenomenon, in which samples crystallised by hydration exhibited a continuous mass loss from room temperature to thermal degradation. In contrast, the precipitated crystalline forms showed loss of bound water mass up to 95ºC, where the thermogram levelled off before thermal degradation. The onset of this plateau corresponds to transition from monohydrate to hemihydrate as determined by WAXS (3). Furthermore, precipitated crystalline samples dehydrated of labile water exhibit a mass loss at 160-180ºC attributed to the release of water from the hemihydrate crystals upon melting (3). These results show that there is a difference in the mobility of the water in the crystals created by different methods.

Another variation in the crystallisation of inulin is found between samples with different molecular weight. DSC of chains with differing chain length, separated by chromatography, shows an increasing melting point with increasing chain length (67). These authors theorize that for shorter chains the relative amount of glucosyl end groups increases increasing the number of defects in the inulin crystal. As molecular weight increases so does the perfection of the crystal allowing for thicker crystals to grow, and a consequent increase in melting temperature (67).

SPECTROSCOPIC ANALYSIS OF INULIN

Nuclear Magnetic Resonance spectroscopy

Nuclear Magnetic Resonance (NMR) spectroscopy is an invaluable tool in the analysis of organic compounds. Polysaccharides are an example of such compounds for which NMR analysis has been extensive (111-113), though the similar chemical environments of most of their detectable groups means that shifts overlap each other to a large extent (75). This effect is worsened in the case of inulin due to the lack of a proton on the anomeric carbon of the fructose component, a proton usually separated from the other related protons so diagnostically useful (75, 112). Efforts to reduce these issues have been attempted by improving resolution and enhancing signals of the NMR using a range of techniques. These include pulsed field gradients for suitably concentrated samples (111), utilisation of 3-D COSY, TOCSY and NOESY to ameliorate the effects of spectral overlap (111) and the use of high temperatures, which makes for longer acquisition times but increases resolution and shifts the signal of the solvent to a less interfering higher field position (112). Solvent selection is also important, deuterium oxide being able to resolve more peaks than deuterated dimethyl sulfoxide (DMSO-d_6), though solubility issues mean that DMSO-d_6 is often used for polymeric forms (112, 114). Accuracy of quantification of NMR analysis can also be improved by ensuring appropriate experimental set-up relative to T1 relaxation and amelioration of nuclear Overhauser effects by using a gated-decoupling pulse sequence (112).

Early experimentation seemed to focus on carbon NMR (90, 113-116), probably because the resolution of proton NMR was too poor with the relatively weak NMR machines then available. Nonetheless, carbon NMR has been important as it can differentiate inulin from other polysaccharides (116) and explore substitution effects on the fructose ring (115, 116), being able to identify and quantify branching (64). It can also differentiate lower oligomers of inulin up to about DP 4. This is because carbon shifts, particularly the fructose carbons closest to the polymer backbone, are sensitive to molecular conformation of the backbone which is dependant on DP (90, 98). Importantly much of this differentiation of peaks remains for the

first few fructose groups adjacent to the glucose unit and do not coalesce as with some other polysaccharides.(98) This makes possible analyses based on these terminal groups (112).

A similar effect described above for the variation in shifts for those carbons attached closest to the polymer backbone in fructose units positioned near the glucose unit is also observed for the attached protons (75). Combining these one-dimensional analyses with two-dimensional NMR has been used to accurately allocate NMR shifts for inulin (75). This assignment has allowed conclusions about the conformation of the structures of various oligomers to be made based on the positioning and movement of shifts for specific protons and carbons, predicting helical structures for larger oligomers (75). Similarly, NMR for each oligomer of DP from 2-12 was conducted in solutions increasing in the concentration of barium (II) cations (99). Addition of Ba^{2+} caused a change in chemical shift for several protons in the inulin chain that increased linearly with increasing salt concentration for a given oligomer. This effect, due to complexing of inulin with Ba^{2+}, was greatest on the chemical shifts for oligomers of DP 4 and 5. It was attributed to these chain lengths having a cyclic conformation in solution that closely matches that of cycloinulo-hexaose, which is known to strongly complex Ba^{2+} (99). From this it can be presumed that longer and shorter oligomers do not have the same ring-like conformation (99).

Fluorescence spectroscopy

Inulin characterised by fluorescence spectroscopy after binding hydrophobic pyrene molecules shows that the critical aggregation concentration (cac) is from 0.07-0.08 mg/ml in aqueous solution (5). This is determined by the reduction in fluorescence during binding between inulin and pyrene in solution. In contrast, the pyrene molecules are less able to find free hydrophobic binding spots in the aggregate and fluorescence remains high(5). The cac was marginally affected by the presence of various salts in the solution (5).

Inulin samples from different sources were analysed by capillary electrophoresis, being detected by a fluorescent tag (117). Difficulties are associated in not knowing how many times a sugar group has been tagged as all the hydroxyl groups are susceptible to the reaction, the primary hydroxyls reacting faster (117). Even so, the authors concluded that inulin from different sources can be identified by this method (117).

Ultraviolet-visual spectroscopy

Though Ultraviolet-Visual (UV-Vis) spectroscopy is of limited direct analytical value for polysaccharides, colorimetric response to reducing chemistry enables quantification of hydrolysed polysaccharides (102, 118, 119). It also allows determination of the amount of reducing ends in unhydrolysed samples (101, 120-127). For inulin, such quantification is important, not just for determination of the stability of inulin samples to reactions involving the reducing end, but also for accurate determination of molecular weight using end group analysis (17, 94, 95, 101). Wight *et al.* (101) used tetrazolium blue to quantify the amount of glucose-free chains in inulin samples so that end group analysis could be used to establish the DP from the HPLC of hydrolysed samples. Perhaps a more common reagent for the colorimetric detection of reducing sugars is dinitrosalicylic acid (DNS) (102, 121, 126) that has also been used to enable simple colorimetric measurement of inulin in mixtures containing other reducing sugars (102). In this case DNS was used to first determine the reducing sugar content of a sample before the amount of inulin was quantified by hydrolysis of the polymer by sulphuric acid in the presence of phenol (102). This research compared the colorimetric results with HPLC methods and found the HPLC method more precise and accurate due to being less affected by interfering compounds (102).

CONCLUSION

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Inulin is a natural polysaccharide with unique physicochemical properties that give it a range of uses in the food and pharmaceutical industry. A broad range of analytical tools has been applied to inulin's characterisation. An increased understanding of the chemistry and behaviour of inulin polymers has led to important new uses as a drug delivery vehicle, immuno-stimulator and vaccine adjuvant.

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