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Note

NMR characterisation of inulin-type fructooligosaccharides as the major water-soluble carbohydrates from *Matricaria maritima* (L.)

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Abstract—By use of ^{1}H and ^{13}C NMR spectroscopy including 2D $^{1}H,^{1}H$ DQF-COSY/TOCSY and $^{1}H,^{13}C$ HMQC/HMBC experiments, the main water-soluble carbohydrate components extracted from leaves of *Matricaria maritima* were identified as oligofructans composed of a linear chain of (2→1)-linked β-D-fructofuranosyl residues specifying an inulin-type structure.

$$\alpha\text{-D-Glc}p\text{-}(1 \rightarrow 2)\text{-}[\beta\text{-D-Fru}f\text{-}(2 \rightarrow 1)\text{-}\beta\text{-D-Fruc}f]_n\text{-}(2 \rightarrow 1)\text{-}\beta\text{-D-Fru}f$$

Keywords: *Matricaria maritima*; Fructans; Inulin

Fructans are fructose-based oligo- or polysaccharides present in many species of higher plants in which they serve as reserve carbohydrates, like the more familiar starch and sucrose.[1] The structural organisation of all known fructans is based on (2→1) or/and (2→6)-linked-β-D-fructofuranosyl units with one internal or external glucosyl residue.[2] Some non-digestible fructans, that is, those containing β-D-(2→1) glycosidic bonds, like inulin and inulin oligomers, potentially present interesting prebiotic properties for both food (human and pets) and non-food applications.[3] There is a growing interest in fructans, and then for fructans-accumulating species, but there is still a lack of information about their biological properties. However, recent advances underlined cough-suppressive and immunological activities of inulin-type oligosaccharides.[4] In addition, anti-tumor activity bioassays showed an inulin-induced inhibition of growth of Lewis pulmonary carcinoma implanted in mice.[5]

Matricaria maritima (L.) belongs to the botanic family of Compositae widely distributed along coastal areas of Brittany. Little information is available about this halophytic plant species and, notably, about key components involved in salt-stress tolerance. Numerous solutes including sugars (fructose, glucose, saccharose), polyols (mannitol, sorbitol, pinitol), quaternary ammonium (glycine betaine, β-alanine betaine) and tertiary sulfonium compounds (dimethylsulfoniopropionate) are known to act as osmolytes.[6] With the aim of better understanding the mechanisms of osmotic adaptation of *M. maritima*, detailed NMR studies were performed on hydroalcoholic extract of aerial parts of this plant species, which led to the identification of inulin-type oligofructans as the main water-soluble carbohydrates.

Hydroalcoholic extraction from powdered dry leaves of *M. maritima* and desalting through ion-exchange resins allowed a semi-purified extract representing ca. 50% of the dry mass of the plant powder to be obtained.

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The ^{1}H NMR spectrum of this extract (MmPE) (Fig. 1a) showed the presence in the anomeric region of two main signals at 5.44 (d, 3.8 Hz) and 5.42 ppm (d, 3.9 Hz), with minor signals at 5.24 (d, 3.2 Hz) and 4.67 ppm (d, 7.5 Hz). Intense signals were observed at 4.27 (d, 8.8 Hz), 4.11 (t, 8.8 Hz), and in the region between 3.68 and 3.98 ppm. HPLC analysis of MmPE revealed the presence, in order of increasing importance, of glucose, fructose and sucrose. This observation was confirmed by the comparison of retention times and co-injections with authentic standards. ^{1}H NMR spectra on the individually collected compounds were also performed, thus confirming the identification and allowing the assignment of the ^{1}H NMR signals at 5.42, 5.24 and 4.67 ppm to H-1 protons of the α-Glc unit of sucrose and the α- and β-anomeric forms of free glucose, respectively. Beside these minor components, the main fraction observed on the HPLC chromatogram showed a retention time shorter than that of sucrose. Taking into account that the specificity of the column is based, in part, on the principle of size exclusion, that is, larger molecules elute earlier than smaller ones, and the global shape of the corresponding peak, it was envisaged that this fraction had molecular weight higher than that of sucrose, and was heterogeneous, probably reflecting different degrees of polymerisation. ^{1}H NMR analysis of the corresponding peak (MmF01) showed the presence of one signal in the anomeric region at 5.44 ppm (d, 3.8 Hz), the other previously identified preponderant signals at 4.27 and 4.11 ppm and those between 3.68 and 3.98 ppm (Fig. 1b). The anomeric region of the *J*-modulated ^{13}C NMR spectrum of MmF01 was more complex with one major signal at 104.3 ppm and three minor signals at 93.6, 104.2 and 104.8 (Fig. 2a). From this spectrum, the carbon atom signal at 93.6 ppm was assigned to an aldose type residue whereas those beyond 100 ppm pointed to ketose residues. From the 2D spectra of MmF01, chemical shifts of the protons and carbons of the preponderant residue were fully assigned and were shown, through a comparison with literature

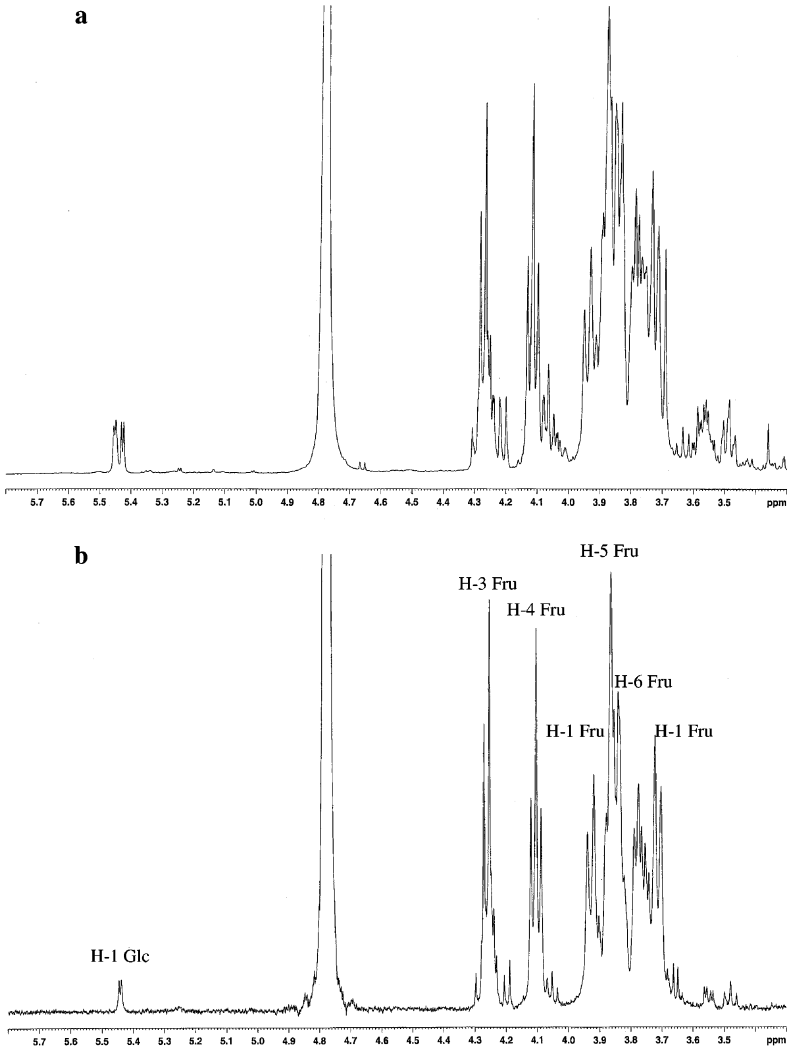

Figure 1. ^{1}H NMR spectra of MmPE (a) and MmF01 (b).

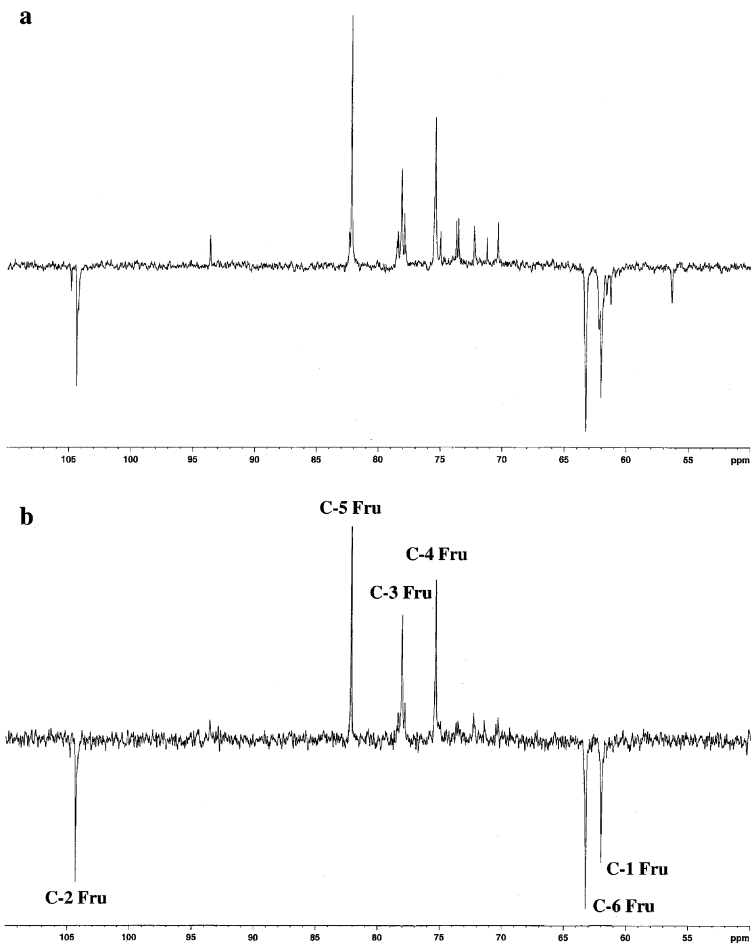

Figure 2. *J*-modulated ^{13}C NMR spectra of MmF01 (a) and MmF02 (b).

data,[7,8] to belong to a D-fructofuranosyl residue with a β-anomeric configuration (Table 1). From these spectra, the spin system corresponding to the residue with $^{1}H/^{13}C$ anomeric signals at 5.44/93.6 ppm was also unambiguously assigned to a D-glucopyranosyl residue with an α-anomeric configuration (Table 1).

Minor residues with ^{13}C anomeric signals at 104.8 and 104.2 ppm were also attributed to fructosyl residues but only approximative chemical shifts were obtained due to signal overlap with the major spin system. Taking into account the generally accepted structures of fructans, these observations allowed to consider a fructan-like structure for MmF01. In addition, proton integration of the H-1 signal of the glucose moiety at 5.44 ppm and the H-3 and/or H-4 signals of the preponderant fructosyl residue at 4.27 and 4.11 ppm, respectively, suggested a mean DP of about 10–11 for this fructan-like structure. A characteristic feature of fructans is the presence of a single glucosyl residue either internal and 6-substituted or located in a terminal position. From the HMBC spectrum, the occurrence of a cross-peak between H-1 of the glycosyl residue and C-2 of a fructosyl residue and the concomitant absence of correlations between C-2 of a fructosyl residue and H-2, 3, 4 or 6 of the glucosyl residue, favoured a terminal position for this latter residue. Moreover, the absence of characteristic downfield shifts for C-6 of the glucosyl residue, that is, absence of signal beyond 65 ppm, was also indicative

Table 1. ^{1}H and ^{13}C NMR chemical shifts of *Matricaria maritima* fructan

Residue[a]	H-1/C-1	H-2/C-2	H-3/C-3	H-4/C-4	H-5/C-5	H-6/C-6
→1)-β-D-Fru*f*-(2→	3.72–3.96	–	4.27	4.11	3.88	~3.79–3.86
	62.0	104.3	78.1	75.4	82.2	63.2
α-D-Glc*p*-(1→	5.44	3.55	~3.79	3.48	3.86	~3.86
	93.6	72.4	73.8	70.5	73.9	~61.3

[a] Due to strong overlapping signals, minor fructosyl signals that is, those belonging to the glucose-linked and terminal residues were omitted.

of the absence of glycosylation at this position. In order to access to the detailed structural organisation of the fructan, i.e., type of linkage between the main fructosyl residues that is, 2→1 and/or 2→6, and, more especially, to determine whether the minor signals represented ramifications or not, graded EtOH precipitation was performed on MmF01 allowing the isolation, inter alia, of a fraction (MmF02) with a mean DP of about 20–22. The *J*-modulated ^{13}C NMR spectrum of MmF02 was rather simple with six main signals at 104.3, 82.2, 78.1, 75.4, 63.2 and 62.0 ppm (Fig. 2b). This observation, combined with the higher DP estimated from integration of the signal at 5.44 and those at 4.27 and/or 4.11 (as described below) in the ^{1}H NMR spectrum of MmF02, was in favour of a linear structure and implied that the fructosyl residues involved in the minor signals were not ramifications on the main chain but probably arose from the sucrose moiety and from the terminal residue of the fructan. Intra- and interresidual connectivities in the HMBC spectrum allow to distinguish between H-1/C-1 and H-6/C-6 hydroxymethyl signals. Furthermore, a strong C-2/H-1 correlation with the concomitant absence of C-2/H-6 correlations and carbons signals beyond 65 ppm (Fig. 3), established a (1 → 2) linkage between fructosyl residues in agreement with an inulin-like structure for MmF02. Moreover, the comparison of ^{1}H and ^{13}C NMR spectra of *M. maritima* fructans with recent work on inulin-type oligofructans reinforced this interpretation.[4,9,10]

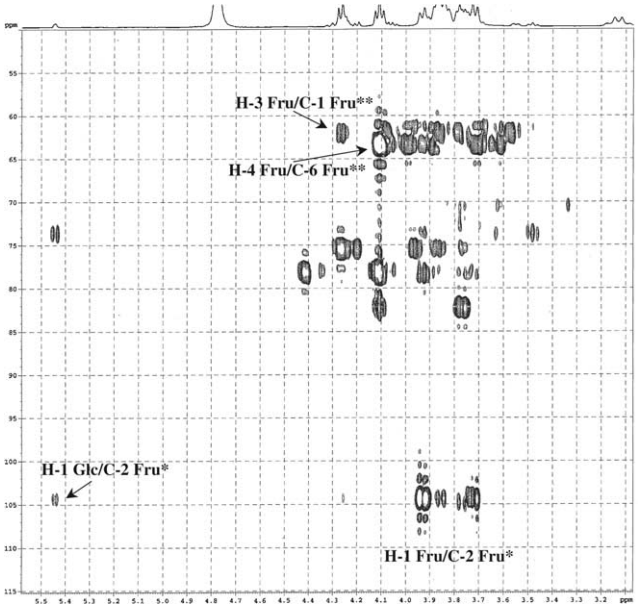

Figure 3. HMBC contour plot of MmF02. *Interresidue connectivities: H-1 Glc/C-2 Fru connotes the cross-peak between H-1 of the glucosyl residue and C-2 of a fructosyl residue, etc.; **intraresidue connectivities: H-3 Fru/C-3 Fru connotes the cross-peak between H-3 of the fructosyl residue and C-3 of the same residue, etc.

Taking into account all the information obtained from the NMR spectra of MmF01 and MmF02, it was possible to establish the presence of inulin-type fructooligosaccharides as the major component of *M. maritima* extracts. It has been suggested that these components could be implicated in the resistance of plants to water deficit caused by drought or low temperature,[11] so these fructans could act as osmoprotectants in this plant species when subjected to salt-stress. Moreover, these results allow *M. maritima* to be considered as a source of inulin-type fructooligosaccharides.

1. Experimental

1.1. Plant material

M. maritima plants were harvested in coastal sites of North Brittany, in mid-winter 2004. Aerial parts of freshly collected samples were rapidly cut-off, washed with deionised water, blotted dry, weighed, stored at −20 °C and then lyophilised. The dry material was ground to yield a fine powder stored under N_2 until use.

1.2. Extraction of the water-soluble carbohydrates

The dry powder (~6.5 g) was extracted with 100 mL of a 7:3 water–EtOH mixture under magnetic stirring for 30 min at room temperature. After centrifugation for 10 min at 5000*g*, the resulting pellet was extracted twice following the same protocol. The supernatants were combined, filtered over glass wool and concentrated by rotary evaporation under diminished pressure to give the crude extract. This extract was further purified by passing successively through AG 50W-X8 (H^+ form, 20–50 mesh, Biorad) and Amberlite IRA-410 (OH^- form obtained by conversion of the Cl^- form with NaOH, 20–50 mesh, Fluka) ion-exchange resins, the whole system being washed with 4 vol of deionised water. The resulting effluent was then lyophilised to give ~2 g of a purified extract used for further analysis.

1.3. Isolation of the highest DP oligofructans

Fractionation was performed on a 20 mg/mL soln of the purified extract by graded EtOH precipitations. Briefly, 1 vol of cold abs EtOH was added and the mixture was kept, after shaking, at −25 °C for about 1 h. The precipitated material was recovered by centrifugation (5 min at 3000*g*) and 1 vol of cold abs EtOH was again added to the resulting supernatant which was also placed at −25 °C for about 1 h. This operation was repeated once more. The last step corresponds to the addition of 8 vol of abs EtOH on the last supernatant. The precipitated material was recovered as described. Each pellet was resolubilised in a minimum of deionised water and frozen

before lyophilisation. The final supernatant was concentrated under diminished pressure in order to remove residual EtOH and lyophilised before NMR analyses.

1.4. HPLC analyses

Identification of the components present in the partially purified extract was performed on a Waters HPLC system consisting of a 717plus autosampler, a 600E pump and the Millenium Chromatography Manager software. The detection system consisted of a refractive index detector (Jasco-930) heated at 40 °C. Injection volumes of 2% solns (w/v) in 5 mg/mL Ca–EDTA in ultra pure water and filtered on Minisart RC 4 0.45 μm (Sartorius), were 50 μL. Separation was performed on a Sugar-Pak (Waters) column (6.5 × 300 mm) with stationary phase composed of a sulfonated styrene divinylbenzene resin in calcium form. The mobile phase, degassed by vacuum filtration through a Millipore HA, was 5 mg/mL Ca–EDTA in ultra pure water. The flow rate, in isocratic mode, was 0.5 mL/min and the column temperature was maintained at 90 °C. Pre-column inserts were used in order to prevent damage to the column. Mono- and disaccharides were identified by comparison of their retention time with authentic standards and confirmed by NMR spectroscopy after collection and accumulation of the corresponding peaks. The earlier eluting, higher DP components were also collected and analysed by NMR after several accumulations, lyophilisation and solubilisation in D_2O.

1.5. NMR studies

Samples were dissolved in 600 μL of 99.95% D_2O. One- and two-dimensional NMR spectra were recorded in D_2O, at 298 K and 500 MHz on a Bruker DRX-500 spectrometer using standard pulse sequences available in the Bruker software (Bruker, Wissembourg, France). Chemical shifts were expressed in ppm relative to 2,2,3,3-tetradeuterio-3-(trimethylsilyl)-propanoic acid sodium salt used as an internal chemical shift reference at 0 ppm. ^1H NMR signals were assigned by use of COSY and TOCSY experiments. ^{13}C resonances were assigned from the ^1H–^{13}C correlations observed in the HMQC spectrum. Intra- and interresidue connectivities were established by means of a HMBC experiment.

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