Inulin Polysaccharide Having Pendant Amino Acids: Synthesis and Characterization

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ABSTRACT: Inulin polysaccharide was esterified with *N*-protected α-amino acids (*N,N′*-di-benzylocarbonyl-L-lysine and *N*-benzylocarbonyl-glycine) under a mild condition (room temperature) and within short reaction times (6 h). The esterification reactions were conducted in the presence of dicyclohexylcarbodiimide and 4-(dimethylamino)pyridine as a catalyst. The optimal reaction time (6 h) was determined by monitoring the concentration of free carboxylic acid of the *N*-protected amino acids during the reaction. The degree of substitution per fructose unit was 0.95 for inulin-lysine and 1.01 for inulin-glycine. The resulting biopolymer was deprotected by catalytic transfer hydrogenation method using 1,4-cyclohexadiene as an effective hydrogen donor. The structures, molecular weight, and thermal properties of the amino acid esters of inulin were determined by Fourier transform infrared spectroscopy, ^{1}H and ^{13}C NMR, UV, viscosity, and dicyclohexylcarbodiimide. This new modified inulin polysaccharide would have the potential as a biomaterial for biomedical applications. © 1998 John Wiley & Sons, Inc. J Appl Polym Sci 70: 953–963, 1998

Key words: inulin; amino acids; dicyclohexylcarbodiimide (DCC); esterification of inulin; biomaterials

INTRODUCTION

Naturally occurring polysaccharides are one of the most abundant and diverse families of biopolymers, and are widely used in various fields.[1] Recently, considerable attention has been focused on the chemical modification of polysaccharides to prepare hydrogels for biomedical applications,[2–4] to improve biological activities,[5] to alter physical and chemical properties,[6] and to be used as drug carriers for the controlled release system.[7–9] The repeating unit of polysaccharides may contain hydroxyl, carboxyl, amino, or sulfate functional groups that provide the sites for the chemical modifications of polysaccharides.

Inulin is a class of polysaccharide composed of β-(2 $\rightarrow$ 1)-linked fructose units. In the food industry, inulin of low molecular weight can be used in fine bakery and confectionery products. In addition to these applications in foods, inulin and fructose may be the raw materials for the synthesis of miscellaneous chemical compounds,[10] particularly in the biomedical field. Currently, inulin uses in medicine include being a test reagent for measuring renal function[11] and a drug carrier in the controlled delivery system.[12,13] In the latter application, a few chemical modifications of inulin have been reported as potential drug carriers, such as the modification of inulin with either epichlorohydrin[14] or succinic

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anhydride[15] to provide reactive sites on inulin for its coupling with drugs.

Covalent bonding of amino acids to polymers in the main chain[16–22] or in the side chain[23–25] are another example of chemical modification of polymers for biomedical use. Because amino acids are naturally occurring compounds, polymers based on amino acids are expected to be nontoxic, biocompatible, and biodegradable. Wirth and Tixier[26] reported the fixation of nitrogenous materials, such as amino acids, to polysaccharides through the use of dialdehydes. Ngo[27] disclosed the coupling of organic ligands, such as amino acids, to a polymeric carrier containing at least one hydroxyl groups by activating the polymeric carrier with 2-fluoro-1-methylpyridinium toluene-4-sulfonate under mild reaction conditions. We have previously reported the covalent attachment of amino acids onto aliphatic polyesters like poly(lactic acid) and poly(glycolic acid).[23] poly(lactic acid) and poly(glycolic acid) were treated with lithium diisopropylamide at a low temperature to generate carbanions on the α-carbons of the polyestered backbone molecules and followed by the coupling of the acid chloride portion of *N*-protected amino acids onto the polyesters backbone chains.

In the present article, we describe a different chemical means to attach amino acids to inulin polysaccharide. We adopted the use of the dicyclohexylcarbodiimide (DCC) method that has frequently been used as a condensing agent for the preparation of esters[28] and peptides[29] in organic/peptide chemistry. Grosse and colleagues[30] reported that syndiotactic poly(2-methallyl alcohol) was esterified with *N*-protected amino acids by the DCC/1-hydroxybenxotriazole method at relatively drastic conditions (50°C, 140 h). Arnold and coworkers[31] prepared poly(carboxylic acid)s bearing pendant cholesteryl esters by using DCC as a coupling agent. In this article, the resulting amino acid esters of inulin would have terminal primary amino groups that could be more reactive toward acylating agents than the hydroxyl groups that are present on the unmodified inulin. This higher reactivity of the amino acid residue of the modified inulin could open a new dimension of potential use of inulin by attaching a variety of biomolecules to the polysaccharide *via* an array of linkages through the pendant amino acids, such as amide, urethane, urea, and secondary amine. In addition, polymers containing amino acid moiety may have many useful applications, such as a starting material for liquid-phase peptide synthesis,[32] chelating agents for metal ions,[33] and promoieties for attaching the biologically active Arginine-Glycine-Asparatic acid (RGD) peptide.[34]

Therefore, this article reports the esterification of inulin with *N*-protected lysine and glycine by the DCC/4-(dimethylamino)pyridine (DMAP) method under mild conditions. A study of the deprotection of *N*-protected amino acids bound to inulin was also investigated by the method of catalytic hydrogenation.

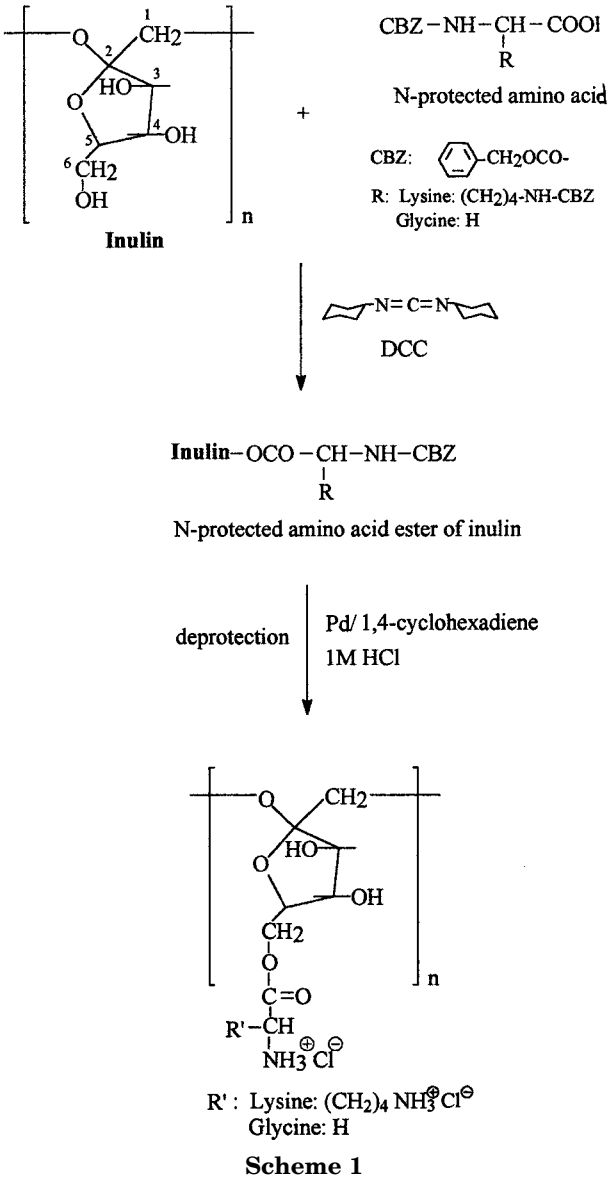
EXPERIMENTAL

Materials

A commercial inulin (from dahlia tubers) was purchased from Sigma Chemical (St. Louis, MO). The protected amino acids [i.e., *N,N′*-di-benzylocarbonyl (CBZ)-L-lysine and *N*-CBZ-glycine, were also purchased from Sigma and used without further purification. DCC, DMAP, and palladium (10 wt %) on activated carbon and 1,4-cyclohexadiene were purchased from Aldrich Chemical (Milwaukee, WI). Pyridine and *N,N′*-dimethylformamide (DMF) were purified by distillation under reduced pressure over calcium hydride and stored over 4A molecular sieves until use. Tetrahydrofuran (THF) was distilled from a sodium/benzophenone under nitrogen. All other solvents and chemicals were obtained from commercial sources and were used without further purification.

Coupling of Amino Acids onto Inulin Polysaccharide

A solution of the *N*-protected amino acids (8 mmol, *N,N′*-di-CBZ-L-lysine or *N*-CBZ-glycine), DMAP (2 mmol), and the inulin (4 mEq of anhydro fructose units) in dried DMF was cooled with stirring in an ice bath. DCC (4 mmol) was added, and the reaction mixture was stirred at 0°C for 2 h and at room temperature for 4 h. Dicyclohexylurea byproduct was filtered out, and the solution was poured into a large amount of cold double distilled water to precipitate the product. The precipitated product was filtered and washed several times with cold distilled water. It was then purified by reprecipitation using DMF as solvent and cold distilled water as precipitant. This purification was repeated twice. Finally, the amino acid esters of inulin were filtered using an aspirator



Scheme 1

and then dried in vacuo at 30°C for 48 h in the presence of phosphorus pentoxide. This covalent attachment of *N*-protected amino acids to inulin through ester bonds was illustrated in Scheme 1.

Deprotection of the Amino Protecting Group of Amino Acid-Coupled Inulin

A 10 wt % palladium-on-charcoal catalyst (4 g) was added to a 30 mL solution of the *N*-protected amino acid ester of inulin (1 g) in DMF. With vigorous stirring, 1,4-cyclohexadiene was slowly added to the mixture. Stirring was continued at 25°C, and the progress of the reaction was monitored by taking thin-layer chromatograms. After 12 h of stirring, the catalyst was removed by filteration and the solution was concentrated to approximately one-fourth the volume under reduced pressure (water spirator). The concentrated solution was then mixed with 1*M* HCl (20 mL) to form a stable formate salt of hydrochloric acid. Finally, the solvent was evaporated under reduced pressure, and deprotected inulin was obtained.

Characterization

Fourier transform infrared (FTIR) spectra were obtained on a Nicolet Magna 560 FTIR spectrophotometer and Nicolet data station with OMNIC 3.1 software with a resolution of 2 cm^{-1} in the region of 4000–500 cm^{-1}. The FTIR spectrum of the original solid inulin was taken as a dispersion in Nujol cast between two KBr plates. All other samples were cast on KBr discs from chloroform solutions. ^{1}H NMR spectra were recorded with a Varian Unity 500 operating at 400 MHz. ^{13}C NMR spectra were obtained at 100 MHz with a Varian VXR 400 spectrometer. All of the chemical shifts were reported in parts per million (ppm) using tetramethylsilane as an internal standard for both ^{1}H and ^{13}C NMR spectra. The sample tube size was 5 mm, with a sample concentration of 10 mg mL^{-1} in $CDCl_3$ or dimethylsulfoxide (DMSO)-d_6. A Cary 5 UV-visible spectrophotometer with 1-cm quartz cuvettes was used to obtain UV spectra at a scanning rate of 60 nm min^{-1}. Pure solvent (chloroform) was used for background subtraction and all spectra were taken at room temperature. Inherent viscosity was measured with DMSO at a concentration of 0.5 g dL^{-1} using Ubbelohde viscometer at 30 ± 0.05°C in a water bath and an extra temperature controller. Thermal properties were obtained by differential scanning calorimetry (DSC) conducted with a Perkin–Elmer DSC-7 under nitrogen purging at a heating rate of 10°C min^{-1}.

Amino Acid Analysis

Amino acid analyses were performed on an Applied Biosystems model 420/130 Derivatizer/Amino Acid Analyzer at the New York State Center for Advanced Technology, Cornell University (Ithaca, NY). The hydrolysis was performed using a Waters Pico Tag Hydrolysis Workstation with 12*N* HCl/propionic at 150°C for 80 min.

Table I Reaction Conditions and Results of Esterification Reactions of Inulin with *N*-Protected Amino Acids in the Presence of DCC and DMAP

	Reaction Conditions							UV
	[OH][a]	[COOH][b]	[DCC]	[DMAP]	Solvent[c]	Yield (%)	D.S.[d]	λ_{max} (nm)
Z-Gly-Inu	0.004	0.008	0.008	0.004	5 mL	72	1.01	260
Z-Lys-Inu	0.004	0.008	0.008	0.004	5 mL	67	0.95	259

[a] Concentration of hydroxyl groups in inulin.
[b] Concentration of carboxylic acid groups in amino acids.
[c] DMF.
[d] Calculated from amino acid analysis.

RESULTS AND DISCUSSION

Coupling of Amino Acids onto Inulin Polysaccharide

In this study, the protected amino acid esters of inulin were prepared by the DCC/DMAP method[35,36] under a mild reaction condition (at room temperature) and a shorter reaction time (6 h). The reaction conditions and the results of esterification reactions of inulin with *N*-protected amino acids are summarized in Table I. A 2-fold stoichiometric amount of the *N*-protected amino acids and DCC and an equimolar amount of DMAP were used for the synthesis of *N*-protected amino acid ester of inulin. Yield was in the range of 67 to 72%, and the degree of substitution of Z-Gly-Inu, Z-Lys-Inu was 1.01 and 0.95, respectively.

The optimal reaction time was determined by monitoring the concentration of free carboxylic acid of the *N*-protected amino acids during the reaction. Measurement of the concentration of free carboxylic acid was done by direct titration of aliquots from the reaction mixture. Figure 1 shows the reduction of the concentrations of the free carboxylic acids with time of reaction. The concentration was continuously decreased, and most esterification reactions were completed in 6 h at room temperature. This indicated that the esterification reaction started instantaneously upon the addition of DCC to form the *O*-acylurea, and the reaction rate was progressively reduced due to a decreasing amount of the available free —COOH in amino acids.

Structural Characterization of Modified Inulin

The structures of the *N*-protected amino acid esters of inulin were characterized by FTIR, ^{1}H NMR, ^{13}C NMR, and UV spectroscopy. As shown in Figure 2, the FTIR spectra of both *N*-CBZ-L-glycine-inulin (Z-Gly-Inu; B) and *N,N′*-di-CBZ-L-lysine-inulin (Z-Lys-Inu; C) showed the characteristic absorption bands of carbamate —NH— at ~ 3328 cm^{-1}, aromatic —CH— bands at 3000–3100 cm^{-1}, carbamate —CO— and amide I bands at 1656 cm^{-1} (Z-Gly-Inu) and 1661 cm^{-1} (Z-Lys-Inu), amide II type bands from carbamate at 1531 cm^{-1} (Z-Gly-Inu) and 1533 cm^{-1} (Z-Lys-Inu). Data also show new absorptions associated with the carbonyl (—CO—) of the ester bond linked

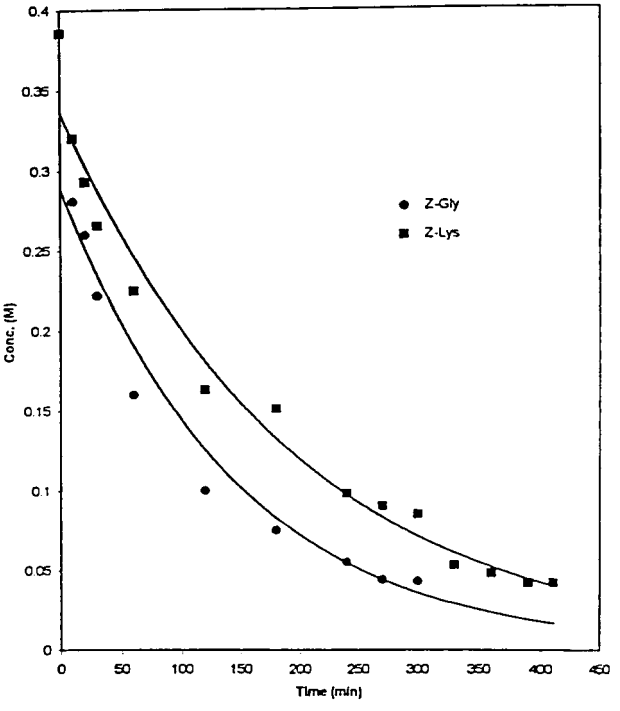


Figure 1 Reduction in concentration of free carboxylic acid of *N*-protected amino acids during their esterification reaction with inulin.

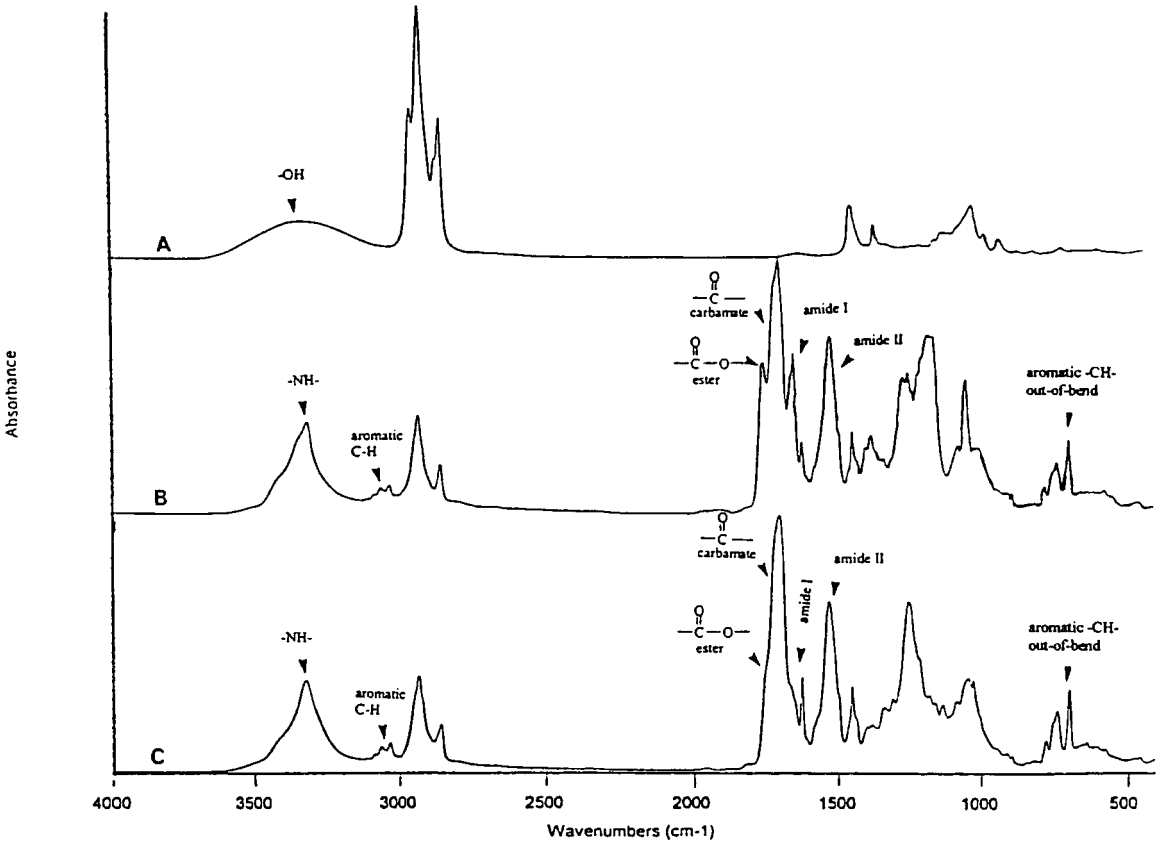


Figure 2 FTIR spectra of inulin and protected amino acid esters of inulin. (A) Inulin; (B) Z-Gly-Inu; (C) Z-Lys-Inu.

between inulin and amino acids at 1758 cm^{-1} (Z-Gly-Inu). However, the carbonyl (—CO—) of the ester bond of the Z-Lys-Inu appears as a broad band due to its overlapping with both amide I and the carbonyl of carbamate bands from the CBZ protecting group.

The ^{1}H NMR spectra of the same esterified inulin [Fig. 3(a,c)] show the complex multiplets in the region δ = 7.23–7.35 due to the protons of aromatic rings of the CBZ protecting group. The peaks at δ = 7.19 and 7.17 can be assigned to the carbamate —NH— protons of Z-Gly-Inu and Z-Lys-Inu, respectively. The peaks for the protons next to phenyl ring of the CBZ protecting group appear at δ = 4.99 (Z-Gly-Inu) and 5.00 (Z-Lys-Inu). There are also broad bands between δ = 3.4 and 5.2, which are due to protons linked to the inulin backbone.[37]

The ^{13}C NMR spectra (Fig. 4) show the characteristic chemical shifts at δ = 170.34 (Z-Gly-Inu) and 173.86 (Z-Lys-Inu) corresponding to the carbonyl carbon atom of ester groups. The peaks δ = 156 can be assigned to the carbonyl carbon atom of carbamate groups. The peaks for the carbons next to phenyl ring of CBZ protecting group appear at δ = 65.57 (Z-Gly-Inu) and 65.27 (Z-Lys-Inu). Also, the signals between δ = 61 and 103 are due to inulin backbone carbon atoms.

The UV spectra (Fig. 5) of Z-Gly-Inu and Z-Lys-Inu also support the attachment of N-CBZ protected amino acids onto the inulin. The largest peaks at 260 nm (Z-Gly-Inu) and 259 nm (Z-Lys-Inu) are due to the aromatic ring of CBZ protecting groups of the amino acids. The conjugated π system of the aromatic ring in the CBZ protecting groups undergo $\pi \rightarrow \pi^*$ transitions are generally characterized by high intensity.[38] Unprotected Gly-Inu or Lys-Inu conjugates do not have UV band.

Amino Acid Analysis

The success of the coupling of the *N*-protected amino acids onto the inulin was further confirmed

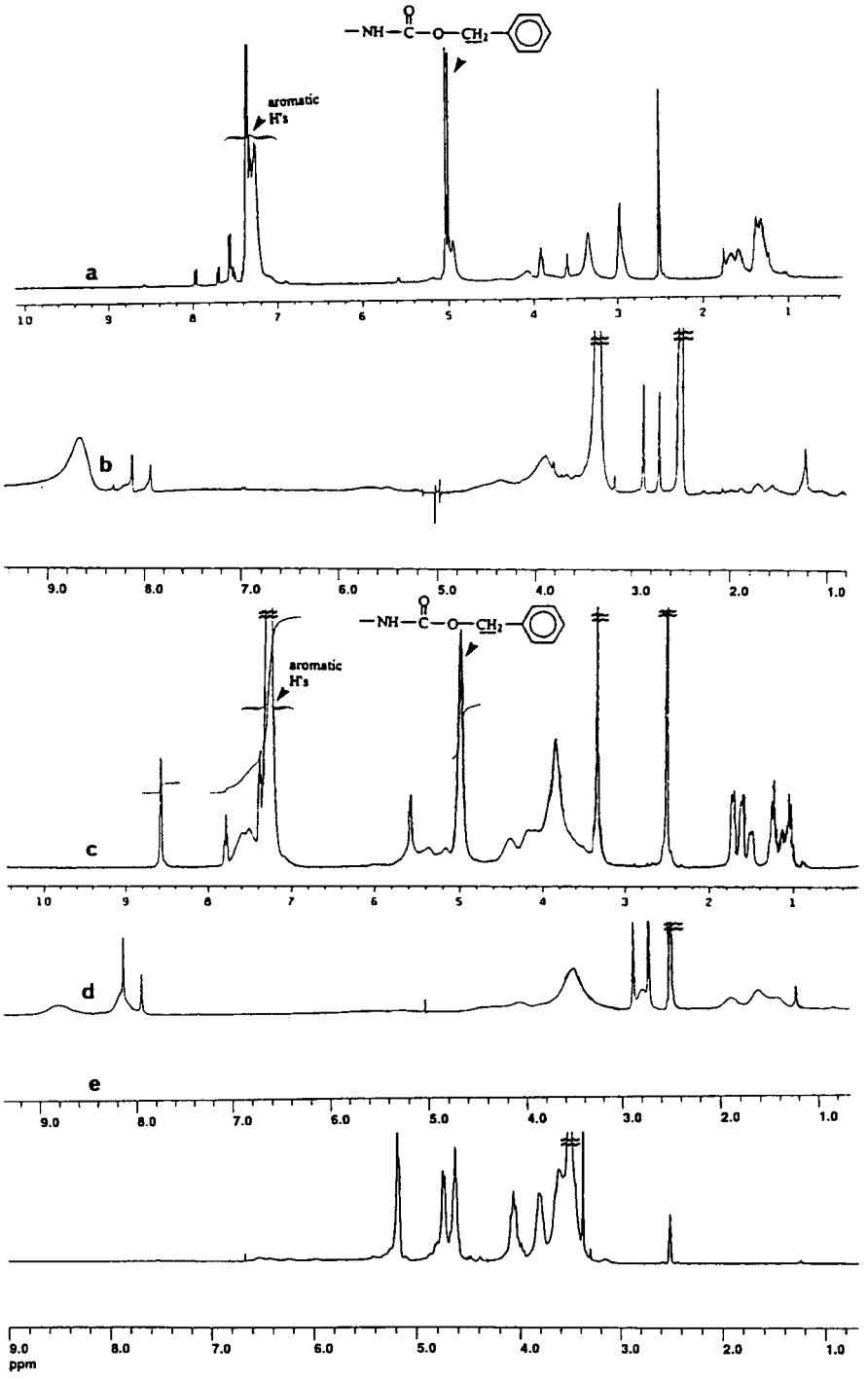


Figure 3 ^{1}H NMR (400 MHz) spectra of the protected and deprotected amino acid esters of inulin in $CDCl_3$ or DMSO-d_6. (a) Z-Gly-Inu; (b) Gly-Inu; (c) Z-Lys-Inu; (d) Lys-Inu; (e) inulin.

by amino acid analysis. Figure 6 shows the results of the amino acid analysis for Z-Gly-Inu (A) and Z-Lys-Inu (B). The inulin molecule contains three hydroxyl groups [one primary: OH(6); two secondary: OH(3) and OH(4)] per fructose unit in the polymer chain. Each of these can react with

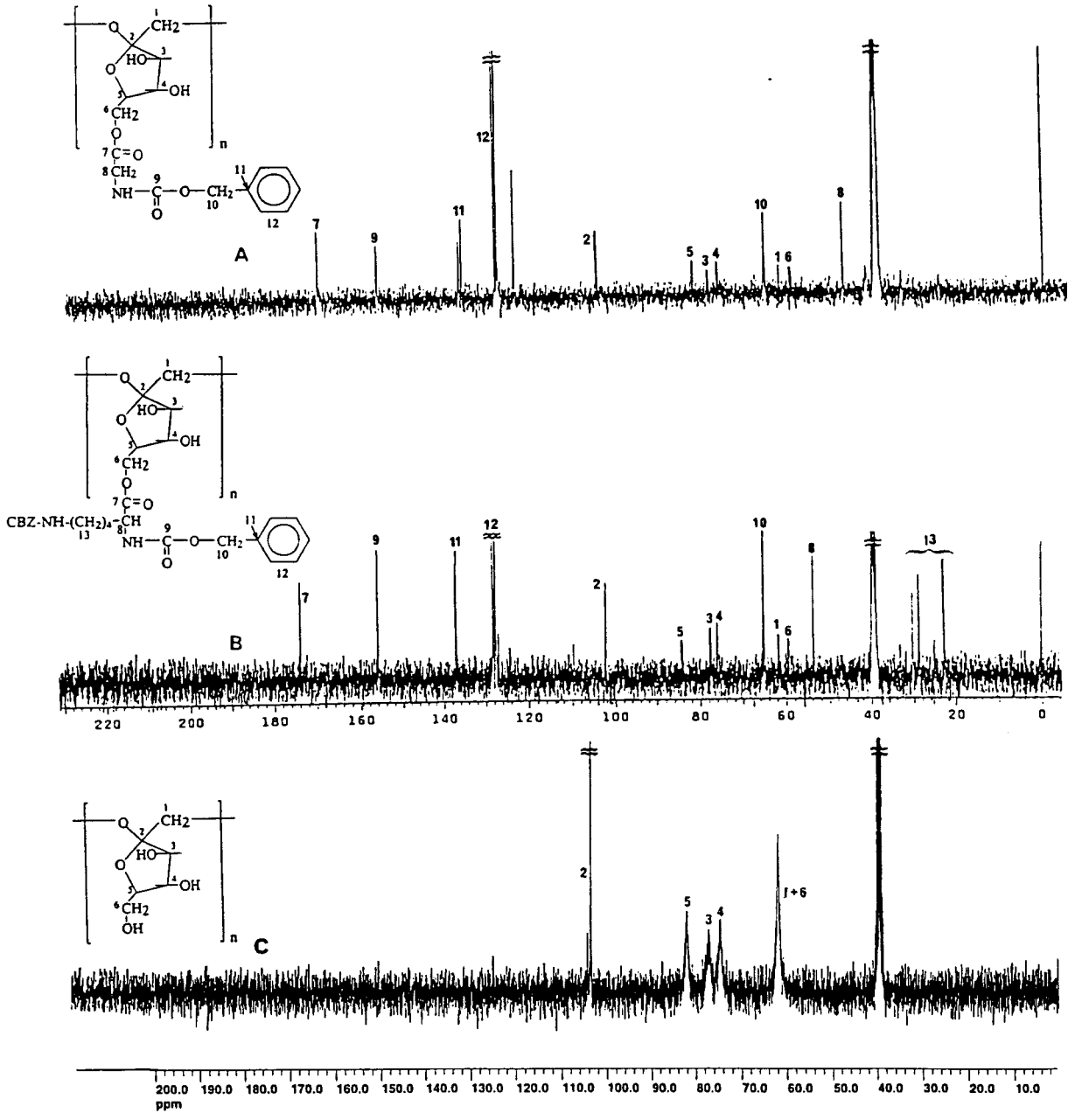


Figure 4 ^{13}C NMR (100 MHz) spectra of the protected amino acid esters of inulin in DMSO-d_6. (A) Z-Gly-Inu; (B) Z-Lys-Inu; (C) inulin.

N-protected amino acids in the same manner; however, their relative reactivities vary considerably. In general, the 1° hydroxyl group reacts much more readily than the two 2° hydroxyl groups because of the stronger nucleophilicity of the 1° hydroxyl group. The degree of substitution (D.S.) was quantitatively determined by amino acid analysis after acid hydrolysis, and the following values were obtained: Z-Gly-Inu, 1360.3 pmol $mL^{-1} \approx$ D.S. = 1.02; Z-Lys-Inu, 854.4 pmol $mL^{-1} \approx$ D.S. = 0.95. If all of the three hydroxyl groups per fructose unit of inulin would be esterified (i.e., a complete esterification), D.S. would be 3.0. Attempts were made in this study to produce the amino acid esters of inulin with D.S. of 2.0 by using the 2 : 1 stoichiometric ratio of the amino acids to

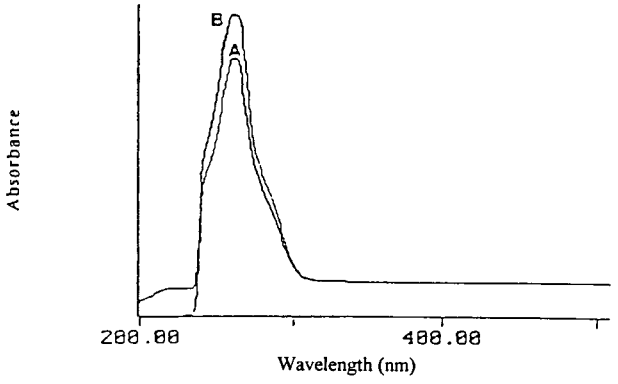


Figure 5 UV spectra of *N*-protected amino acid esters of inulin. (A) Z-Gly-Inu; (B) Z-Lys-Inu in chloroform.

the available hydroxyl groups of inulin. In both lysine and glycine, the resulting D.S. was half of the target D.S. (D.S. = 2). The low viscous solution medium would be required to free some of the —OH functional groups of inulin from their inter- or intramolecular hydrogen bonding interactions for their esterification with the *N*-protected amino acids. Therefore, a higher D.S. should be achieved by using a larger amount of solvent. The D.S. of Z-Gly-Inu was slightly higher than that of Z-Lys-Inu. This result may be explained by steric hindrance that the reactivity of the hydroxyl groups in Z-Lys-Inu was more sterically hindered due to its two bulky CBZ protecting groups.

Solubility Properties

The solubility behavior of amino acid esters of inulin is given in Table II. The modified inulins (Z-Gly-Inu and Z-Lys-Inu) were soluble at room temperature in polar solvents, such as THF, $CHCl_3$, and methylene chloride (MC), whereas the unmodified inulin was not dissolved in these solvents. The deprotected amino acid esters of inulin (Gly-Inu and Lys-Inu) were well dissolved in aprotic polar solvents (DMF and DMSO) at room temperature and soluble in $CHCl_3$ and MC at their boiling temperatures. But, they were insoluble in THF.

Deprotection of Amino Protecting Group of Amino Acid-Coupled Inulin

The CBZ protecting group is usually removed by solvolysis with HBr/HOAc i.e., acetic acid as the solvent.[39] However, chain scissions of polymers may happen due to strong acids required for deprotection. In fact, Idelson and Blout[40] reported the breakdown of the poly(γ-benzyl-L-glutamate) backbone during a prolonged deprotection reaction by comparing the molecular weight of the polymer samples before and after deprotection treatment.

To avoid this problem, we adopted another deprotection method (catalytic transfer hydrogenation) generally used in peptide chemistry[41] to regenerate free amine without degrading the inulin biopolymer. 1,4-Cyclohexadiene was used as an effective hydrogen donor under mild conditions. Palladium overactivated carbon (10 wt %) was used as the catalyst for the hydrogenolysis of the aromatic CBZ protecting group.

Structural analysis of the deprotected amino acid esters of inulin by ^1H NMR spectroscopy [Fig. 3(b,d)] revealed that the most distinctive feature of the deprotected inulin was the absence of the aromatic absorptions at 7.2–7.35 ppm, indicating the complete removal of the CBZ protecting group. Progress of the deprotection reaction could readily be monitored by the degree of the disappearance of the aromatic proton resonances of the CBZ group. At room temperature, the complete removal of the CBZ group required ~ 12 h.

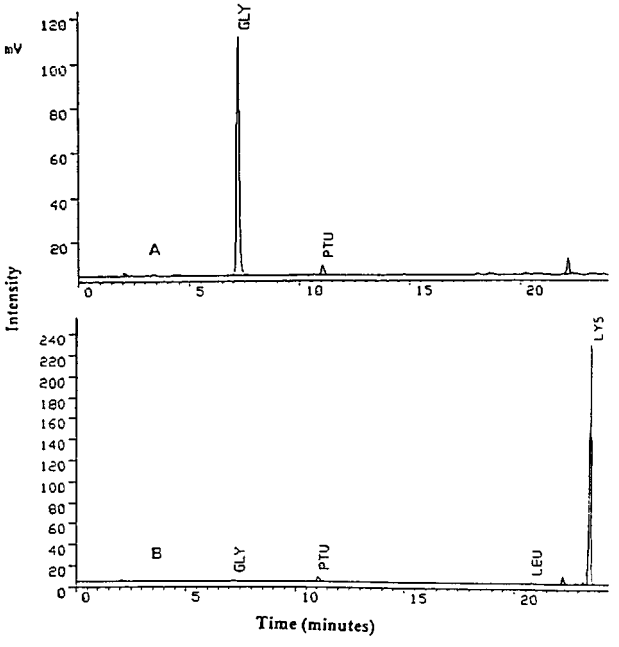


Figure 6 Amino acid analysis of *N*-protected amino acid esters of inulin. (A) Z-Gly-Inu; (B) Z-Lys-Inu.

Table II Solubilities of Z-Gly-Inu, Z-Lys-Inu, Gly-Inu, Lys-Inu, and Unmodified Inulin

	Samples[a]				
Solvents	Z-Gly-Inu	Z-Lys-Inu	Gly-Inu	Lys-Inu	Inulin
DMF	++	++	++	++	++
Pyridine	++	++	+−	+−	++
DMSO	++	++	++	++	++
$CHCl_3$	++	++	+	+	−
MC	++	++	+	+	−
THF	++	++	−	−	−
Ether	−	−	−	−	−
Hexane	−	−	−	−	−
MeOH[b]	−	−	−	−	−
Water	−	−	−	−	−

Solubility: ++, soluble at room temperature; +, soluble in hot solvent; +−, partially soluble; −, insoluble.
[a] Z-Gly-Inu: CBZ-protected glycine ester of inulin; Z-Lys-Inu: CBZ-protected lysine ester of inulin: Gly-Inu; deprotected glycine ester of inulin; Lys-Inu: deprotected lysine ester of inulin.
[b] MeOH, methanol.

Inherent Viscosity

Table III shows that the inherent viscosities of the modified inulins (Z-Gly-Inu and Z-Lys-Inu) were slightly increased due to the pendant amino acids groups. Also, the deprotected inulins (Gly-Inu and Lys-Inu) had higher inherent viscosity than that of the protected inulin (Z-Gly-Inu and Z-Lys-Inu). This result might be attributed to the hydrogen—bond interactions between —OH of inulin and the free amino groups. In addition, this result indicated that there was no chain cleavage during the deprotection reaction.

Thermal Analysis

The melting temperatures, T_m, of the unmodified inulin, Z-Gly-Inu, and Z-Lys-Inu were 186.9, 135.2, and 122.3°C, respectively (Fig. 7). The T_m of the unmodified inulin shifted to a significantly lower temperature (ΔT ranged from 51 to 64°C) after the attachment of the pendant amino acids groups. This may be due to the partial replacement of hydroxyl groups of inulin by the bulkier amino acids groups, leading to a reduction not only in its hydrogen bonding capability and but also in the formation of crystalline structure. The T_m of Z-Lys-Inu was slightly lower than that of Z-Gly-Inu, and may be due to the more bulky lysine group that would disrupt the close compactness of molecules. There was a small shallow shoulder peak ~ 150°C, in addition to the main melting peak (186.9°C) in the unmodified inulin. It is well known that the appearance of multiple melting peaks of a polymer is frequently associated with the heterogeneity in crystalline morphology, such as the presence of crystallities of different sizes or perfection. The small lower melting peak in the unmodified inulin might be thus be attributed to the presence of smaller crystallites. However, no glass transition temperatures were observed in all DSC curves.

CONCLUSIONS

Amino acid esters of inulin were prepared by esterification of inulin with *N*-protected (L)-α-

Table III Inherent Viscosities of Unmodified and Modified Inulins

	Inulin	Z-Gly-Inu	Gly-Inu	Z-Lys-Inu	Lys-Inu
Inherent viscosities ($dL\ g^{-1}$)	0.08	0.18	0.24	0.13	0.21

Measured with DMSO at a concentration of 0.5 g dL^{-1} at 30°C.

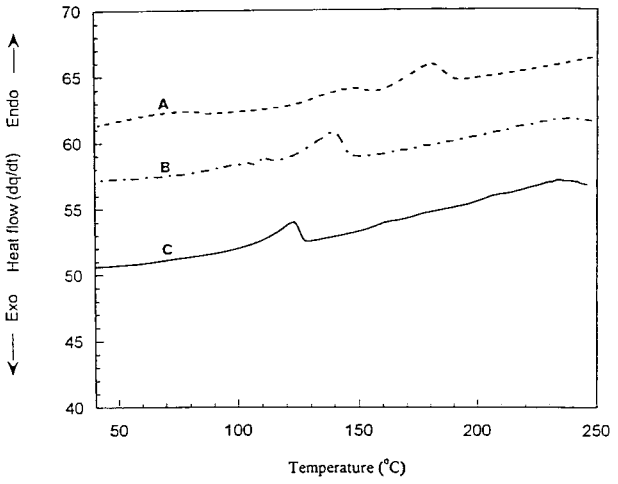


Figure 7 DSC thermogram of *N*-protected amino acid esters of inulin. (A) Unmodified inulin; (B) Z-Gly-Inu; (C) Z-Lys-Inu.

amino acids (*N,N′*-di-CBZ-L-lysine and *N*-CBZ-glycine) using a DCC/DMAP method under a mild reaction condition (room temperature) and shorter reaction time (6 h). The coupling reaction with DCC/DMAP was identified as a mild and efficient chemical method for immobilization of amino acids to inulin polysaccharide. The structures of the resulting biopolymers were confirmed by FTIR, ^{1}H NMR, ^{13}C NMR, and UV spectrophotometers. The results obtained showed that the esterification of inulin by *N*-protected amino acids *via* the DCC/DMAP method was practically quantitative. Based on amino acid analysis, the D.S. per fructose unit was 1.02 for Z-Gly-Inu and 0.95 for Z-Lys-Inu. After removal of the CBZ protecting group by catalytic transfer hydrogenation, Gly-Inu and Lys-Inu were obtained. The inherent viscosities of Z-Gly-Inu and Z-Lys-Inu were 0.18 and 0.13 dL g^{-1}, respectively. The melting temperatures were 135.2°C for Z-Gly-Inu and 122.3°C for Z-Lys-Inu. The pendant amino groups of these deprotected amino acid esters of inulin could facilitate the attachment of cross-linkers, various drugs, or any other biologically active compounds for a variety of biomedical applications.

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