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Analysis of *N*-glycans of pathological tau: possible occurrence of aberrant processing of tau in Alzheimer's disease

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Abstract In a previous study [Wang et al. (1996) Nat. Med. 2, 871–875], Wang et al. found (i) that abnormally hyperphosphorylated tau (AD P-tau) isolated from Alzheimer's disease (AD) brain as paired helical filaments (PHF)-tau and as cytosolic AD P-tau but not tau from normal brain were stained by lectins, and (ii) that on in vitro deglycosylation the PHF untwisted into sheets of thin straight filaments, suggesting that tau only in AD brains is glycosylated. To elucidate the primary structure of *N*-glycans, we comparatively analyzed the *N*-glycan structures obtained from PHF-tau and AD P-tau. More than half of *N*-glycans found in PHF-tau and AD P-tau were different. High mannose-type sugar chains and truncated *N*-glycans were found in both taus in addition to a small amount of sialylated bi- and triantennary sugar chains. More truncated glycans were richer in PHF-tau than AD P-tau. This enrichment of more truncated glycans in PHF might be involved in promoting the assembly and or stabilizing the pathological fibrils in AD. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Biochemical Societies.

Key words: Alzheimer's disease; *N*-Glycan; Tau; Glycosylation; Neurofibrillary tangle

1. Introduction

Alzheimer's disease (AD) is the most common cause of dementia in the elderly population. Although the pathogenesis of the disease is not yet understood, AD is characterized by the presence of two histopathological hallmark brain lesions, extracellular deposits of β-amyloid in neuritic plaques and intracellular neurofibrillary tangles. AD is characterized by a specific type of neuronal degeneration (called 'neurofibrillary degeneration'), in which the neuronal cytoskeleton is progressively disrupted and displaced by the appearance of bundles of paired helical filaments (PHFs), the neurofibrillary tangles. Neurons with neurofibrillary tangles lack microtubules, and microtubule assembly from AD brain cytosol is not observed [1]. PHFs are comprised mainly of the microtubule-associated protein tau in an abnormally hyperphosphorylated state [2–4]. Tau in PHF (PHF-tau) is different from that in normal neurons. In AD brain, in addition to PHF, there is a cytosolic pool of the abnormally hyperphosphorylated tau (AD P-tau) as amorphous aggregates [5]. Compared with normal tau, which contains two to three phosphate groups, the AD P-tau contains 5–9 mol of phosphate per mol of the protein [5]. This pool of abnormal tau, which is soluble in physiological buffer conditions, disrupts microtubules and inhibits their assembly in vitro [6–9]. Evidence from several studies has indicated that the hyperphosphorylation of tau is responsible for its loss of biological activity and its resistance to proteolytic degradation, and probably plays a key role in neurofibrillary degeneration in AD.

In a previous study [10], we found that PHF-tau and AD P-tau but not normal tau were stained by lectins and that on in vitro deglycosylation the PHF untwisted into thick straight filaments, suggesting that tau in only AD brain was glycosylated. Deglycosylation plus dephosphorylation, but not deglycosylation alone, of AD P-tau and tau from PHF tangles restored their microtubule polymerization activity [8,9]. The glycosylation of PHF in AD brain was also confirmed immunocytochemically by lectin binding [11]. Thus, glycosylation appeared to be responsible for the maintenance of the PHF structure. To elucidate the relationship between glycosylation of tau and the pathogenesis of AD, information on the exact carbohydrate structures of the pathological tau is required. In this study, we comparatively analyzed the primary structure of *N*-glycans of PHF-tau and AD P-tau.

2. Materials and methods

2.1. Enzymes

Arthrobacter ureafaciens sialidase and bovine epididymal α-fucosidase were purchased from Nacalai Tesque (Kyoto, Japan), and from Sigma Chemicals Co. (St. Louis, MO, USA), respectively. Diplococcal β-galactosidase and β-*N*-acetylhexosaminidase were purchased from Roche Molecular Biochemicals (Mannheim, Germany). Jack bean β-*N*-acetylhexosaminidase and α-mannosidase, and snail β-mannosidase were purchased from Seikagaku Co. (Tokyo, Japan). *Aspergillus saitoi* α-mannosidase I was purified according to Amano and Kobata [12].

2.2. Purification of normal tau, AD P-tau and PHF-tau

Histopathologically confirmed AD brains obtained between 3 and 5 h post-mortem and stored at −75°C were used. The human tissue was obtained from the Brain Resource Center McLean Hospital, Belmont, MA, USA. AD P-tau was isolated by the method of Köpke et al. [5]. Bovine tau was isolated as described [4] for acid-soluble tau (tau isolated from a $HClO_4$ brain extract) and chromatography on a phosphocellulose (cellulose phosphate P11, Whatman) column.

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Abbreviations: 2AB, 2-aminobenzamide; AD, Alzheimer's disease; AD P, AD hyperphosphorylated; ER, endoplasmic reticulum; gu, glucose unit; MALDI-FTMS, matrix-assisted laser desorption ionization Fourier transform mass spectrometry; OST, oligosaccharyl transferase; PHF, paired helical filament; PNGase, peptide: *N*-glycanase

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AD P-tau was prepared from the 27 000×*g* to 200 000×*g* fraction of the AD brain homogenate. This fraction contains soluble abnormally phosphorylated tau (AD P-tau) and individual PHFs. For the isolation of AD P-tau from PHFs, the above fraction was extracted in 8 M urea, followed by acid precipitation, chromatography on a phosphocellulose column and dialysis against MES buffer as described previously [5]. PHF-tau was isolated from AD brains as described previously [13]. Briefly, cerebral cortex, cleaned free of white matter and meninges, was fractionated by a combination of sieving through nylon bolting cloth, treatment with 2% sodium dodecyl sulfate (SDS), and by discontinuous sucrose density gradients and glass beads chromatography. Protein was estimated as previously described [14]. The purified taus thus obtained gave several polypeptides on SDS–PAGE, and all were reactive on Western blots with the tau polyclonal and monoclonal antibodies (Fig. 1). Rabbit antibody 92e [7] and monoclonal antibody Tau-1 (after alkaline phosphatase treatment of the blots; [3]) recognize total tau.

2.3. 2-Aminobenzamide (2AB) derivatization of N-glycans released from tau

Three purified taus (bovine tau, 165 μg; PHF-tau, 120 μg; AD P-tau, 150 μg) were thoroughly dried over P_2O_5 in vacuo and subjected to hydrazinolysis at 100°C for 9 h. Liberated oligosaccharides were *N*-acetylated and purified as described previously [15]. This procedure releases the *N*-linked sugar chains of glycoproteins quantitatively as oligosaccharides.

The total *N*-glycans obtained above were labeled with 2AB as described previously [16]. To detect the 2AB-labeled oligosaccharides, fluorescence emission was monitored at 420 nm with excitation at 330 nm. Since any 2AB-labeled oligosaccharides could not be obtained from bovine tau, further study was not performed.

2.4. Oligosaccharides

A total of 10 oligosaccharides were obtained (Table 1). G2, G2F and GN2FB were obtained from lipocalin-type prostaglandin D synthase purified from human urine by hydrazinolysis and derivatized by 2AB as described above [16]. G3 was obtained from fetuin [17], and M9 and M5 were obtained from human IgM [18], respectively. M3 and M3F were obtained by digestion of G2 and G2F with a mixture of diplococcal β-galactosidase and β-*N*-acetylhexosaminidase, respectively. M1 and M1F were obtained by digestion of M3 and M3F with jack bean α-mannosidase, respectively.

2.5. Analytical methods

Column chromatographies and other experimental procedures used in this work have been described in the previous papers [16]. Exoglycosidase digestion was performed according to the methods described previously [16].

Molecular mass of the 2AB-labeled oligosaccharides was determined by matrix-assisted laser desorption ionization Fourier transform mass spectrometry (MALDI-FTMS). 2,5-Dihydroxybenzoic acid, which was purchased from Sigma Aldrich, was used as the matrix. It was dissolved to a concentration of 10 mg/ml of 30% aqueous ethanol. Dried 2AB-labeled oligosaccharide was dissolved in the matrix solution (2 pmol/μl). Aliquots of the resulting mixtures (5 μl) were placed onto probe tips and dried at room temperature. The molecular masses of the oligosaccharides were determined using a BioAPEX 47e (4.7-T, Bruker Daltonics GmbH, Bremen, Germany) FTMS.

3. Results

3.1. Fractionation of oligosaccharides by anion-exchange column chromatography

The 2AB-labeled oligosaccharide mixtures obtained from AD P-tau and PHF-tau were separated into a neutral (N) and acidic fraction (A) by anion-exchange chromatography on a Mono Q HR5/5 column (Fig. 2). By exhaustive *A. ureafaciens* sialidase digestion, a part of acidic fraction was converted into neutral oligosaccharides, indicating that sialic acids are included as the acidic residues in these oligosaccharides. The neutral oligosaccharide fraction obtained from fraction A was named fraction AN. Components not digested by sialidase may contain other anionic residues such as sulfated sugars and uronic acids, although no further analysis was performed due to the limited amounts of sample. Therefore, fractions N and AN were subjected to structural analysis as below.

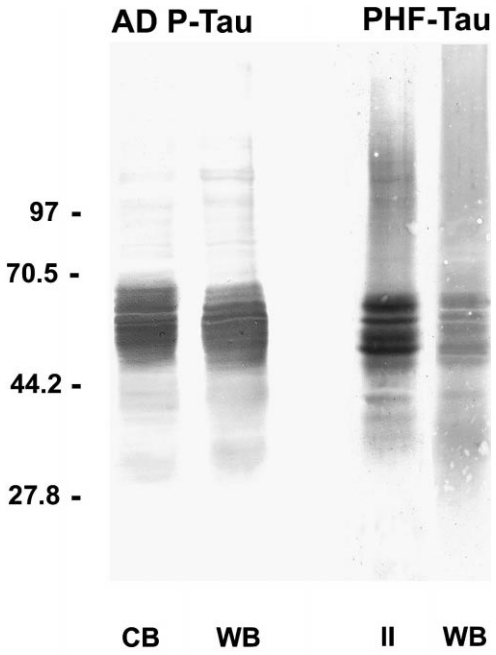


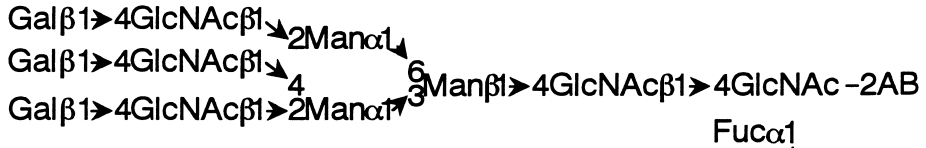
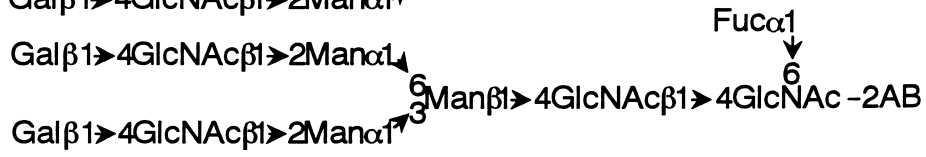
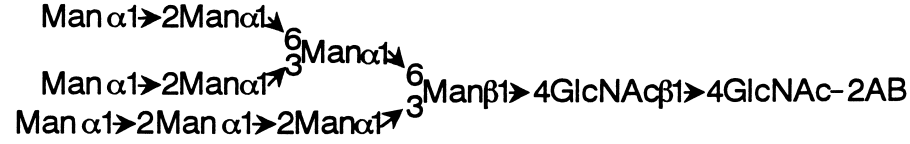
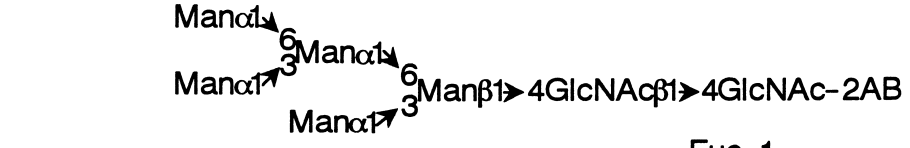
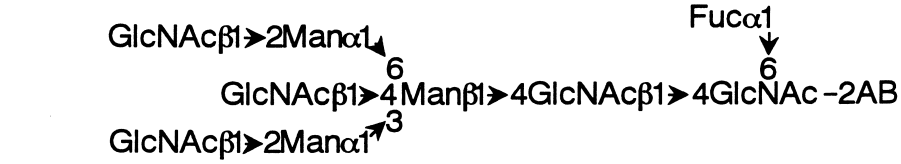
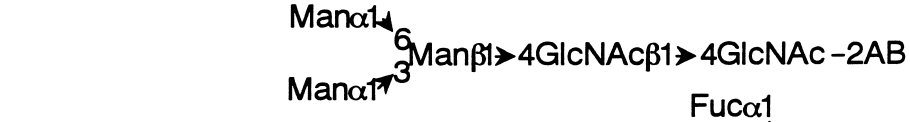
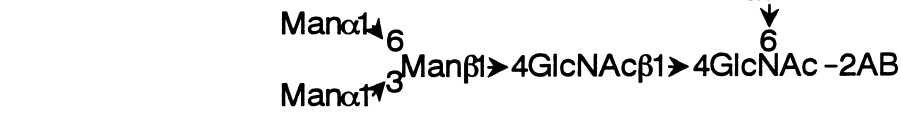
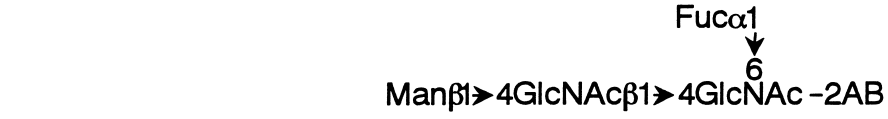
Fig. 1. SDS–PAGE and Western blots of purified PHF-tau and AD P-tau used for the analysis of the primary structure of *N*-glycans. AD P-tau, and PHF-tau (8 μg/lane for staining; and 4 μg/lane for Western blots) were subjected to 7.5–15%, 5–15% SDS–PAGE, respectively, transferred to a PVDF membrane, and either stained with dye or immunostained with a polyclonal antibody 92e to tau (in the case of PHF-tau) or with tau monoclonal antibody Tau-1 to the alkaline phosphatase-treated membrane (in the case of AD P-tau) as described previously [8]; the staining of PHF-tau and AD P-tau with lectins was shown previously [10]. CB, Coomassie blue staining; II, India ink staining; WB, Western blots. Molecular weight markers were prestained phosphorylase b (97 kDa), bovine serum albumin (70.5 kDa), ovalbumin (44.2 kDa) and carbonic anhydrase (27.8 kDa) from Gibco BRL/Life Technologies.

3.2. Fractionation of the neutral oligosaccharides by normal phase high performance liquid chromatography (HPLC)

When fractions N and AN from AD P-tau and PHF-tau were subjected to normal phase HPLC, finally 12 peaks were obtained as shown in Fig. 3. It should be noted that peaks d, e, f, g and l were only detected in AD P-tau and that peaks h and i were only in PHF-tau. Remaining five peaks were found in both samples. In order to determine the anomeric configuration and monosaccharide sequences of each component in Fig. 3, they were subjected to sequential exoglycosidase digestion, and the reaction products at each step were analyzed by normal phase HPLC (Fig. 4). Schematic diagram of sequential glycosidase digestions is shown in Fig. 5.

Information on the molecular weights of these components is helpful for the interpretation of the results obtained by HPLC as above and sequential exoglycosidase digestion as will be described later. Due to the limited amounts of sample, the molecular weights of component a and component b were determined by MALDI-FTMS. The observed $[M+Na]^+$ ion

Table 1
Structures of authentic oligosaccharides used in this study

Abbreviation	Structure
G3	
G2F	
G2	
M9	
M5	
GN2FB	
M3	
M3F	
M1	
M1F	

peaks of component a and component b were at *m/z* 1377.6 and 1808.7, respectively. The observed molecular mass values were consistent with those expected: 2AB-labeled five mannose-type sugar chains (Hex_5·HexNAc·HexNAc-2AB, 1377), and 2AB-labeled fucosylated bisected non-galactosyl-biantennary ones (Hex_3·$HexNAc_4$·DeoxyHex·HexNAc-2AB, 1808).

3.3. Structural studies

3.3.1. Components j and k. As shown in Fig. 3, component j and component k were eluted at the same positions as authentic G2 (7.3 glucose units (gu)) and G2F (7.9 gu) on normal phase HPLC, respectively. These results suggested that component j and component k are a complex-type non-fucosylated and fucosylated biantennary sugar chains, respectively. In order to confirm these findings, the component j and component k were subjected to sequential exoglycosidase digestion.

When component j was incubated with diplococcal β-galactosidase, which cleaves the Galβ1→4GlcNAc linkage only [19], two galactose residues were removed completely from

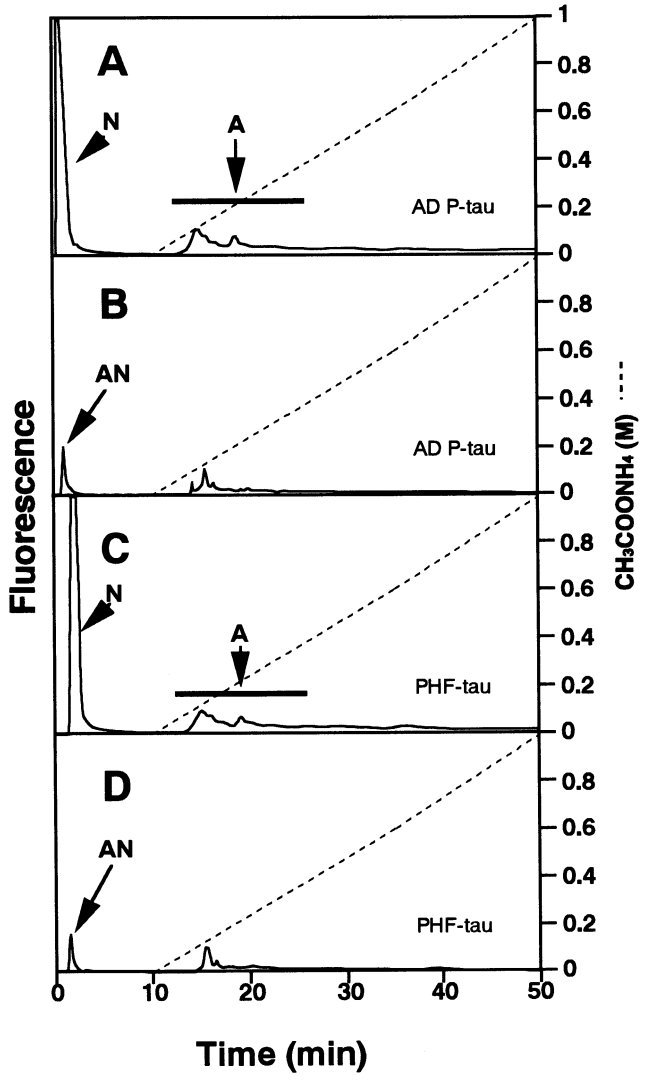


Fig. 2. Anion-exchange column chromatography of 2AB-labeled *N*-glycans from AD P-tau and PHF-tau. 2AB-labeled oligosaccharides were subject to HPLC using a Mono Q HR5/5 column. After elution of the neutral oligosaccharides with 10 ml of water, the acidic oligosaccharides were eluted with a 0–600 mM gradient of ammonium acetate, pH 4.0, at a flow rate of 1 ml/min at room temperature (dotted line). (A) Oligosaccharides from AD P-tau; (B) following de-sialylation of fraction A in (A) by digestion with *A. ureafaciens* sialidase; (C) oligosaccharides from PHF-tau; (D) following de-sialylation of fraction A in (C) by the same enzymatic treatment. N, neutral glycans; A, acidic glycans; AN, acidic neutral glycans. To detect the 2AB-labeled oligosaccharides, fluorescence emission was monitored at 420 nm with excitation at 330 nm.

the component j (Fig. 4A). When the component in Fig. 4A was incubated with diplococcal β-*N*-acetylhexosaminidase, which cleaves the GlcNAcβ1→2Man linkage only [20], it was converted to a component with the same mobility as authentic M3 (4.5 gu) with the release of two *N*-acetylglucosamine residues (Fig. 4B). The component in Fig. 4B was confirmed by sequential digestion with jack bean α-mannosidase, β-mannosidase, and jack bean β-*N*-acetylhexosaminidase.

On the other hand, sequential exoglycosidase digestion of component k gave a series of products which were larger in size by approximately 0.5 gu than those in the case of component j (dotted lines in Fig. 4A,B, respectively). The dotted-line component in Fig. 4B, which was eluted at the same position as authentic M3F (5.0 gu) was confirmed by sequential digestion with jack bean α-mannosidase followed by β-mannosidase, jack bean β-*N*-acetylhexosaminidase, and α-fucosidase. Based on these results, the proposed structures of

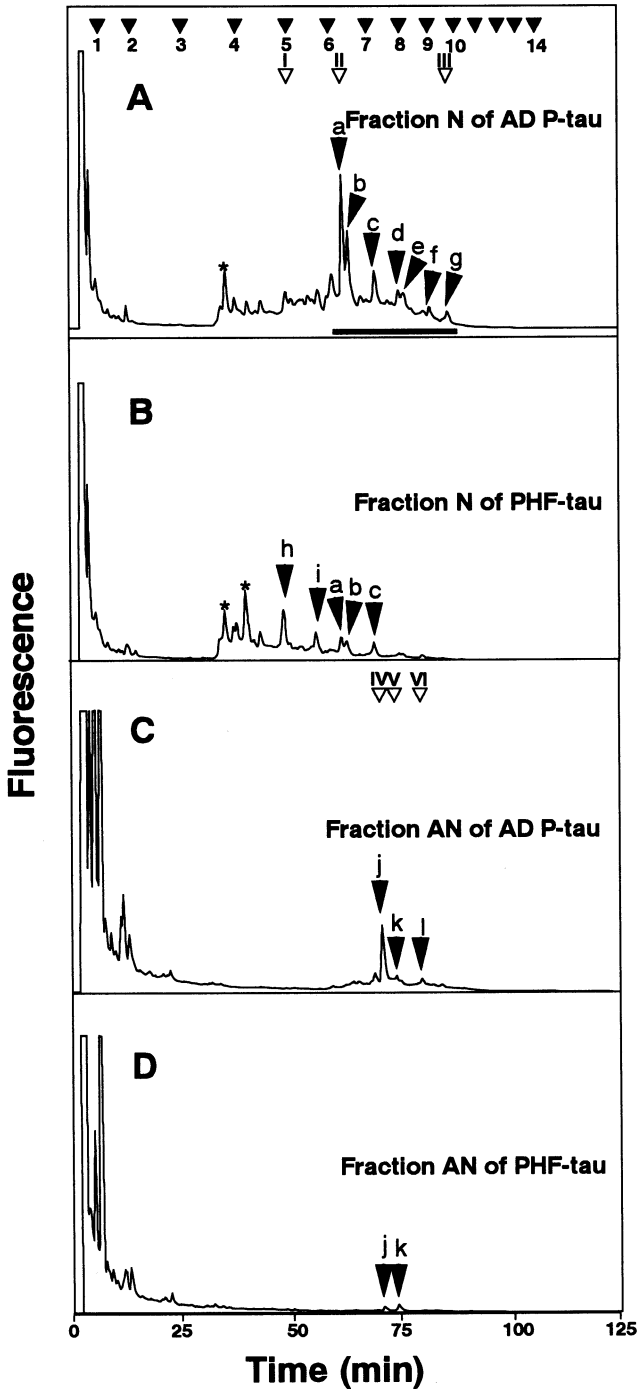
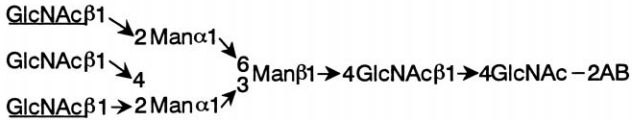


Fig. 3. Normal phase HPLC of components in fractions N and AN from Fig. 2. (A) Fraction N of AD P-tau; (B) fraction N of PHF-tau; (C) fraction AN of AD P-tau; (D) fraction AN of PHF-tau. Each fraction was applied to a GlycoSep-N column and eluted in a 250 mM ammonium acetate–acetonitrile gradient solvent system at a flow rate of 1 ml/min at 30°C. The 250 mM acetate–acetonitrile ratio was changed linearly from 20:80 to 53:47 (v/v) during 132 min. Peaks were assigned gu values by comparison with the 2AB-labeled glucose oligomer ladder shown at the top of A. The open triangles indicate the elution positions of the 2AB-labeled authentic oligosaccharide standard, I, M3F; II, M5; III, M9; IV, G2; V, G2F; VI, G3. Asterisks indicate contaminating materials.

component j and component k were concluded to be shown in Table 2.

3.3.2. Component l. On normal phase HPLC, the component l migrated to the same position as authentic G3 (Fig. 3C). The component l released three galactose residues by diplococcal β-galactosidase digestion (Fig. 4C) and three *N*-acetylglucosamine residues by sequential jack bean β-*N*-acetylhexosaminidase digestion (Fig. 4D). The component at this stage moved to the same position as authentic M3 (4.5 gu). These results indicated that it is a non-fucosylated triantennary oligosaccharide. When digested with diplococcal β-*N*-acetylhexosaminidase, the component in Fig. 4C released two *N*-acetylglucosamine residues (Fig. 4D, dotted line). The diplococcal enzyme can release two underlined *N*-acetylglucosamine residues from the following oligosaccharides [20]:



Based on the results, the structure of component l was proposed to be shown in Table 2.

3.3.3. Components a, c, e, f, and g. When components in fraction N (underlined in Fig. 3A) were incubated with *A. saitoi* α-mannosidase I, which cleaves the Manα1→2Man linkage only [21], components c, e, f, and g were converted to component a, which was eluted at the same position as authentic M5, indicating that only these four components contain Manα1→2Man groups (Fig. 4E). By comparison of the mobilities of the peaks before and after the enzymatic digestion, it was concluded that components c, e, f, and g have one, two, three, and four Manα1→2 residues linked to component a, respectively. However, components b and d were resistant to this enzymatic digestion (Fig. 4E). While structural study of component b will be described later, detailed structural information of component d could not be obtained due to the limited amounts of sample. It should be noted, however, that component d was susceptible to α-fucosidase and jack bean β-*N*-acetylhexosaminidase digestions. Component a which showed the same mobility as authentic

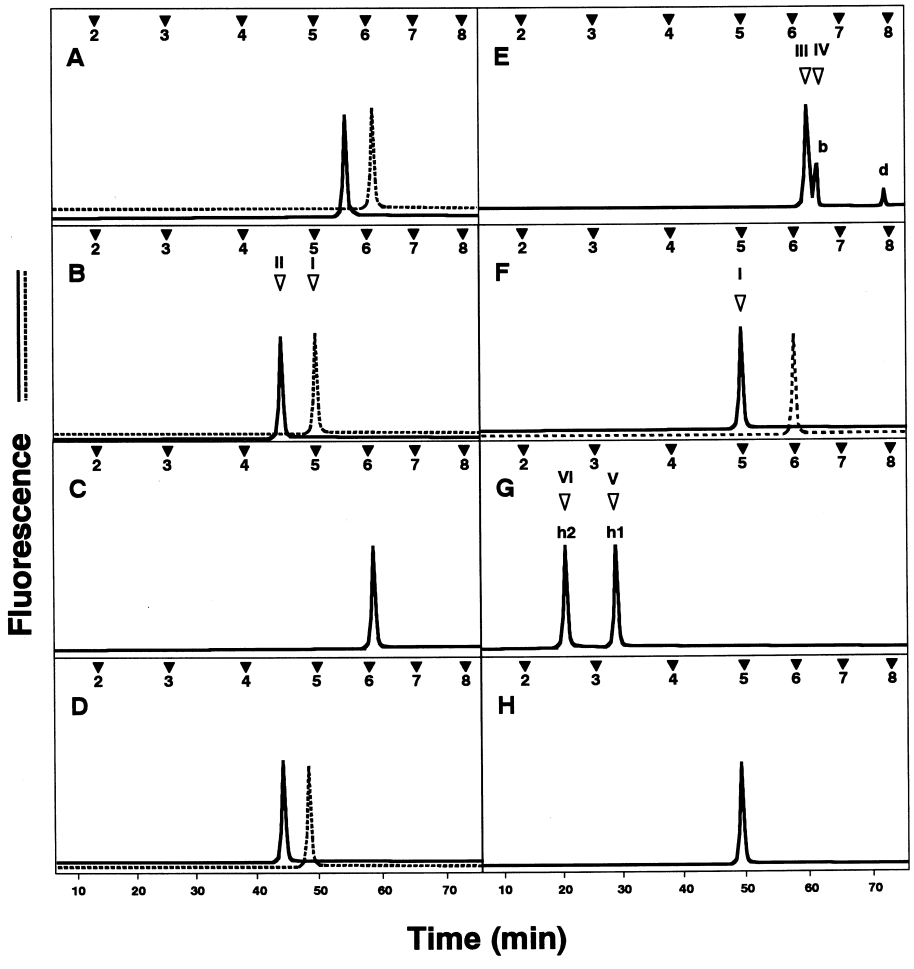


Fig. 4. Sequential glycosidase digestion of each component fractionated by normal phase HPLC. (A) Component j (solid line) and component k (dotted line) from Fig. 3 after digestion with diplococcal β-galactosidase; (B) the solid-line and dotted-line components from (A) after digestion with diplococcal β-*N*-acetylhexosaminidase; (C) component l from Fig. 3D after digestion with diplococcal β-galactosidase; (D) the component from (C) after digestion with jack bean β-*N*-acetylhexosaminidase (solid line) and with diplococcal β-*N*-acetylhexosaminidase (dotted line); (E) the components underlined in Fig. 3A after digestion with *A. saitoi* α-mannosidase I; (F) component b from (E) after digestion with jack bean β-*N*-acetylhexosaminidase (solid line) or with diplococcal β-*N*-acetylhexosaminidase (dotted line); (G) peak h in Fig. 3B after digestion with jack bean α-mannosidase; (H) component i from Fig. 3B after digestion with diplococcal β-*N*-acetylhexosaminidase. The open triangles indicate the elution positions of the 2AB-labeled authentic oligosaccharide standard, I, M3F; II, M3; III, M5; IV, GN2FB; V, M1F; VI, M1. The solid triangles show the position of the 2AB-labeled glucose oligomers as in Fig. 3.

M5 was confirmed by sequential digestion with jack bean α-mannosidase, β-mannosidase, and jack bean β-*N*-acetylhexosaminidase. On the other hand, only component c in fraction N of PHF-tau (Fig. 3B) was digestible by *A. saitoi* α-mannosidase I, releasing a single mannose residue (data not shown). Therefore, components a, c, e, f, and g are a series of high mannose-type sugar chains as shown in Table 2.

3.3.4. Component b. As shown in Fig. 4E, *A. saitoi* α-mannosidase I resistant component b was eluted at the same position as authentic GN2FB (6.5 gu), suggesting that these are complex-type fucosylated bisected non-galactosyl-biantennary oligosaccharide.

To confirm the findings, component b was subjected to sequential digestion. Component b released three *N*-acetylglucosamine residues by jack bean β-*N*-acetylhexosaminidase digestion and co-eluted with authentic standard M3F (5.0 gu) (Fig. 4F). On the other hand, the component b released only one *N*-acetylglucosamine residue by diplococcal β-*N*-acetylhexosaminidase digestion (6.0 gu) (Fig. 4F, dotted line). These results indicated that the structure of the component b was GlcNAcβ1→2Manα1→6(GlcNAcβ1→2Manα1→3)-(GlcNAcβ1→4)Manβ1→4GlcNAcβ1→4(Fucα1→6)GlcNAc-2AB. As reported previously [20], diplococcal β-*N*-acetylhexosaminidase cleaves only the underlined β-*N*-acetylglucosamine residue from the above oligosaccharide. Based on these results together with the molecular mass data, the proposed structure of component b was summarized in Table 2.

3.3.5. Components h and i. As shown in Fig. 3B, component h seemed to be eluted as a single peak on normal phase HPLC. However, when this peak was incubated with jack bean α-mannosidase, two components, h1 and h2, appeared (Fig. 4G). Component h1 was co-eluted at the same position as authentic M1F (3.3 gu) and component h2 was as authentic M1 (2.6 gu), respectively. By comparison of the mobilities of the peaks before and after the enzymatic digestion, it was concluded that peak h was a mixture of a component having two α-mannose residues linked to component h1 and another component having three α-mannose residues linked to component h2, respectively. The structure of h2 was confirmed by sequential digestion with snail β-mannosidase followed by jack bean β-*N*-acetylhexosaminidase, and that of h1 was also done by the same enzymatic digestion followed by α-fucosidase digestion. Based on these results, the proposed structures of components in peak h are shown in Table 2.

Component i (5.8 gu) released only one *N*-acetylglucosamine residue by diplococcal β-*N*-acetylhexosaminidase digestion (5.0 gu) (Fig. 4H). The results of sequential exoglycosidase digestions of the component in Fig. 4H were the same as authentic M3F. Therefore, component i was fucosylated mono *N*-acetylglucosaminylated trimannosyl core, as shown in Table 2.

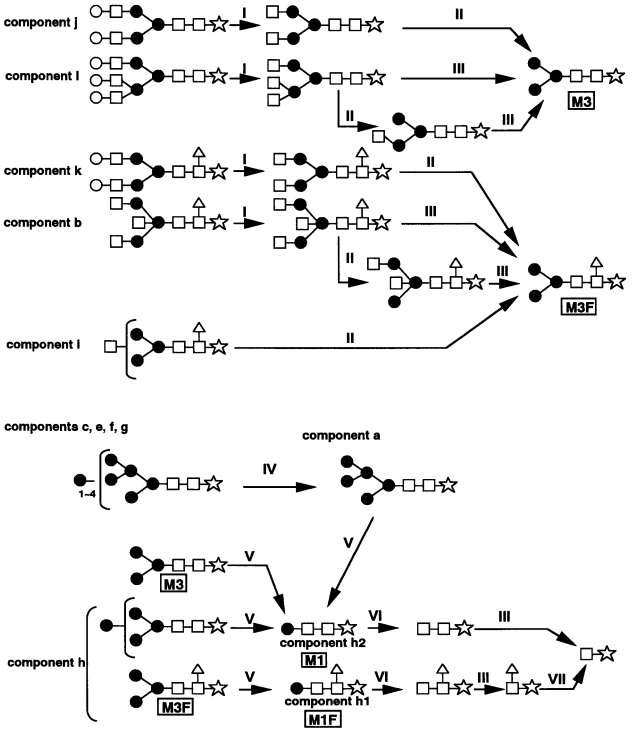


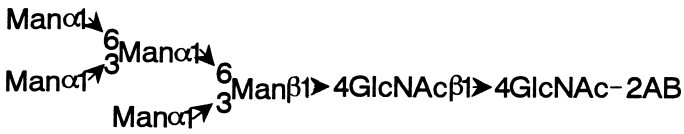
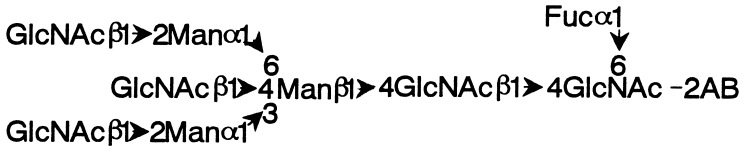
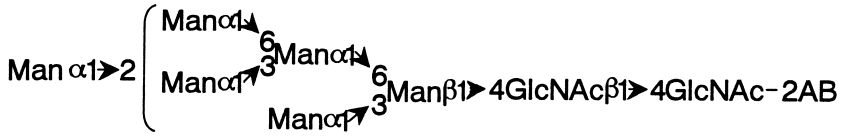
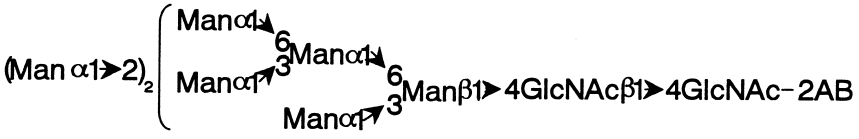
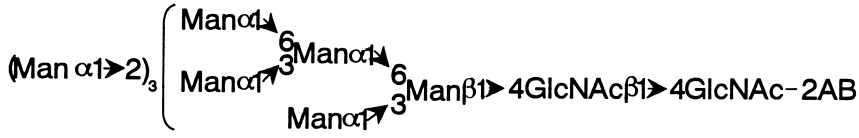
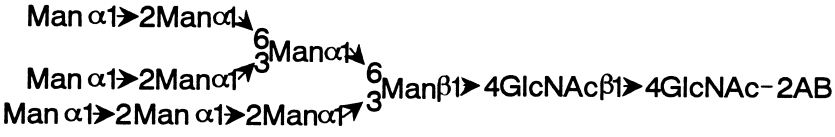
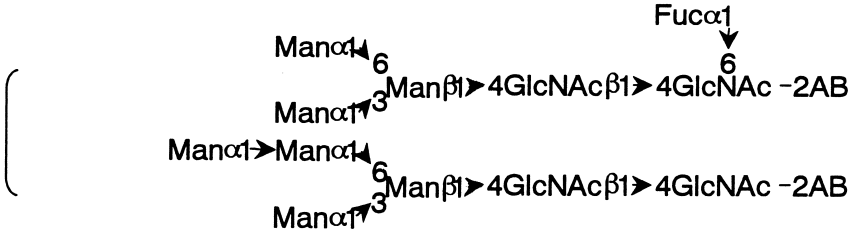
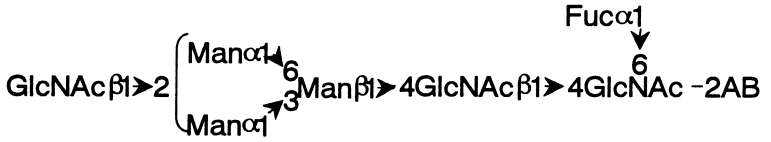
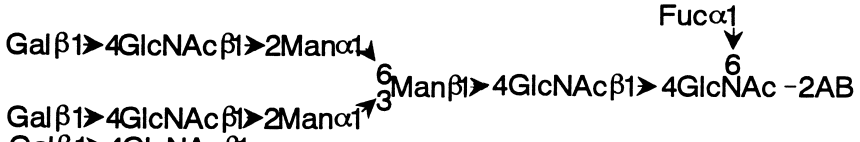
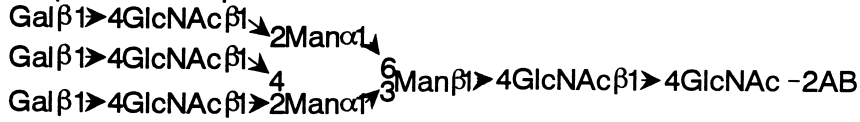
Fig. 5. Schematic diagram of sequential exoglycosidase digestions used to determine the structures of the *N*-glycans. Symbols used for the monosaccharides units and 2AB are: ○, galactose; □, *N*-acetylglucosamine; ●, mannose; △, fucose; ☆, 2AB. The exoglycosidases used are: I, diplococcal β-galactosidase; II, diplococcal β-*N*-acetylhexosaminidase; III, jack bean β-*N*-acetylhexosaminidase; IV, *A. saitoi* α-mannosidase I; V, jack bean α-mannosidase; VI, snail β-mannosidase; VII, bovine epididymal α-fucosidase.

4. Discussion

In a previous study, Wang et al. [10] found that normal tau was not stained by lectin binding on Western blots, suggesting that the glycosylation of tau is not a normal physiological modification. The absence of *N*-glycans on normal tau is confirmed in the present study by a chemical method, hydrazinolysis, which is well known to release the *N*-glycans of glycoproteins quantitatively as oligosaccharides [15]. This finding is also consistent with a previous study which showed that bovine tau was not *N*-glycosylated on the basis of the susceptibility to peptide: *N*-glycanase (PNGase) [22]. On the other hand, the recovery of *N*-linked oligosaccharides from PHF-tau and AD P-tau by hydrazinolysis confirmed the glycosylation of PHF-tau and AD P-tau shown previously by lectin binding on Western blots [10] and immunocytochemically by lectin binding in AD brain [11].

One of 'brain-type' *N*-glycan structures [23–26], truncated, fucosylated, bisected biantennary sugar chain (component b in Table 2), was found in both PHF-tau and AD P-tau. A series of high mannose-type sugar chains were also detected in both taus, while compositional difference was found. The presence of high mannose-type sugar chains is consistent with a previous study in which AD P-tau and PHF-tau were positively stained by a lectin, *Galanthus nivalis* agglutinin (GNA) [10]. It is known that GNA is a mannose binding lectin and exhibits high affinity for the branched trisaccharide Manα1→6(Manα1→3)Man [27]. When compared between AD P-tau and PHF-tau, the small glycans were richer in PHF-tau than in AD P-tau; e.g. components h and i were found only in PHF-tau. AD P-tau is probably a precursor form of PHF tau [5]. Tangles are long lived as compared to AD P-tau. The enrichment of small glycans in PHF probably represents a more advanced stage of processing of glycans in these long lived structures. Since the glycan moiety of a glycoprotein modifies the properties of the protein [28], subtle structural change of *N*-glycan may induce conformational change. The changes in the glycans composition from AD P-tau to PHF-tau might reduce structural stability to PHF.

Table 2
Proposed structures and their percent molar ratios of *N*-glycans obtained from AD P-tau and PHF-tau

Component	Structure	AD P-tau	PHF-tau
a		26.4	18.0
b		18.0	14.0
c		15.1	16.6
e		10.7	—*
f		5.7	—
g		5.9	—
h		—	15.9
		—	13.2
i		—	20.8
j		6.9	0.4
k		1.9	1.1
l		0.9	—

*Not detected. The percentage molar ratios of *N*-glycans in AD P-tau and PHF-tau were determined by fluorescence intensity.

The appearance of *N*-glycosylated tau in patients with AD may be relevant to the understanding of the pathology of this disease. It is known that human tau has three potential *N*-glycosylation sites (Asn^{167}-Ala-Thr, Asn^{359}-Ile-Thr, and Asn^{410}-Val-Ser; amino acids are numbered according to the longest isoform of human brain tau [29]). Although the AD-dependent mechanism by which tau becomes *N*-glycosylated remains to be elucidated, several possibilities could be considered. The first possibility is that the intracellular localization of tau may change in AD, resulting in production of *N*-glycosylated species of tau due to the altered accessibility to *N*-glycan processing enzymes. The addition of *N*-glycans in proteins is catalyzed by an oligosaccharyl transferase (OST) [30], whose active site is considered to be located in the lumenal side of the endoplasmic reticulum (ER) and the processing enzymes acting in the latter steps are also in the lumen of the ER and Golgi apparatus. The possibility described is supported by the fact that some of tau in AD brains was associated with ribosomes on the rough ER [31]. Additionally, we must also consider the possibility of abnormality of import and export system of proteins across the membrane of the ER [32], probably due to a membrane abnormality in AD neurons. Since abnormalities of membrane phospholipids in brain and membrane fluidity have been reported in AD [33,34], it may affect the import and export system of proteins across the membrane of the ER. A second possibility is that the activity of an OST might increase in aberrant neurons of AD. The OST is thought to associate closely with translation and generally to have access only to nascent chains as they emerge from the ribosome, resulting in co-translational *N*-glycosylation [30]. However, recently it has been shown that even after completion of translation, the proteins in the lumen of the ER are accessible to OST [35]. Such a post-translational *N*-glycosylation has been observed in unusual circumstances such as on small acceptor tripeptide [36], on non-glycoinsulin receptor generated by tunicamycin treatment or by glucose deprivation-readdition [37,38], and with heat-induced prompt glycosylation of the chaperone protein calreticulin [39]. The phenomenon of post-translational *N*-glycosylation likely applies to tau protein. A third possibility is that the activity of an uncharacterized cytosolic PNGase, which could cleave the glycan moieties from glycoproteins [40,41], might be altered in AD neurons. Recent studies have suggested that misfolded proteins themselves are exported across the ER membrane to the cytosol before degradation and that cytosolic proteasomes are responsible for this degradation process [32]. In some cases, it has been reported that the glycoproteins are deglycosylated by the action of PNGase prior to proteolytic degradation by proteasomes [42–46]. Therefore, defect of deglycosylation may cause the accumulation of abnormal tau. Although details of the mechanism of cytosolic *N*-glycosylation are not clear, *N*-glycosylation of cytosolic protein like tau might occur due to the intracellular perturbation in AD neurons.

It is noteworthy that the presence of *N*-glycan in the cytosolic region has been reported; the Na^+ pump α subunit is a glycoprotein with its cytosolic domain *N*-glycosylated [47]. Furthermore, it is of interest that these *N*-glycans may be GlcNAc-terminating ones because they are galactosylated by using galactosyltransferase, a specific enzyme which attaches galactose to terminal GlcNAc residues, although fine structural study should be awaited. Appearance of truncated *N*-glycans may be relevant to the presence of many glycosidases including sialidase and β-galactosidase in the cytosol [48,49], although occurrence of incomplete glycan processing cannot be eliminated.

Other post-translational modifications of PHF-tau have been reported. For example, PHF-tau is both non-enzymatically glycated [50] and ubiquitinated [51,52]. Recently, an up-regulation of *O*-GlcNAc in AD brains [53–55] and the presence of *O*-GlcNAc on tau have also been reported [22]. It is suggested that *O*-GlcNAcylation is an important regulatory modification that might have a reciprocal relationship with phosphorylation [56]. In the present study *O*-linked GlcNAc in tau was not found by the hydrazine treatment which has been reported to result in the partial degradation of *O*-glycans [57].

Understanding of the mechanism of the neurofibrillary degeneration is critical to devising a rational, therapeutic treatment of AD. Elucidation of aberrant *N*-glycosylation of tau might lead us to a greater understanding of the pathogenesis of AD.

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