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Basic Subunit of Glutelin, Rice Major Storage Protein, has N-Linked Sugar Side Chain

Tadashi KISHIMOTO, Hidetaka HORI, Mayumi WATANABE and Toshiaki MITSUI*

Graduate School of Natural Science and Technology, Niigata University, Ikarashi 2-nocho 8050, Niigata 950-2181, Japan
**Corresponding author* E-mail address: t.mitsui@agr.niigata-u.ac.jp

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Abstract

The basic subunit of glutelin from rice dry seeds were shown to have N-linked sugar chains by lectin staining with PHA-E_4, WGA and Con A. $NaIO_4$ and N-glycanase treatments also provided the evidences of N-linked sugar chains. The purified basic subunit was applied to NEPHGE and eight isoforms of it were separated. The fractionated eight isoforms were shown to be nearly homogeneous by C_{18}-silica column chromatography. Each was subject to lectin staining. The eight isoforms were hybridized with PNA, PHA-E_4 and Con A. This lectin staining indicated that each of the isoforms of the basic subunit had N-linked sugar chain. This is the first report showing the occurrence of N-linked sugar chain in isoforms of glutelin basic subunit.

1. Introduction

During rice seed development, glutelin is synthesized as 57 kDa pro-glutelin in endoplasmic reticulum (ER). Then it is transferred to the vacuole via the Golgi complex and accumulated as protein body II (PBII), which is formed from the vacuolar component (Yamagata and Tanaka, 1986; Li *et al.*, 1992). In vacuole, the 57 kDa pro-glutelin is proteolytically digested into two subunits, i.e., acidic and basic subunits having molecular sizes of 22 to 23 kDa and 37 to 39 kDa, respectively (Yamagata *et al.*, 1982; Sarker *et al.*, 1986). Both subunits are composed of isoforms, which have been thought to assemble for producing the hexamer of glutelin (Sugimoto *et al.*, 1986). Biosynthetic pathway of glutelin resembles to that of 11S globulin (Muntz, 1998) and 30-40% of the amino acid sequence of glutelin was identical to those of 11S globulin (Zaho *et al.*, 1983). The 11S globulin is also composed of acidic and basic subunits (Wen *et al.*, 1985).

Carboxy-terminal of certain proteins such as barley lectins locating in vacuole is known to determine sorting of those proteins to vacuole (Bednarek *et al.*, 1990). Furthermore, N-linked sugar chain was shown to localize on the C-terminal and influence the sorting speed of the proteins to vacuole (Wilkins *et al.*, 1990). According to Wen *et al.* (1989), interestingly, one N-glycosylation site, NES, is located at the C-terminal of glutelin. Thus, if this site is really saturated with sugar chain, this N-linked sugar chain is thought to affect the sorting speed or even sorting destination. However, nothing has been resolved so far regarding the N-glycosylation of glutelin. On the other hand, glutelin has been thought to contain O-glycosylation site in the C-terminal according to nucleotide sequence analyses (Wen *et al.*, 1989). In deed, we found O-linked mucin-type sugar chain, Galβ1-3GalNAc, in the basic subunit of glutelin (Kishimoto *et al.*, 1999).

Glycosylation of the proteins was thought to give them hydrophilicity (Lamport, 1980) and stabilize them against proteolytic degradation (Faye and Chrispeels, 1989). Therefore, the sugar chain of the glutelin may play important physiological roles in biosynthesis of glutelin and proteolytic degradation. But obviously the solubility of proteins cannot be simply attributed to only the sugar chains, following is a good evidence to prove this, thus although 11S globulin and glutelin resemble each other, and former does not have sugar chain (Zhao, *et al.*, 1983), solubility of globulin was substantially higher compared to that of glutelin. As pointed above, nonetheless, the N-glycosylation may play various roles in the biosynthesis and biodegradation of glutelin as well as O-linked sugar side chains. To address these subjects, it is very important to characterize the glycosylation of glutelin.

We purified the glutelin basic subunits and their

eight isoforms and all the components were found to contain N-linked sugar chains.

Here we furnish the proof that the basic subunit of glutelin and its isoforms have N-linked sugar chains. As far as we know, this is the first report to show the presence of N-linked sugar chain in the isoforms of basic subunit of glutelin.

2. Materials and Methods

2.1 Extraction of rice glutelin

Twelve g of de-hulled, dry seed of rice (*Oryza sativa* L. caltivar Koshihikari) were ground to a fine powder, and various rice proteins, such as albumin, prolamin, and globulin were sequentially extracted with 5 mM Tris-HCl, pH 7.5, 70% (V/V), n-butanol and Tris-HCl, pH 7.5, containing 1M NaCl. Each extraction was done with the extraction solutions with reciprocal shaking for 30 min at 110 strokes/min, 70 mm amplitude. The suspensions were centrifuged (17,000 g, 10 min) to recover the proteins in the supernatant fractions. The precipitates were rinsed with distilled water and extracted twice with 0.1% and 1% lactic acid each for 10 min. After the centrifugation the two supernatants containing glutelin were combined and dialyzed against distilled water overnight at 4 ℃. One hundred and fifteen mg of insoluble precipitates resulted during the dialysis and were recovered by centrifugation. The precipitates were suspended with distilled water and subjected to lectin staining, proteolytic digestion and column chromatography after solubilization with 6 M urea.

2.2 SDS-PAGE, lectin staining and NEPHGE of glutelin subunits

Glutelin, subunits and isoforms were analyzed by SDS-PAGE, Laemmli (1970). The SDS-PAGE plates were electroblotted onto polyvinylidene difluoride (PVDF) membrane or nitrocellulose filter (NCF) and detected with erythroagglutinating phytohem agglutinin (PHA-E_4), wheat germ agglutinin (WGA), concanavalin A (Con A), peanut aggulutinin (PNA) conjugated with peroxidase, Mitsui *et al.* (1990), or with enhanced chemi-luminescence kits (ECL, Amersham, Boston MA, USA). The basic subunit was applied to non-equilibrium pH gel electrophoresis (NEPHGE), as described by Abe *et al.* (1996). The protein bands were detected by Coomassie Brilliant Blue (CBB).

2.3 N- and O-glycanase digestion and $NaIO_4$ treatment of the glutelin basic subunit

The glutelin basic subunit blotted onto PVDF membrane was hydrolyzed with 20 mM $NaIO_4$ at 4 ℃ for 4 and 48 h to remove the sugar side chains, Hearn *et al.* (1996). In the glycanase digestions, the PVDF membrane were incubated with either 10 unit N-glycanase or 25 mUnit O-glycanase in 0.1 M Tris-HCl, pH 8.6 and 0.1 M Tris-malate, pH 6.0 containing 0.1% Nonidet P-40 for 24 h at 37 ℃. The treated membranes were stained with lectins as above.

2.4 Bio Gel P-4 column chromatography of the glycopeptide of glutelin digested by N- and O-glycanase

The suspension containing about 60 mg of glutelin was incubated with 600 μg of trypsin pH 8.0 at 37 ℃ for 24 h in 10 mM phosphate buffer, and digested with 100 μg of *Staphylococcus aureus* V8 protease at 37 ℃ for 24 h. The digest was treated with N- or O-glycanase as described above. The resultant glycopeptides were fractionated by a Bio Gel P-4 column chromatography (1.0 × 86 cm) at 50 ℃ and an aliquot of each fraction was blotted onto PVDF membrane to stain with WGA.

2.5 Preparation of glutelin basic subunit by CM Sephadex C-50 column chromatography

The suspension containing about 50 mg of glutelin was adjusted to pH 8.5 with 10 mM Tris-HCl containing 6 M urea, 50 mM 2-mercaptoethanol, 0.1 mM PMSF and 1 mM EDTA and allowed to stand over night at 4 ℃. The suspension was applied to a CM Sephadex C-50 column (1.0 × 10 cm) equilibrated with the same buffer as above and the column was washed with 100 m*l* of the same buffer but containing 0.1 M NaCl to elute acid subunits. The basic subunit was then eluted with 10 mM Tris-NaOH containing the same chemicals as above but at pH 11.4. The eluate containing basic subunit was then adjusted to pH 8.5 with 1 M HCl for re-chromatography on CM Sephadex C-50. The sample was eluted again with the same 10 mM Tris-HCl buffer but containing 500 mM NaCl. We carefully employed these procedures of re-chromatography to avoid aggregation of the basic subunit during preparation, otherwise, it was difficult to remove trace amounts of contaminated acidic subunit from the basic subunit fraction. The eluate containing about 16 mg of basic subunits were dialyzed against distilled water containing 1 mM 2-mercaptoethanol.

2.6 Purification of the basic subunit isoforms.

The dialysate of the basic subunit from CM Sephadex C-50 column chromatography was substituted for 2% (V/V) acetonitrile (CH_3CN) in 0.1% (V/V) trifluoro acetic acid (TFA) and applied to a

HPLC with a C_{18} column (Cap cell pak SG300, 4.6 × 150 mm, Shiseidou Co., Kyoto Japan). The basic subunits were fractionated by biphasically increasing CH_3CN in 0.1% TFA. Thus the CH_3CN concentration was increased from 2 to 37.5% for 30 min and further to 60% for 50 min. All isoforms of the basic subunits except number 2 and 3, were recovered and re-chromatographed twice with the same conditions. The two isoforms, No 2 and 3, were also re-chromatographed but the increase of the solvent was 2 - 37.5% for 30 min and 37.5%-41.5% for 80 min. Eight isoforms were finally recovered as protein amount of 28.7% from glutelin extract.

3. Results

3.1 Lectin staining of the basic and acidic subunits

Glutelin was extracted from dry rice seed, analyzed by SDS-PAGE under reducing conditions and was shown to be composed of polypeptides with apparent molecular size of 21-22 kDa, 32-34 kDa and 54 kDa (**Fig. 1A**, lane 1). These profiles were almost the same as that of glutelin and its subunits shown in Yamagata *et al.*, (1982). To prove whether the glutelins were glycosylated, they were incubated with various lectins such as Con A, PHA-E_4, WGA and PNA conjugated with peroxidase. These lectins all bound to the polypeptides electroblotted onto NCF but in a somewhat different fashion from each other. Thus, the acidic and basic subunits and 54 kDa polypeptide were stained with Con A, PHA-E_4 and WGA (**Fig. 1A**, lanes 2, 3 and 4). The basic subunits, however, obviously had higher affinity to the lectins than either the acidic subunit or 54 kDa polypeptide. On the other hand, PNA bound to only basic subunits (**Fig. 1A**, lane 5). PHA-E_4 and WGA have been thought to have affinity with bisecting GlcNAc (Yamamoto *et al.*, 1981; Yamashita *et al.*, 1983) and Con A has affinity with high mannose structure of sugar side chains. Furthermore, PNA has been shown to have a high specificity to Galβ1-3GalNAc structure localized on O-linked sugar chains (Lotan *et al.*, 1975). Therefore, these results clearly suggest that glutelin was most likely glycosylated.

To demonstrate if the four lectins recognized specifically a sugar moiety of the basic subunit, the sugar side chains were destroyed. The basic subunits were blotted onto PVDF membranes after the SDS-PAGE, and were treated with sodium periodate (Hearn *et al.*, 1996). The 48 h-treatment by periodate significantly reduced the binding of Con A, PHA-E_4, WGA and PNA to the basic subunit (**Fig. 1B**). The loss of the binding between the

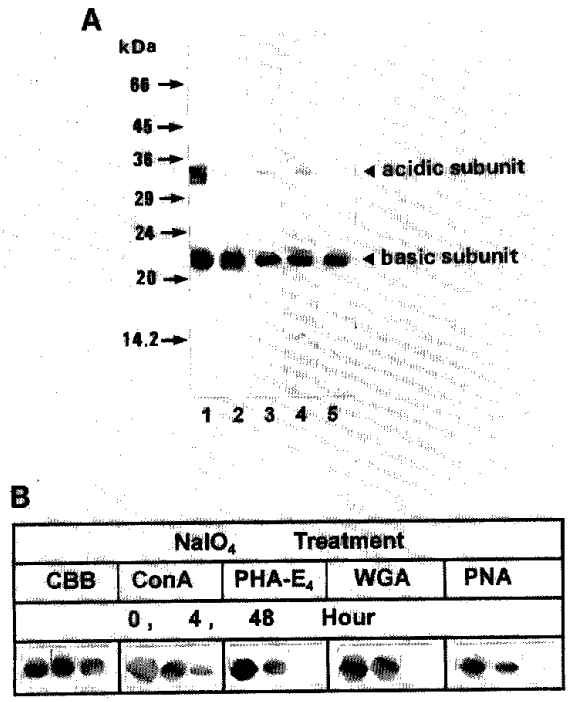

Fig. 1 Hybridization of basic subunit of glutelin with various lectins. Glutelin and its subunits were extracted and analyzed by SDS-PAGE. (A) The proteins were blotted onto PVDF membrane and bound to Con A (lane 2); PHA-E_4 (lane 3); WGA (lane 4) and PNA (lane 5). The proteins were stained with CBB (lane 1). (B) The basic subunits separated by SDS-PAGE were blotted onto PVDF membrane and treated with $NaIO_4$ for 4 h and 48 h. The samples treated were hybridized with Con A (lanes 4-6), PHA-E_4 (lanes 7-9), WGA (lanes 10-12) and PNA (lanes 13-15). The subunits treated were stained with CBB (lanes 1-3).

lectins and basic subunit was not due to a loss of the protein since the staining with CBB was not changed. These results suggested that the glutelin basic subunit contained both N-linked and O-linked sugar chains which were susceptible to periodate oxidation.

3.2 Effects of N-glycanase digestion of the glutelin on the affinity with the lectins

The basic and acidic subunits separated by SDS-PAGE were electroblotted onto NCF, treated with N-glycanase and stained with Amido Black, Con A, PHA-E4, WGA and PNA (**Fig. 2**, upper picture). The digestion almost completely eliminated the binding of Con A, PHA-E_4 and WGA to the subunits (Upper picture, corresponding columns marked +). The treatment, however, did not change the binding with PNA (Column PNA , + and -). Protein staining with Amido Black was not changed

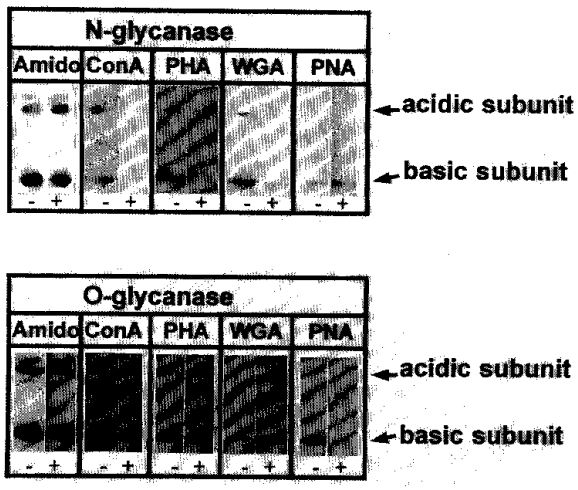

Fig. 2 The effect of N- and O-glycanase digestion on the hybridization between acidic and basic subunits and various lectins. The basic and acidic subunits separated by SDS-PAGE were electroblotted onto NCF and treated with N-glycanase (upper picture) or O-glycanase (lower picture) for 48 h. They were analyzed with the lectins, and were also stained with Amido Black. The symbols + and - represented treated and non-treated, respectively.

by the treatment (Column Amido, + and -). These results suggest that Con A, PHA-E_4 and WGA bound to the sugar chains, which were removed by N-glycanase.

To check if the binding was specific to N-linked sugar chains, the basic subunit was digested with O-glycanase and stained with the lectins (**Fig. 2**, lower picture). The lectin staining was opposite to that of N-glycanase treated. Thus, the O-glycanase treatment reduced the binding with only PNA (Column PNA, + and -). Amido Black staining again was not changed by the O-glycanase treatments (Column Amido, + and -). These results clearly support that glutelin was glycosylated with N-linked sugar chains.

3.3 Bio Gel P-4 column chromatography of glutelin-peptides de-glycosylated by N-glycanase

The suspension containing glutelin was proteolytically digested with trypsin and V8. The digests were further treated with N-glycanase to remove N-linked sugar chains and resulted peptides were analyzed by Bio Gel P-4 column chromatography (**Fig. 3**). The elution of part of the peptides was delayed in the chromatography on the digestion with N-glycanase. Especially, the shift was significant in the smaller sized peptides compared to that of the larger one. The peptides were also digested with O-glycanase and analyzed the same way. The treatment shifted the elution profile again, but the

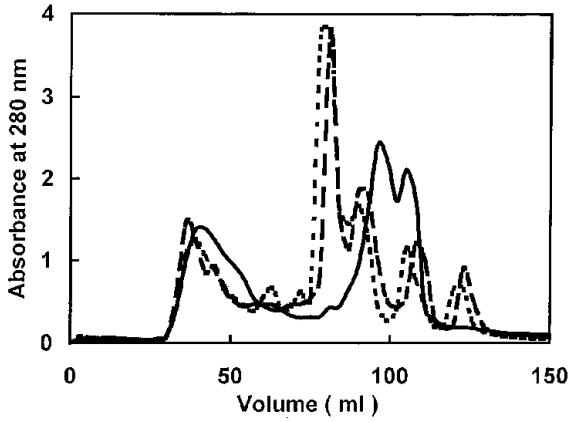

Fig. 3 Bio Gel P-4 column chromatography of glutelin-peptides treated with N- and O-glycanases. Glutelin was digested with trypsin and V8 and resulted peptides were further treated with N-glycanase (— —) and O-glycanase (——). Those treated samples were fractionated by Bio Gel P-4 column chromatography and elution profiles were compared to that of no-treated glutelin-peptide (—·—).

delay was less than that of N-glycanase treated. These results suggest that the sugar side chains were cleaved from glutelin by either N-glycanase or O-glycanase digestions. N-glycanase was thought to be able to release N-linked sugar chains, having no α-fucose residue bound to C-3 of GlcNAc, linked to peptidyl Asn (Tretter *et al.*, 1991). On the other hand, O-glycanase has been thought to digest the linkage between GalNAc and Ser/Thr in the disaccharide, Galβ-1,3GalNAc-Ser/Thr (Umemoto *et al.*, 1977). Thus, the difference of the shift made by the enzyme treatments in the column chromatography may reflect the fact that either the population of the N-linked sugar chains was larger than that of the O-linked or the molecular size of the sugar side chain chopped off by N-glycanase was larger than that by O-glycanase.

Each fraction was fractionated after the treatment with N-glycanase by Bio Gel P-4 column and dot blotted to check the affinity with WGA. The N-glycanase digestion resulted in loss of WGA binding to the peptide on the membrane (data not shown).

3.4 Separation of basic subunit isoforms

The basic subunit purified from the solubilized glutelin was further fractionated by a CM Sephadex C-50 column chromatography with a step-wise elution. The basic subunit was analyzed by SDS-PAGE. Thus, in the first CM C-50 chromatography, the washings with Tris-HCl buffer containing 0.1 mM NaCl mainly harbored the acid

subunit (**Fig. 4A**, lane 3). In addition to the 32-34 kDa subunit, a 24 kDa polypeptide was also contained in this fraction (lane 3). But none of the lectins such as PNA and Con A interacted with the 24 kDa-peptide (data not shown). On the other hand, the eluate in the second CM C-50 chromatography contained only the basic subunit (lane 4).

The purified basic subunit was analyzed with a NEPHGE and the eight bands were detected in the electrophoresis (**Fig. 4B**).

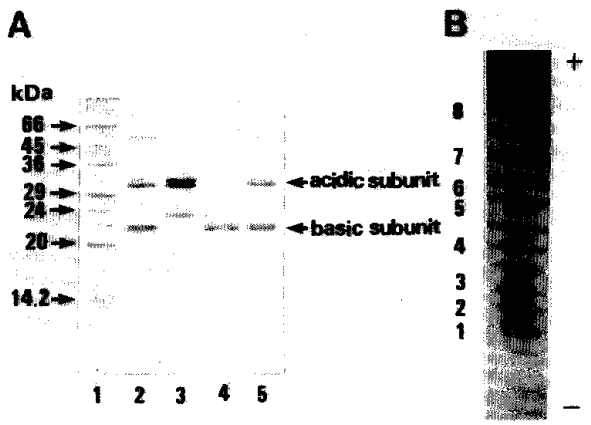

Fig. 4 SDS-PAGE of the subunits purified by CM Sephadex C-50 column chromatography (A) and NEPHGE of the basic subunit (B). A: Lanes 2 and 5: solubilized glutelin. Lane 3: eluate from the CM-50 contained acidic subunit. Lane 4: eluate from the CM-50 column contained basic subunit (for detail see Materials and Methods).
B: The basic subunit recovered in the second eluate of the Tris-buffer (lane 4) was analyzed by a NEPHGE.

3.5 Purification of basic subunit isoforms by C_{18}-Silica column chromatography

To recover the eight isoforms shown in NEPHGE (**Fig. 4B**), the basic subunit purified by CM Sephadex was further fractionated by C_{18}-silica column chromatography and eight peaks were detected (**Fig. 5A**). The peaks were individually recovered and applied to the same column twice to obtain each single peak. By these two re-chromatographies, eight, almost homogeneous, peaks were recovered (**Fig. 5B**). The homogeneity of the peaks was further checked by capillary electrophoresis and they were shown to be nearly homogeneous (Data not shown). The eight peaks from the C_{18} column re-chromatography were checked whether they were glycosylated or not using Con A, PHA-E_4 and PNA. All eight isoforms were shown to be recognized by the lectins, as shown in **Fig. 6**. This result clearly suggests that all of them were glycosylated with N-linked sugar chain(s).

The content of each isoform in the basic subunit was calculated using the integrated individual peak area in C_{18}-chromatography (**Table 1**). The isoforms of 2 and 5 were the major elements and they

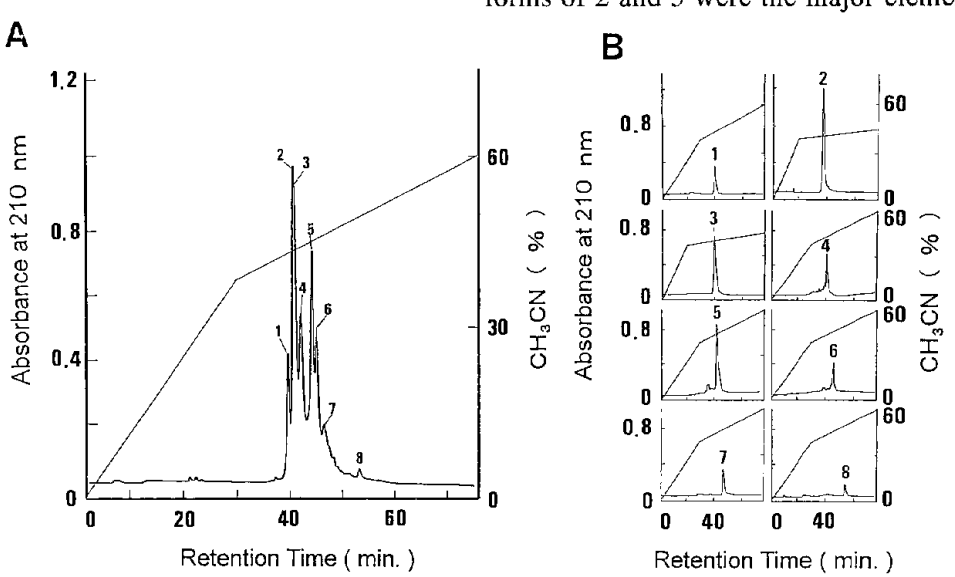

Fig. 5 C_{18}-Silica column chromatography of basic subunit purified by CM Sephadex C-50.
A: The basic subunit was recovered from the CM Sephadex C-50 column chromatography and applied to HPLC with the C_{18} column (Cap cell Pak SG300). The sample was eluted with biphasic increasing of CH_3CN in 0.1 % TFA. The gradient of CH_3CN in first phase was 2-37.5 % for 30 min. In the second phase, the gradient was increased to 60 % for 50 min.
B: The eight peaks shown in panel A were recovered individually and chromatographed twice with the same column and condition. Peaks 2 and 3 were differently purified as shown in Materials and Methods. The numbers of the peaks in both panels were corresponded each other.

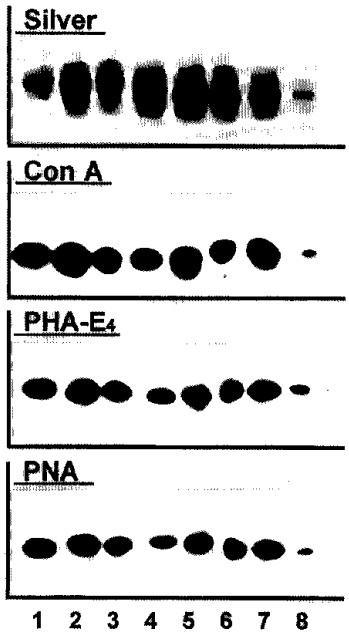

Fig. 6 H telin basic subunit with various lectins. The eight isoforms isolated by C_{18}-Silica column chromatography were blotted onto PVDF membrane and bound to Con A, PNA and PHA-E_4. They were also visualized with silver stain. The numbers 1-8 were corresponded to that of peaks shown in Fig. 5.

Table 1. Contents of eight isoforms in the glutelin basic subunit

# of Isoforms	Contents of Isoforms (% weight)
1	7.8
2	24.4
3	17.4
4	12.2
5	20.1
6	10.1
7	7.2
8	0.8
Total (%)	100

occupied about half the weight of the glutelin.

4. Discussion

We presented that the basic subunit of rice glutelin had N-linked sugar chains. Furthermore, the lectins, we used to proof the occurrence, suggested that the glutelin basic subunit may be glycosylated with either high mannose- and complex-type sugar chains. Indeed N-linked sugar chains of glutelin showed sensitivity toward $NaIO_4$ and N-glycanase but not to O-glycanase (**Fig. 1**, **2** and **3**). N-glycanase cleaves the N-linked sugar chains which contain even β-1,2-linked Xyl residue such as Man-α-1,6(Xyl β-1,2)Man β-1,4GlcNAc β-1,4GlcNAc-Asn (Tretter *et al.*, 1991), but it can not chop off the sugar chain containing Fuc residue which links to inner most GlcNAc with α-1,3 bond (Leiter *et al.*, 1999). Interestingly, the Fuc residue linking with α-1,6 does not change the susceptibility toward the N-glycanase. Therefore, since N-glycanase released the sugar chain, N-linked sugar chain of glutelin may not contain the Fuc residue linking α1-3 bond.

The N-linked sugar chains of glycoproteins in plants are grouped into two types. Thus, one is complex type sugar chain, which is represented following structure;

Man α-1,3(Xyl β-1,2)Man β-1,4(Fuc α-1,3)GlcNAc-β-1,4GlcNAc or

Man α-1,3(Man α 1-6)(Xyl β-1,2)Man β-1,4GlcNAc-β-1,4(Fuc α-1,3)GlcNAc

(Lerouge, *et al.*, 1998). The other is the ordinary high mannose-type sugar chains described elsewhere (Elbein, 1979; Hori and Elbein, 1982; Kornfeld and Kornfeld, 1985; Tanner and Lehle, 1987).

The glutelin basic subunit bound to PHA-E_4 and WGA. Interestingly, these lectins are known to recognize bisecting GlcNAc residue linked to inner most Man residue of the core structure by β-1,4 linkage (Yamamoto *et al.*, 1981; Yamashita *et al.*, 1983). This branched structure having bisecting GlcNAc residue can be seen often in mammalian glycoproteins such as immunoglobulin G (Takahashi *et al.*, 1987). Thus the result contends that the modification resulting in the bisecting GlcNAc may be taking place in glutelin as well as mammalian cells. If this is the case, since the all isoforms from basic subunit were recognized by PHA-E_4 (**Fig. 6**), each isoform composing basic subunit may have sugar chain containing bisecting GlcNAc.

Plant storage proteins such as phytohemagglutinin, phaseolin, Con A, β-conglycinin and barley lectin have N-linked sugar chains. The glycosylation of some of those proteins were inhibited by tunicamycin or genetic engineering for elucidating the roles of N-linked sugar chains. The roles of N-linked sugar chains were suggested to increase the solubility of the proteins, maintain stable structure of the proteins, promote right folding and protect the proteins from proteolytic breakdown (Chrispeels, 1991). Furthermore, the carbohydrate moieties were shown to not affect the processing of phytohemagglutinin (Voelker *et al.*, 1989), destination of the protein sorted and assembly of subunits for constructing mature proteins, but they reduced the speed of transport of the proteins from ER to the Golgi apparatus and assembly of the

subunits in barley lectin and phaseolin (Wilkins *et al.*, 1990; Ceriotti *et al.*, 1995; Vitale *et al.*, 1995). Moreover, bisecting GlcNAc in N-linked sugar chain inhibited the transport of a certain protein to plasma membrane as so called negative sorting signal (Sultan *et al.*, 1997).

On the other hand, the N-glycosylation of glutelin has long been enigma, thus, the various suggestions or facts cited above are not available in glutelin.

We clearly presented the evidences showing occurring of N-linked sugar chains in glutelin in this report and moreover we obtained the suggestion that some of these sugar chains contained bisecting GlcNAc. It is of interest to know these N-linked sugar chain or especially one containing bisecting GlcNAc have what kind of roles.

These are especially important to sketch the biosynthesis of glutelin. Glutelin basic subunits are very hydrophobic, but isoforms being composed of the subunits showed good solubility to the extent of 1 mg ml^{-1} of water. When different types of isoform were mixed, however, they coagulated. The mixing of basic and acidic subunits also resulted in the aggregation (Data not shown). These *in vitro* experiments suggested that sugar side chain may provide hydrophilicity to each isoforms and this may be convenient for sorting of the pro-glutelin, 57 kDa peptide, from ER to vacuole. Thus, the 57 kDa pro-glutelin must be composed of the same kind of isoforms, since as mentioned different type of isoforms coagulated.

On the other hand, *in vitro* mixing of basic and acidic subunits of 11S globulin which has no sugar chain also resulted in re-association of 11S hexamer (Dickinson *et al.*, 1989). Thus the mechanism of coagulation in both glutelin and 11S globulin should be the same and physicochemical interaction between peptides is taking places. In the vacuole, isoforms composing the basic subunit and may be acidic subunit also will be shuffled to be insoluble hexamer according to the physicochemical reactions.

Hence, the roles of sugar side chains of glutelin in its biosynthesis must be uncovered.

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