Characterization of low molecular weight starch granule associated protein in common wheat by proteomic approaches

Nazrul Islam, Motoko Takaoka, Hidenori Sassa, Hiroshi Kawasaki, Hisashi Hirano

SUMMARY

Starch granule proteins (SGPs) were extracted from wheat seeds and characterized using proteomic approaches: two dimensional electrophoresis (2-DE), 40 cm long gradient sodium dodecyl sulfate (SDS) gel electrophoresis and electrospray ionization mass spectrometry. The majority of the low molecular weight (LMW) SGPs, especially in the range of 14-30 kDa, was located on the surface of starch granule, and only a small number of LMW SGPs of molecular weight range from 30 to 55 kDa was found within starch granules. The surface SGPs were mostly expressed in acidic, whereas the integral SGPs were expressed basic regions of 2-DE. Gene location of one surface SGP of approximately 14 kDa was assigned on short arm of chromosome 4B. One of the LMW SGPs was identified by LC-ESI MS/MS endochitinase (30 kDa). Intensity of this proteins was found to decrease during seed germination and seed maturation.

Key words: starch granule, protein, wheat, proteomic approach.

INTRODUCTION

In addition to storage proteins, wheat seeds contain some other minor proteins tightly associated with starch granules, and is commonly known as starch granule proteins (SGPs). The SGPs are biologically distinct from the storage proteins and are either tightly bound on the surface of starch, the surface SGP, or located inside the starch granules, the integral SGP. These proteins are also considered to have substantial contribution in the bread making quality of wheat flour. Based on extraction procedure and electrophoretic mobility, Greenwell and Schofield detected ten surface SGPs with molecular weight ranged from 5 to 149 kDa, and five integral SGPs with molecular weight ranged from 59 to 149 kDa. Among the integral SGPs, the 60 kDa protein, more commonly known as the Waxy protein, has been studied in detail. Substantial information on the genetic control of the integral SGPs having molecular masses higher than the waxy proteins are also available from the studies elsewhere. However, there is a little known about low molecular weight SGPs with the exception of a 15 kDa surface SGP, the friabilin.

Identification of SGPs and other proteins in wheat and other cereals has commonly been performed by a gas-phase (Edman) sequencing method. But this method is neither a rapid nor a sensitive; it needs at least pmol amount of protein for identification. Moreover, if the N-terminal amino groups of proteins are modified, we can not determine the N-terminal amino acid sequences by Edman degradation. Although the high molecular weight integral SGPs are well characterised by using this method because of their higher abundance, the characterization of LMW SGPs was always a challenge until the recent development of protein identification techniques. Recent development of proteomics techniques such as 2-DE electrophoresis, high resolution long gradient gel, image analyzer and soft ionization mass spectrometry (MS) has provided a new range of useful tools for in depth characterization of proteins. 2-DE which analyses complex mixtures of denatured proteins based on two independent criteria, charge (pI) and molecular weight (Mr), has become a powerful technique of choice for detecting and quantifying protein in plant proteome research. Matrix-assisted laser desorption/ionization time of flight mass spectrometry analysis of proteins,
although considered fast and specific, presents however a high mass resolution, it is still poor compared with other techniques such as electrospray mass spectrometry (ESI-MS). It has been also reported that the LC ESI MS can be used to identify protein and to determine its molecular weight with accuracy range of 0.1 to 0.01% even if there is a small amount protein, fmol, present in the sample. To our knowledge, application of these novel techniques has not been performed to the investigation of LMW SGPs. In our current research, we aim to separate the SGPs in common wheat by using high resolution long gradient gel and 2-DE, and characterized the LMW SGPs by LC-ESI MS.

MATERIALS AND METHODS

Plant materials
Ditelocentric lines (DT) of common wheat (Triticum aestivum L.) cv Chinese Spring were originally produced by Sears, and have been maintained in the gene bank of Kihara Institute for Biological Research, Yokohama City University, Japan. The codes designated in DT lines are the chromosomal arm present in the lines, and thus, DT-1BS, for example, indicates the plant lacking whole long arm of chromosome 1B. Two varieties of waxy wheat Mochiotome and TNCMH-2 were provided by Dr. M. Yamamori (National Institute of Agrobiological Resources, Tsukuba, Japan). Developing endosperms were collected from the varieties grown in the experimental farm of our institute. In germination study, seeds were surface sterilized and placed in petridishes containing wet blotting paper. Germinating seeds were colleted on 2nd and 3rd days after germination.

Extraction of Starch
Endosperms were dissected from seeds and ground to obtain flour. Proteins were extracted according to the procedures described by Takaoka et al. and Sulaiman and Morrison with slight modification. A portion of the flour (60 mg) was homogenized in water with a mortar and pestle, macerated and kept at 4°C for overnight. In the following morning, the dough was kneaded by tooth pick and the milky washing water is strained through a fine bolting cloth. The milky water was then centrifuged at 15000 g for 10 min. The sediment of crude starch was suspended to 300 µl of SDS sample buffer. After a vortexing briefly, the suspension was placed in water bath heated at 100°C for 10 min and cooled on ice for 1 h. After thawing at room temperature, the gelatinized starch was centrifuged at 15000×g for 10 min, and the supernatant was transferred to a fresh tube. This fraction is considered as integral SGP.

Proteins from both the fractions, surface and integral SGPs, were precipitated on ice for 1 h after the addition of 3 volumes of cool acetone. The samples were centrifuged, and the pellet containing the starch granule proteins was washed with cool acetone twice with water and then air dried overnight. In the following morning, the hard pellet was suspended to 300 µl of SDS sample buffer. After a vortexing briefly, the suspension was placed in water bath heated at 100°C for 10 min and cooled on ice for 1 h. After thawing at room temperature, the gelatinized starch was centrifuged at 15000×g for 10 min, and the supernatant was transferred to a fresh tube. This fraction is considered as integral SGP.

Electrophoresis
Both surface and integral SGPs were separated in long gradient SDS gel (10 to 20%, 0.9 mm) (Daichi Pure Chemicals Co.) and in 10 cm SDS PAGE (10%/17%). For 2-DE, sample-solutions (40 µl) were loaded on to the acidic side of the isoelectric focusing (IEF) rod gels (pH 4–11) for the 1st dimension, and anodic and cathodic electrode solutions were filled in the upper and lower electrode chambers, respectively. SDS-PAGE in the second dimension was performed with 17% separation and 5% stacking gels. Protein spots in 2-DE gels were visualized by silver staining. Each sample was run at least two times.

Protein identification by ESI-MS/MS
Proteins separated by 2-DE were digested in gels with trypsin (for details see our previous publications). The digested peptide fragments were injected to a capillary liquid chromatography (Waters) equipped with a pre-column, LC packing Pepmap (320 µm i.d. x 1 mm), and an analytical column, LC Packing Pepmap (75 µm i.d. x 150 mm). After desalting, the peptides were eluted by gradient flow of acetonitrile and water containing 0.1% (v/v) formic acid and then injected into Q-TOF MS (Q-Tof Ultima, Micromass, UK) through nano LC probe (ESI). Data acquisition was performed through MassLynx, and peptide sequence analyses were done by BioLynx (Micromass, UK). The sequence information was submitted to SWISS-PROT and EMBL protein and peptide databases for identification.

Extraction of Surface and Integral SGP
Sixty milligrams of the dried starch was suspended in 200 µl of SDS sample buffer containing 60 mM Tris-HCl, pH 6.8, 10% (w/v) sodium dodecyl sulfate (SDS), 3% (v/v) β-mercaptoethanol and 10% (v/v) glycerol, vortexed for 30 min and then placed in a water bath heated at 30°C for 1 h. The sample was then centrifuged at 15000×g for 10 min, and the supernatant was transferred to a fresh tube. The extraction procedure was repeated one more time, and the supernatant was added to the previous fraction. This fraction is considered as surface SGP. The sediment was washed with cool acetone twice with water and then air dried overnight. In the following morning, the hard pellet was suspended to 300 µl of SDS sample buffer. After a vortexing briefly, the suspension was placed in water bath heated at 100°C for 10 min and cooled on ice for 1 h. After thawing at room temperature, the gelatinized starch was centrifuged at 15000×g for 10 min, and the supernatant was transferred to a fresh tube. This fraction is considered as integral SGP.

Materials and methods

Electrophoresis
Both surface and integral SGPs were separated in long gradient SDS gel (10 to 20%, 0.9 mm) (Daichi Pure Chemicals Co.) and in 10 cm SDS PAGE (10%/17%). For 2-DE, sample-solutions (40 µl) were loaded on to the acidic side of the isoelectric focusing (IEF) rod gels (pH 4–11) for the 1st dimension, and anodic and cathodic electrode solutions were filled in the upper and lower electrode chambers, respectively. SDS-PAGE in the second dimension was performed with 17% separation and 5% stacking gels. Protein spots in 2-DE gels were visualized by silver staining. Each sample was run at least two times.

Protein identification by ESI-MS/MS
Proteins separated by 2-DE were digested in gels with trypsin (for details see our previous publications). The digested peptide fragments were injected to a capillary liquid chromatography (Waters) equipped with a pre-column, LC packing Pepmap (320 µm i.d. x 1 mm), and an analytical column, LC Packing Pepmap (75 µm i.d. x 150 mm). After desalting, the peptides were eluted by gradient flow of acetonitrile and water containing 0.1% (v/v) formic acid and then injected into Q-TOF MS (Q-Tof Ultima, Micromass, UK) through nano LC probe (ESI). Data acquisition was performed through MassLynx, and peptide sequence analyses were done by BioLynx (Micromass, UK). The sequence information was submitted to SWISS-PROT and EMBL protein and peptide databases for identification.
RESULTS AND DISCUSSION

Starch purification and protein extraction

Over the last few years, the procedures for extraction of starch granules from wheat flour have changed a very little, although this procedure is considered to have great importance to the separation of SGPs from storage proteins in the starch fraction\textsuperscript{11}. Traditionally, starch granules are extracted from flour by gluten washing, washing flour with water to remove the starch from the insoluble storage proteins\textsuperscript{15,16}. The cell debris is then removed by distilled water followed by filtration and/or centrifugation steps. In an attempt to purify starch by this procedure in our investigation, we found a substantial quantities of residual storage proteins with the surface SGP (data not shown). To avoid contamination of residual storage proteins, we have modified the procedures reported earlier (for details see materials and methods). This has enabled us to extract starch with higher degree of purification.

SGPs are commonly extracted either by NaCl solution\textsuperscript{17} or by 2 to 5% SDS detergent. Rahman et al.\textsuperscript{10} has clearly demonstrated that the buffer containing 10% SDS extracted a substantially higher yield of SGPs compared to the lower concentration (2 to 5%). In addition to extraction reagents, temperature during extraction is also reported to have substantial influence on extraction of proteins from starch granules. Gough et al.\textsuperscript{19} chose an extraction temperature of 50°C in presence of SDS, because they felt that, at this temperature, the surface SGPs would be extracted but the starch granules themselves would not be disrupted. On the other hand, Schofield and Greenwell\textsuperscript{20} reported that the extraction temperature above 30°C forced some of the integral protein to come out from the starch granules. Based on these information, we adopted extraction temperature of 30°C for surface SGPs and 100°C for integral SGPs in presence of 10% SDS.

Surface and integral proteins separation by 40 cm gradient and 2-DE gels

Although the 2-DE, which analyses complex mixtures of denatured proteins based on two independent criteria-charge (pI) and molecular weight (Mr), has become a powerful technique of choice for detecting protein in plant proteome research, it has several challenges yet to be made. For example, it is difficult to resolve highly acidic or basic proteins by 2-DE, and proteins having pI beyond the range of 2-DE resolution remain undetected. On the other hand, the SDS PAGE (10% or 17%) which has traditionally been used for protein separation is reported inefficient in identifying proteins with very close molecular weight. Recently, Daiichi Pure Chemicals Co., Japan, has introduced a 40 cm long gradient gel which are reported to be efficient in separating proteins with close molecular weight. Using this gradient gel, we found a clear separation of both the surface and integral proteins (Fig. 1 B and C).

As shown in Fig. 1, there was a clear separation of SGP-2 and SGP-3 in long gradient gel (B) compared to the 15 cm SDS PAGE (C). Some of the bands, for example 30 kDa, appeared as a single band in SDS PAGE (C) separated into 2 to 3 bands in long gradient gel (B), indicating the robustness of long gradient gel in protein separation. Using this gel, we detected about 70 protein bands on the surface, and about 15 protein bands inside the starch granule. These bands correspond to proteins with molecular weight ranging from 5 to 149 kDa for surface and 15 to 149 for integral SGPs. Out of the 70 surface SGPs, only two of the proteins have received intensive investigation, the 15 kDa friabilin/puro-indolines\textsuperscript{21} and 30 kDa glycoprotein\textsuperscript{20}, but information for the rest of the proteins is not available. It is interesting to note here that many proteins ranging from 5 to 30 kDa are located on the surface of starch granule. These proteins are believed to have significant role on starch-protein interactions. Recently Baldwin\textsuperscript{11} have indicated that the LMW surface SGPs play a fundamental role as a first barrier to processes such as granule hydration, enzyme attack and chemical reaction with the modifying agents. One of the LMW surface SGPs, about 16 kDa, shown by arrow mark in Fig. 1A, disappeared in 4BL, suggesting that gene encoding this protein is located on the short arm of 4B chromosome. When a band/spot disappeared due to the missing of one chromosomal arm or region, it is assumed that the structural gene controlling the protein is located in that part of the chromosome\textsuperscript{22–24}. The same principle is also applied to localize enzyme loci.

Unlike the surface SGPs, no protein band was observed from 5 to 30 kDa region except one at 15 kDa (Fig. 1B). On the other hand, seven LMW SGPs were detected in molecular weight range of 29 to 59 kDa region. Of the seven bands, three bands (marked by arrow) were either disappeared or their expressions were greatly reduced in the waxy lines (Wt and Wm), indicating that the expression of these proteins is related to the expression of waxy proteins.

Analysis of SGPs by 2-DE (Fig. 2) revealed that the surface SGPs were mostly expressed in acidic, whereas the integral SGPs were expressed in basic regions, suggesting that integral SGPs are basic and most of the surface SGPs are acidic proteins. The 2-DE pattern also indicates that the proteome map of surface SGPs were quite distinct from that of the storage proteins (see our previous publications for proteome map of storage proteins\textsuperscript{22–24}).

Identification of integral LMW SGPs

A substantial research has been performed in characterizing integral SGPs specially the 60 kDa starch synthase, commonly known as Waxy protein or SGSS\textsuperscript{1,2,4}. This protein constitutes the major starch granule associated proteins in wheat seed. In addition to the 60 kDa SGSS, wheat seed also contains five other high molecular weight proteins of 115, 108, 100 and 92 kDa\textsuperscript{3,5}. These proteins...
are considered as isoforms of SGBSS. Because of the higher abundance, the high molecular weight integral SGPs (60 kDa and above) are well characterised, but the characterization of LMW SGPs was always a challenge because of their low abundance until the recent development of protein identification techniques such as soft ionization mass spectrometry. As shown in Fig. 1, there are some LMW integral SGPs which have not been studied probably because of their low abundance. The presence of LMW integral SGPs are also evident from Figures reported by Yamamori and Endo4) and Nakamura et al.5).

Using LC ESI-MS/MS, we identified two LMW integral proteins (Fig. 3). Out of the four bands detected, the band-1 is most abundant and identified as endochitinase (30 kDa). This protein is widely distributed among higher plants, and it functions as a defence against any invasion. The presence of this protein inside starch granules is not clear. The second band was identified as β-amylase inhibitor (16 kDa) (Fig. 3).

**LMW SGPs expression in developing and in germinating seeds**

The expression patterns of LMW integral SGPs were studied in developing and in germinating seeds of common wheat. Results revealed that, in germinating seeds, the intensity of endochitinase (30 kDa) was found to decrease during seed germination (Fig. 4). Similarly, the intensity of endochitinase (30 kDa) had decreased substantially during grain development. These results suggest a possible involvement of the protein in mobilizing energy for metabolic requirements. Further studies concerning their specific role in metabolic mechanism is necessary.

**CONCLUDING REMARKS**

It is clear from our studies that the application of novel proteomic techniques such as two dimensional electrophoresis, long gradient gel electrophoresis and electrospray ionization mass spectrometry is very useful to study the low abundance LMW integral SGPs. Two LMW SGPs, endochitinase (30 kDa), and α-amylase inhibitor (16 kDa), identified for the first time by LC-ESI MS/MS from our study will assist cereal scientists to elucidate their possible roles in bread making quality or in other metabolic pathways. Therefore application of proteomic approach to study LMW integral SGPs in association with knowledge of other abundant SGPs could help cereal scientists to modify starches for a new properties.
Fig. 2. 2-DE patterns of surface and integral SGPs in Chinese Spring (A) and in Norin61.

Fig. 3. Peptide sequence and identification of LMW SGP bands by LC ESI MS/MS. Peptide sequence of band 2 is shown in the figure, and other information are listed in table.
Fig. 4. Expression pattern of integral starch garnule proteins in developing (A) and in germinating (B) seeds of common wheat separated by 10 and 17% SDS PAGE respectively.

REFERENCES


