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Synthesis of Gluten-Forming Polypeptides. 1. Biosynthesis of Gliadins and Glutenin Subunits

TIBOR ABONYI,[†] ISTVÁN KIRÁLY,[§] SÁNDOR TÖMÖSKÖZI,[†] ORSOLYA BATICZ,[†] ADRIENN GUÓTH,[†] SZILVESZTER GERGELY,[†] ÉVA SCHOLZ,[†] DEMETER LÁSZTITY,[§] AND RADOMIR LÁSZTITY*[,†]

Department of Biochemistry and Food Technology, Budapest University of Technology and Economics, P.O. Box 91, Budapest H-1521, Hungary, and Department of Plant Physiology, Eötvös Loránd University Budapest, Pázmány Péter sétány 2, Budapest H-1117, Hungary

Five winter wheat cultivars—GK Öthalom (HMW-GS composition 2*, 7+8, 5+10), Ukrainka (1, 7+8, 5+10), Palotás (2*, 7+9, 5+10), Ködmön (2*, 7+8, 5+10), and Csongrád (2*, 7+9, 2+12)—grown in Hungary and harvested in the year 2005 were studied. The biosynthesis of gluten-forming polypeptides was followed starting at the 12th day after anthesis to the 53rd. Fresh kernel weight, moisture, and dry matter content of fresh kernels and gliadin and glutenin contents were determined. Gliadin components, total amounts of HMW and LMW polypeptides, and individual HMW polypeptides were determined using a RP-HPLC technique. Although considerable quantitative differences were observed concerning the content of total protein, gliadin, glutenin, and individual gluten-forming polypeptides, the character of accumulation of protein components—determined on the basis protein mass/kernel—was the same for the all of the cultivars studied and could be presented by a sigmoid curve. Small quantities of the gliadin and glutenin monomers may be detected in early stages of kernel development, but the bulk of these proteins is synthesized in later stages of development. It is generally suggested by specialists that the formation and accumulation of glutenin polymers starts later than the synthesis of monomers. Experimental data presented in this paper confirm this suggestion and show that in the first phase of protein synthesis the monomers are in "free" form; polymeric glutenin is detected only later. HMW glutenin subunits are synthesized synchronously, and quantitatively the polypeptides coded by chromosomes D and B dominate.

KEYWORDS: Gluten; biosynthesis of gluten proteins; gliadin; glutenin; HMW; LMW; RP-HPLC

INTRODUCTION

The preparation of gluten and the description of its properties were published by Beccari as early as the 18th century, and the first systematic description of proteins of wheat endosperm was made by Osborne (*1*) in the 20th century. Because it has been shown that gluten may be fractionated to gliadin (soluble in 70% alcohol) and glutenin (dissoluble in dilute alkali or acid), the study of correlation between protein composition of kernel and wheat quality remained an important part of cereal research (*2*, *3*).

In particular, breeders were interested in finding relatively simple methods of prediction of final wheat quality in the early stages of the breeding process. Early research concentrated on the ratio of macrofractions (e.g., gliadin/glutenin ratio), and later the gliadin polypeptides, the genes coding for these polypeptides, and the variety identification became increasingly a focus for studies. At present, prediction based on high molecular weight (HMW) glutenin subunits is widely accepted and used (*4*).

Although this latter approach to quality prediction is successfully applied in many countries in wheat breeding (*5*−*7*), the often observed low correlation (and even contradiction) between predicted and practical quality (*8*−*10*) and the fluctuation of the quality of the same variety (*11*, *12*) suggest that further studies are needed to improve the reliability of prediction.

Due to the central role of wheat storage proteins in the determination of quality performance, it may be suggested that two factors may be responsible for the fluctuation of quality of the same variety: (i) quantitative differences in translation of genetic information and consequently changes in total protein content and ratios of different gluten-forming polypeptides and/or (ii) differences in interactions of components forming gluten complexes due to changing conditions (e.g., by environmental effects). A possibility that the same mixture of polypeptides could yield glutens of different rheological properties was experimentally confirmed by Lasztity (*13*). These differences could be due to changing interactions of polypeptides. A mixture of polypeptides obtained by the reduction of gluten was

* Author to whom correspondence should be addressed (fax 361-463-3855; e-mail lasztity@mail.bme.hu).
[†] Budapest University of Technology and Economics.
[§] Eötvös Loránd University Budapest.

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reoxidized. Depending on the conditions of reoxidation, glutens of quite different qualities were obtained, although the initial mixture contained the same polypeptides in the same ratio. Improved understanding of processes leading to gluten formation in vivo needs a detailed study of the synthesis and interactions during the development of wheat kernel. On the basis of research work started about 40 years ago by Graham and Morton (*14*), today we have a general overview of the process of biosynthesis of wheat proteins (*15*). The proteins are synthesized on ribosomes attached to the ER and pass into the lumen with the cleavage of an N-terminal signal peptide. Folding and disulfide bond formation are considered to occur within the lumen. It is supposed that these processes are assisted by specific proteins, enzymes (e.g., protein disulfide isomerase), and molecular chaperons. The gluten proteins are deposited in the developing endosperm cells in discrete protein bodies, which have been reported to disappear during the advanced stages of kernel development, and a continuous protein matrix surrounding the starch granules will be formed. In the last phase of ripening the disruption of protein bodies is complete, as is apparent from the presence of a continuous protein matrix.

The synthesis of macrofractions (albumin, globulin, gliadin, glutenin) during kernel development was studied by several authors (*16*−*18*). However, relatively few data are available about the synthesis of individual gluten-forming polypeptides, about the mechanism of their interactions, for example, polymerization, and about the factors influencing the type of interactions (*11*, *18*−*24*). Although the majority of researchers detected gliadin polypeptides and glutenin subunits even in the early stage of maturation, the views differ concerning the sequence and dynamic of their synthesis. According to some researchers the synthesis of glutenin subunits starts earlier (*25*, *26*), whereas according to others (*17*, *27*, *28*) the sequence of the beginning of synthesis is gliadin−glutenin subunits−glutenin.

Within the framework of the research project realized in our laboratory the dynamics of the synthesis of individual gluten-forming polypeptides and their interactions resulting in gluten formation were studied. In this paper the results connected with the synthesis of gluten-forming polypeptides will be summarized.

MATERIALS AND METHODS

Materials. Five winter wheat cultivars−GK-Öthalom (HMW-GS composition 2*, 7+8, 5+10), Ukrainka (1, 7+8, 5+10), Palotás (2*, 7+9, 5+10), Ködmön (2*, 7+8, 5+10), and Csongrád (2*, 7+9, 2+12)−known in Hungary were used in this experiment.

The cultivars were grown at the experimental field of the Agricultural Research Institute of the Hungarian Academy of Sciences (Martonvásár, Hungary) and at the farm of the National Institute for Agricultural Quality Control (Monor, Hungary). On the first day of anthesis, each spike was tagged and dated. Spikes were collected at 2−3 day interval starting at the 12th day after anthesis up to the 53rd day. Fresh grains were taken under the median part of the ear. Part of the fresh grains was used for the determination of average kernel weight and moisture content. The other part of the fresh grains was freeze-dried and kept at −18 °C.

Methods. *Determination of Average Fresh Kernel Weight.* One hundred kernels were weighed, and the average weight was calculated. *Moisture content* was determined by drying of a homogenized fresh grain sample at 105 ± 2 °C.

Extraction of Gliadins and Sample Preparation for Chromatography. Fifty milligrams of the lyophilized samples was extracted with 70 v/v % ethanol (containing 0.1 μL/mL propiophenon retention standard) by shaking for 30 min (750 rpm, IKA Vibrax, IKA Ltd.). After extraction, the suspension was filtered into sample vials on a 45 μm PVDF filter. The filtrate was directly injected into the chromatograph.

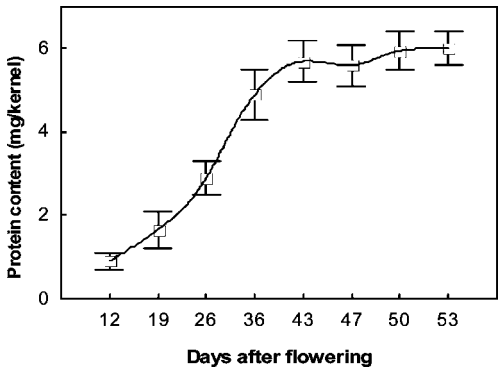

Figure 1. Protein accumulation during grain development (average values of five cultivars; bars show the minimal an maximal measured values).

Extraction of Glutenin and Preparation of Samples for Separation of Glutenin Subunits. Fifty milligrams of lyophilized samples was extracted with 50 v/v % 1-propanol. The suspension was centrifuged (10000*g*, for 5 min). Pellets were redissolved in 1-propanol−urea−DTT (PUD) buffer and incubated in a water bath (60 °C) for 1 h. After the addition of 10 mL of 4-vinylpyridine, the samples were incubated for an additional 15 min and then centrifuged (12000*g*) for 10 min. Before the chromatographic separation, the samples were filtered through a 45 μm PVDV filter.

RP-HPLC Separation of Gliadins and Glutenin Subunits. Modified and parameter-optimized methods were used for the separation of glutenin subunits and gliadins based on the works of Marchylo et al. (*29*), Wieser et al. (*30*), and Larroque et al. (*31*). The analytical separations were carried out using a Perkin-Elmer LC 200 DAD-HPLC system equipped with a quaternary pump, an autosampler, a column thermostat (65 and 50 °C), and a Vydac C18 (TP 104, Hesperia Ltd.) analytical column; 0.1% TFA in AN/W gradient elution was used. The gliadins were divided into three arbitrary subgroups, based on hydrophobicity, as shown in **Figure 3**. Total HMW and LMW and individual HMW subunits were determined.

Nitrogen Determination. The nitrogen content of seed samples was determined according to the Dumas−Pregl method, using a LECO FP-528 autoanalyzer (LECO Instruments).

Statistics. The Total-Chrom software package (Perkin-Elmer Instruments) was used for processing the chromatograms and Stastitica for Windows 7.0 (StatSoft) for statistical evaluation.

RESULTS

Total Protein Accumulation. The lyophilized material was used for all analyses. Protein contents were calculated on both dry matter basis and on the basis of the quantity of accumulated protein per kernel. Although quantitatively significant differences were observed between cultivars for protein content (bearing in mind the precision and reproducibility of protein analysis), the character of protein accumulation was similar (**Figure 1**). The figure shows the minimum and maximum values of measured data and the mean. The character of accumulation may be presented with sigmoid curves. The relative differences are higher in the early period of grain development and lower at the end of the ripening period. Changes occur in the order of varieties concerning quantity of accumulated protein. This observation may be connected with differences in the rate of protein synthesis or eventually with differences in kernel weight. In this experiment, bearing in mind that the wheats were grown under the same conditions, agrotechnical and meteorological factors, as causes of differences, may be excluded. It was found by analyzing the accumulation of other gluten-forming polypeptides that such a statement is valid for these components, too. The absolute

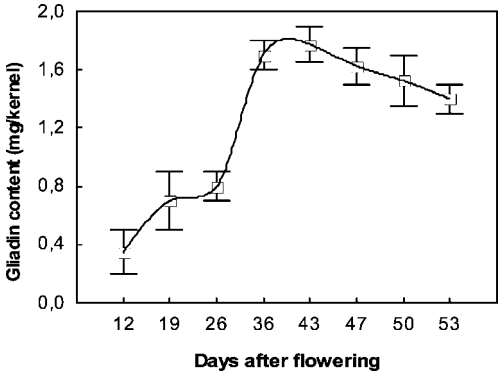

Figure 2. Gliadin accumulation during grain development (average values of five cultivars; bars show the minimal and maximal measured values).

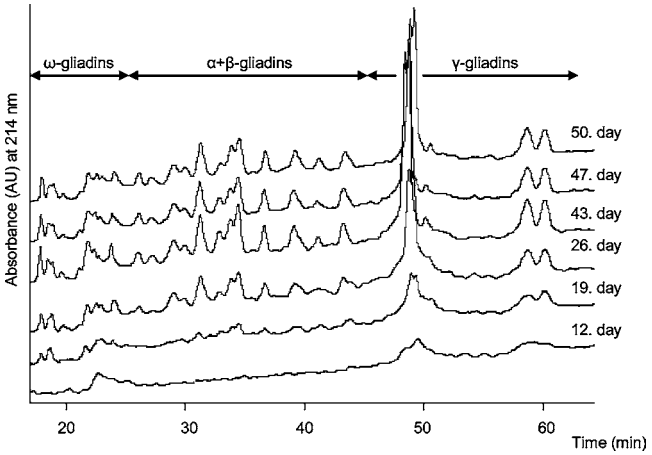

Figure 3. RP-HPLC chromatogram of gliadins.

protein quantity/kernel showed a slow increase over the first few days after anthesis. At about 25−35 days after anthesis a more rapid protein accumulation occurs, and in the final period of grain development stagnation may be observed.

Gliadins. The synthesis of the first significant amounts of gliadin starts at 15−16 days after anthesis. This is followed by a moderate increase in the period between 20 and 26 days after anthesis and later by a rapid accumulation until a maximum at 40−43 days. Finally, a moderate decrease may be observed (**Figure 2**).

The RP-HPLC separation of gliadin components allowed the accumulation of gliadin subfractions differing in hydrophobicity to be studied. Three gliadin subfractions (Gli-1, Gli-2, Gli-3) were distinguished, which consists probably of γ-, $\alpha+\beta$-, and ω-gliadins on the basis of hydrophobicity as shown in **Figure 3**. Similar separation of different types of gliadins was applied by Larroque et al. (*31*) and Wieser and Seilmayer (*32*) in a study of the effect of N fertilization on gliadins. Such division into groups of gliadins may be problematic due to the fact [mentioned also in paper of Wieser and Seilmeier (*32*)] that small quantities of some other proteins may be extracted by ethanol or that gliadin-like proteins remained unextracted. Nevertheless, we can speak about subgroups of gliadin-type proteins. Two subfractions (possibly $\alpha+\beta$- and γ-gliadins) show a similar curve of accumulation (**Figure 4**.). The gliadin subfraction eluted first (corresponding possibly to the less hydrophobic ω-gliadins) started to accumulate earlier than the other two types of gliadin. However, the rate of its synthesis decreased and its amount remained practically unchanged at 26 days after anthesis.

Group of Glutenin Polypeptides. The total quantity of glutenin subunits and the quantity of the sum of HMW- and

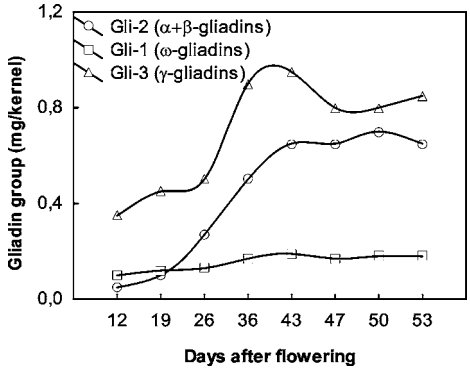

Figure 4. Accumulation of gliadin groups (cv. Öthalom).

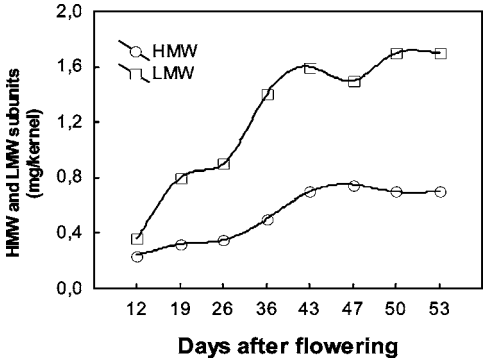

Figure 5. Accumulation of HMW- and LMW-GS (cv. Öthalom).

LMW-glutenin subunits may be characterized, independently of the wheat variety studied within the framework of this research project, with a sigmoid curve calculating the quantity/kernel values. The data calculated for the cultivar Öthalom are presented in **Figure 5**.

With regard to the individual HMW polypeptides, a typical chromatogram is shown in **Figure 6**. It may be stated that all of the subunits are synthesized synchronously. However, differences were observed between subunits in quantities (**Figure 7**). Lower quantities of subunits coded on chromosome A and higher ones on chromosomes D and B are characteristic. It should be noted that separation of some subunits coded on chromosome B1 and those coded on chromosome D1 (e.g., Bx7 and Dx5) is difficult, and so only approximate quantitive differentiation may be achieved. Our results concerning the synthesis of LMW subunits are in agreement with the results of Kaczkowski et al. (*25*) suggesting that the synthesis of these subunits starts earlier than that of HMW subunits.

Preliminary results of measurements of changes of polymeric glutenin content suggest that in the early stages of kernel development the HMW and LMW subnunits are present partly in monomeric (or oligomeric, soluble) form.

DISCUSSION

Results of accumulation of total protein confirm the sigmoid character of the curve presenting absolute protein/kernel values depending on time after anthesis. (If the protein concentration in total dry matter is calculated, the highest value occurs in the early stage of grain development before the start of intensive synthesis of carbohydrates. The further shape of the protein concentration curve is influenced by the ratio of intensity of the synthesis of the two main chemical components of wheat grain.) With regard to similar character of protein accumulation curves, it should be noted that all cultivars studied were hard winter wheats grown under the same agrotechnical and meteo-

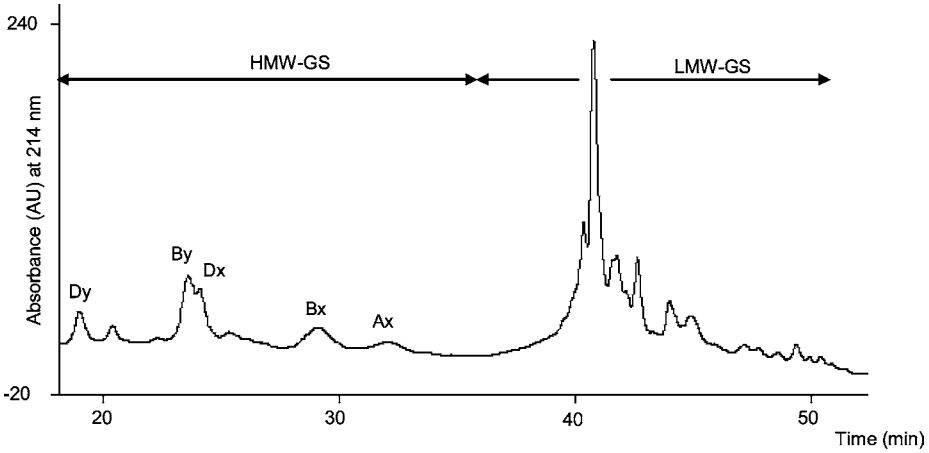

Figure 6. RP-HPLC chromatogram of HMW-GS (cv. Öthalom).

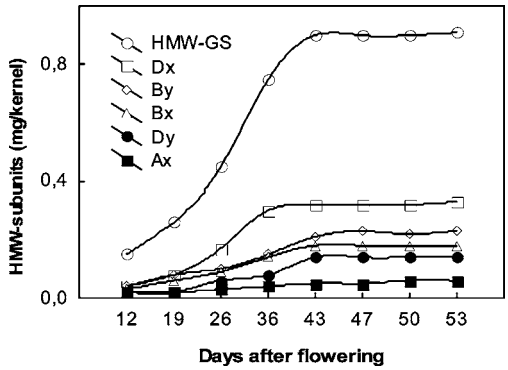

Figure 7. Accumulation of individual HMW-GS (cv. Öthalom).

rological conditions, so the effects of the latter factors were not expressed in the results. The majority of cultivars had the HMW-GS D-5+10 (typical for high-quality wheats) except cultivar Csongrád, which had HMW-GS D-2+12 (typical for lower quality varieties). However, no significant differences were found between Csongrád and Palotás, two nearly isogenic cultivars. This confirms the view that differences between varieties with HMWs 5+10 and HMWs 2+12 are not associated with total protein or glutenin content but with the ratio and timing of the synthesis of highly polymeric glutenin (*21*, *22*).

On the basis of the physiology of wheat it may be expected that in the early stage of kernel development (cell division and extension period) the protein content consists of metabolic and structural proteins and no gluten-forming storage proteins are present. However, the results presented in this paper confirm the views that the gluten-forming polypeptides, although in low concentrations, are synthesized even in this early stage of kernel development. Their presence was confirmed also by other separation techniques such as capillary electrophoresis (*33*) and using specific monoclonal antibodies (*26*). Stone and Nicolas (*21*) also reported that monomers (different from albumins and globulins) were synthesized in the early stage of development. It may be also noted that according to observations (*15*, *34*) coordinate accumulation of transcripts from the HMW-GS, LMW-GS, and α-, γ-, and ω-gliadin gene families occurs early in grain development. This observation also suggests the early synthesis of gluten-forming polypeptides. With respect to the question of the sequence of synthesis of gluten-forming polypeptides, our results could not give a definitive answer. This is because we started with a study of kernel development 12 days after anthesis. At this time all identified polypeptides were present, although in different concentration levels. It is interesting that the synthesis of LMW subunits is in the early stage more intensive than that of HMW subunits, because according to the views of several researchers the formation of polymeric glutenin starts with the synthesis of a backbone from HMW subunits, which will be later coupled with micelles of LMW subunits. Reverse-transcriptase polymerase reactions revealed that individual HMW-GS genes and at least seven LMW-GS genes exhibited identical patterns of temporal regulation (*34*).

The total quantity of gliadin polypeptides did not show significant differences between varieties. This is connected probably with the fact that all of the varieties are winter wheats, grown at the same region and under the same agrotechnical conditions. The slight decrease of gliadin quantity in the final period of ripening may be explained by solubility changes caused by aggregation and interaction with polar lipids.

In contrast to total gliadin fraction, our knowledge concerning the biosynthesis of different types of gliadins (α-, β-, γ-, and ω-gliadins) is poor. The available publications report experiments connected with the effect of N fertilization and sulfur deficiency primarily on the accumulation of ω-gliadin. Increased N supply and sulfur deficiency increased the accumulation of ω-gliadins in comparison to other types of gliadins (*35*−*37*). Recently, efforts have been made to obtain transgenic wheat varieties poor in α-gliadin (*38*, *39*). We separated the gliadin fraction into three arbitrary groups differing in hydrophobicity. Although we did not make a detailed electrophoretic examination of the three subgroups, on the basis of a similar separation technique reported by other researchers (*29*, *30*, *39*) we suggest that these subgroups consists mainly of α-, β-, γ-, and ω-gliadins, respectively. Our results show that quantitatively the more hydrophobic α-, β-, and γ-gliadins (sulfur-rich gliadins) dominate. The difference in timing of the rate of accumulation of gliadin groups is interesting. Whether this a general trend or valid only for this small group of hard winter wheat cultivars widely grown in Hungary could be clarified by further investigations.

Preliminary results of investigations in our laboratory suggest that in the early stage of kernel development part of the subunits are present in monomeric (oligomeric) form. Is this connected with a limiting concentration of subunits needed for the start of polymerization, or do other factors, such as the redox status of the kernel (*40*), PDI, and regulatory protein, play a role that needs further investigations?

With regard to the HMW subunits it may be concluded that these are synthesized synchronously. Quantitatively, the subunits coded by chromosomes D and B dominate.

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