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Development and Characterization of Anti-gliadin Polyclonal Antibody in Wheat

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ABSTRACT

Immunological method has been applied in biochemical genetic analysis of seed storage proteins. We developed and characterized anti-gliadin polyclonal antibody (AGPab) specific to gliadin fractions whose quality and quantity were known to be associated with wheat end-use quality. Reactions of anti-gliadin polyclonal antibody (AGPab) to gliadin were linearly decreased as AGPab and antigen were diluted. Dot-blot and immunoblot assay showed that produced AGPab specifically reacted to gliadin and mainly α-, β-, and γ-gliadin subunits. Enzyme-linked immunosorbent assay (ELISA) was applied for quantification of gliadins in Korean wheat cultivars and breeding lines by using AGPab. High reactions between AGPab and gliadins were found in wheat cultivars Olmil and Olgeurumil. Significant difference of optical densities for alcohol soluble proteins among crop species was found, as wheat showed the highest value (0.697) followed by rye (0.295), and barley (0.066).

Keywords : wheat (*Triticum aestivum* L.), biochemical genetics, gliadin, polyclonal antibody.

Immunological methods have been widely used for protein detection and quantification with their convenience, high levels of sensitivity and specificity. High structural relationships in gliadins and similarities between the majority of wheat and rye alcohol soluble proteins were found by polyclonal antibody (Booth & Ewart., 1970; Ewart, 1983). The fact that wheat low-molecular weight glutenin subunits (LMW-GS) had immunological homology with gliadins and high molecular weight glutenin subunits (HMW-GS) was reported by using monoclonal antibodies (Skerrit & Lisa, 1990). Wheat-rye chromosomal translocations and wheat dough extensibility were screened by enzyme linked immunosorbent assay and immunoblotting (Grabosch et al., 1993; Andrew & Skerritt, 1996). ELISA enables us to detect presence or absence of specific proteins and to measure quality and quantity of specific proteins of interest (Seo et al., 1995). Immunological methods have been used extensively for not only plant itself but also plant pathogens (Margret et al., 1985; Skerrit & Lisa, 1990; Miura et al., 1994).

The endosperm of common wheat (*Triticum aestivum* L.) contains proteins that are the components of gluten, one of the most intricate and naturally occurring protein complexes. According to Osborne (1907), gluten can be divided into gliadin and glutenin by its solubility characteristics. Gliadin is a large monomeric protein fraction and soluble by 70~90% alcohol/water (V/V). Gliadin is important in end-use qualities such as dough-making, loaf volume and viscosity (Leisle et al., 1981; Peleg, 1994; Fido et al., 1997). Gliadin subunits are suited for cultivar identification because of their near-constant expression under various environmental conditions and their have strong relationship with wheat quality (Kosmolak et al., 1980).

Characterization and nomenclature of gliadin were proposed on the basis of gel electrophoretic mobility as α-, β- γ- and ω- gliadins (Bushuk Zilliman, 1978). Jackson et al. (1983) demonstrated that genes coding most α- and β- gliadins are located on the short arms of homoeologous group 6 chromosomes, whereas genes for most of ω- and γ-gliadins are on the short arms of chromosomes 1A, 1B, and 1D.

The purposes of this study is to develop and characterize polyclonal antibodies specific to gliadin fractions. Anti-gliadin polyclonal antibody (AGPab) was used for evaluating Korean wheat cultivars and breeding lines by using ELISA. Also, the AGPab analysis was employed to identify different crop species.

MATERIALS AND METHODS

Plant materials

Wheat (*Triticum aestivum.* L.) samples were kindly provided from the National Crop Experimental Station at Suwon. Eleven cultivars and seven breeding lines were grown at Korea University research farm at Deokso during 1997~1998. Seeds were harvested and milled by Buhler experimental mill for the laboratory experiments. Other grain samples, triticale, rye, and barley were also provided by the National

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Crop Experiment Station at Suwon. The designation of wheat lines used in antibody production or immunoassay are listed in Table 1.

Antigen Preparation and Production of Anti-Gliadin polyclonal Antibody (AGPab)

Unreduced gliadin was obtained from a wheat cultivar, "Alchanmil" by extraction of flour with 70% ethanol. After centrifugation at 20,000 g, the supernatant was transferred to a new 1.5 ml Eppendorf tube and freeze dried.

Antisera to gliadins were prepared for immunization. Although the gliadins are known to be insoluble in neutral salt solutions, low concentration of gliadin components could be suspended homogeneously in physiological saline solution. 1 mg of gliadin was homogeneously suspended and stirred in 1 ml of phosphate buffered saline solution (PBS, 140 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, pH 7.4). Freund's complete adjuvant (0.5 ml) was added to the suspension and mixed. New Zealand white Rabbits were injected intramuscularly by antigen preparations. After 14 days, seven subcutaneous booster injections with 1 ml protein solution (5 mg/ml) emulsified in incomplete Freund's adjuvant were applied. Quality of antibody was evaluated by taking few quantities of serum periodically. The rabbits were bled when serum gave the highest response to antigen. Serum was collected after blood clotting and stored at -20℃ with 0.01% sodium azide.

One-Dimensional SDS-PAGE (1D SDS-PAGE)

Methods of protein separation, reduction, and alkylation by SDS-PAGE followed protocols of Graybosch et al. (1993).

Immunoblot Analysis

After protein fractionation by electrophoresis, a trans-blot semi-dry electrophoretic transfer cell (Bio-Rad) was constructed. Whatman filter papers covering nitrocellulose (MSI) membrane and gel were completely soaked by transfer buffer (25 mM Trizma Base, 192 mM Glycine, and 20% Methanol). Proteins of prepared blotted gel were transferred to 0.45 micron nitrocellulose membrane using a semi-dry transfer cell at constant voltage (25 V) for 1 hour (Gershoni and Palade, 1983). After transfer, membrane was incubated and dried overnight at 37℃. Membrane was blocked by 2% bovine serum albumin (BSA) dissolved in TBS (100 mM Trizma Base, 0.15 M Sodium Chloride, pH 7.4) with constant agitation using a rocker at 28℃. Membrane was incubated with primary antibody (anti-gliadin polyclonal antibody) diluted 1/100 in incubation buffer (100 mM Trizma Base, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 90 minutes at 25℃. Membrane was washed by 3 changes of TBS-Tween (0.2% Tween-20 in TBS) at 15 minutes and incubated in alkaline-phosphatase labelled goat anti-rabbit IgG (Sigma) diluted 1/1000 (V/V) in incubation buffer for 1 hour at 28℃. Following three washes of membrane by TBS-Tween as above, reacted protein bands were detected by incubation in 1% 5-bromo-4 chloro-3-indolylphosphate p-toluidine solution (V/V) dissolved in dimethyl sulfoxide (DMSO) making 3.3% (W/V) and 1% nitroblue tetrazolium chloride solution (V/V) dissolved in 70% DMSO making 1.7% (W/V) in incubation buffer. After 10 minutes of incubation, color reactions were stopped by dropping 0.25 M EDTA. Blotted membrane was dried in dark area after reaction was stopped.

Enzyme-linked immunosorbent assay (ELISA)

Gliadins in 25 mg of flour samples were extracted with 1 ml 70% ethanol by vortexing for 1 hour. After centrifugation at 20,000 g for 1 minute, supernatant was transferred to a new 1.5 ml Eppendorf tube. Each gliadin extractant was diluted to 1/625, and 30 μl of antigen (gliadin) dilutent was loaded in each microtitrate plate well and dried overnight in incubator set at 37℃. After plate was dried, plate was blocked by 200 μl of 2% (W/V) BSA dissolved in phosphate-buffered saline solution (PBS, 136.9 mM NaCl, 2.7 mM KCl, 10.1 mM Na_2HPO_4, pH 7.4) for 90 minutes (Kocna, 1995). After blocking, the solution was removed and the plate was washed three times by using 0.2% (V/V) PBS-Tween solution. After washing, 50 μl of anti-gliadin polyclonal antibody solution, diluted with PBS by 1/100, was applied to each well. After incubation at 37℃ for 90 minutes, the anti-gliadin polyclonal antibody solution was removed and the plate was washed three times with PBS-Tween. Wells were incubated with 50 μl alkaline-phosphatase labelled goat anti-rabbit IgG (Sigma) diluted with 1/7500 in PBS for 90 minutes at 37℃. After plate was washed as described above, 200 μl of 0.05% (W/V) p-nitrophenylphosphate in substrate buffer (2 M Diethanolamine, 2 mM $MgCl_2$, pH 9.7 diluted with H_2O) was added to each well for alkaline-phosphatase and substrate reaction. After 20 minutes of incubation, optical densities of the alkaline phosphatase reaction products were scanned at 405 nm by using a microtitration plate reader (Bio-Rad).

RESULTS

Antibody and Antigen Reaction

In order to evaluate antibody production in rabbit, about 10 ml of blood samples were taken from vein in rabbit ears and tested by dot blot method. Seven days after the final injection, collected serum gave the highest response to antigen. Dot blot results of anti-gliadin polyclonal antibody collected at 7 days after final injection are shown in Figure 1A and 1B. As gliadin (antigen) concentrations decreased, the degree of anti-gliadin polyclonal antibody (AGPab) reaction was reduced. The control region in which only Bovine Serum Albumin (BSA) was applied did not show any reaction with AGPab. This result indicated that produced AGPab could bind specifically to gliadin but not to BSA. The result of dot blot assay also confirmed that 7 days after final antigen injection was the proper time for blood-gathering (Fig. 1A and 1B).

Different dilutions of AGPab reacted to antigens prepared by mechanical mixtures were observed, i) to evaluate specific binding of different concentrations of antibody to different ranges of antigen concentrations, and ii) to find optimum concentrations of antibody and antigen for further application (Fig. 2). Reactions of AGPab to gliadin were linearly decreased as AGPab and antigen were diluted. There was high coefficient of determination (r^2 = 0.989) for antibody dilution of 1/200 between antigen concentrations from $1/5^7$ to $1/5^3$ and antigen concentration between $1/5^7$ and $1/5^3$ gave high coefficient of determination(r^2= 0.992). This result showed that the range of antibody dilution of 1/200 was appropriate for further use in wheat quality

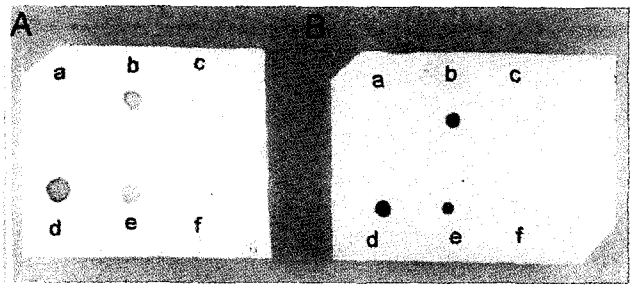

Fig 1A and 1B. Dot blot result of anti-gliadin polyclonal antibody collected 7 days after final injection to different concentrations of gliadins. Serum concentration was 1/1000. 1A (with Tween-20) and 1B (without Tween-20); a: control (no-gliadin), b: gliadin dilution of 0.5 X, c: gliadin dilution of 0.1 X, d: straight gliadin solution (1 X), e: gliadin dilution of 0.25 X, and f: gliadin dilution of 0.05 X.

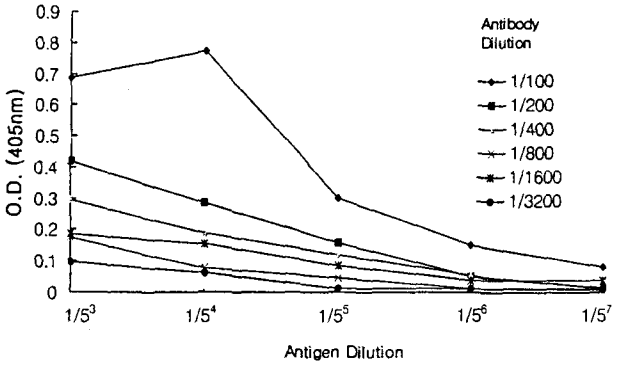

Fig 2. Reaction of different concentrations of anti-gliadin polyclonal antibody on different concentrations of gliadin solutions. Coefficient of determination ranges between antigen dilutions from $1/5^7$ to $1/5^4$ for antibody dilutions of 1/100 (r^2 = 0.974), 1/200 (r^2 = 0.989), 1/400 (r^2 = 0.965), 1/800 (r^2 = 0.938), 1/1600 (r^2 = 0.858) and 1/3200 (r^2 = 0.814).

breeding programs. Other reactions from different concentrations of antibodies and antigens gave higher coefficient of determination than 0.96 for the antigen ranges from $1/5^7$ to $1/5^3$. Therefore, AGPab could be applied in quantification of gliadin which was known to affect wheat end-use qualities.

Characterization of Anti-Gliadin Polyclonal Antibody (AGPab)

After 70% ethanol extractable proteins were loaded and separated in two gels in the same conditions, one gel was silver-stained and the other gel was used for immunoblotting for characterizing anti-gliadin polyclonal antibody (Fig. 3A and 3B). SDS-PAGE of proteins extracted from different crop species and different denaturation treatments were assigned in each lane. Different alcohol soluble protein subunits were found among different crop species (Fig. 3A). Immunoblot results by using AGPab showed that AGPab mainly bound to α-, β-, and γ-gliadins (Bushuk and Zillman, 1978). There was no antibody reaction with hordein proteins. Although binding was not as strong as found in gliadins, proteins extracted from cultivated triticale bound to AGPab. This result was thought to be associated with chromosomal composition of triticale, in that, genomic group of AABB in triticale came from durum wheat which had identical genes for seed storage protein with common wheat. Smear binding pattern of secalin extracted from rye might be associated with similar nature of epitopes provided by high molecular weight secalins. The low

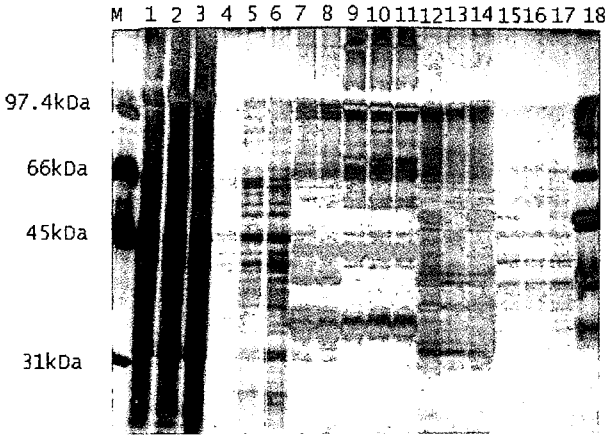

Fig 3A. SDS-PAGE of seed storage proteins in different crop species. Lane 1-3: unreduced glutenins for Alchanmil, Urimil, Tapdongmil, respectively; land 4-6; reduced glutenins for Alchanmil, Urimil, Tapdongmil, respectively; lane 7-8: unreduced triticale prolamins for Suwon 18 and Suwon 24; lane 9-11: unreduced rye prolamins for Geuruhomil, Homil 21, Homil 23, respectively; lane 12-14: unreduced gliadins for Alchanmil, Urimil, Tapdongmil, respectively; lane 15-17: reduced gliadins for Alchanmil, Urimil, Tapdongmil, respectively; lane 18: unreduced hordeins for Gangbori, M: molecular size marker.

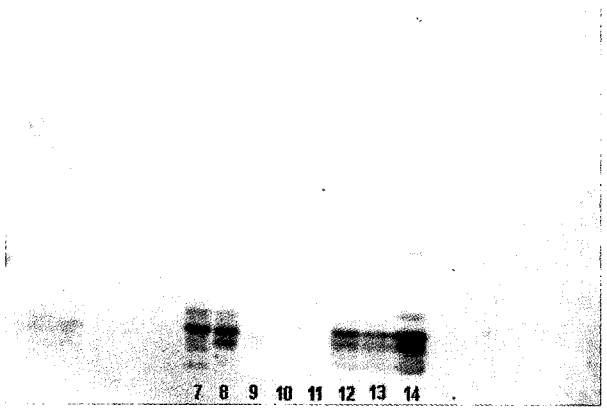

Fig 3B. Immunoblot of seed storage proteins in different crop species. Lanes and sources are the same as shown in Fig. 3A.

bind of triticale proteins in the similar locations to secalin binding region could be thought to be ascribed to rye secalin gene expression in triticale. Once gliadin was denatured, AGPab did not react to reduced proteins. Both reduced and unreduced glutenin proteins showed that they did not provide epitopes to AGPab.

SDS-PAGE and Immunoblotting in Korean wheats

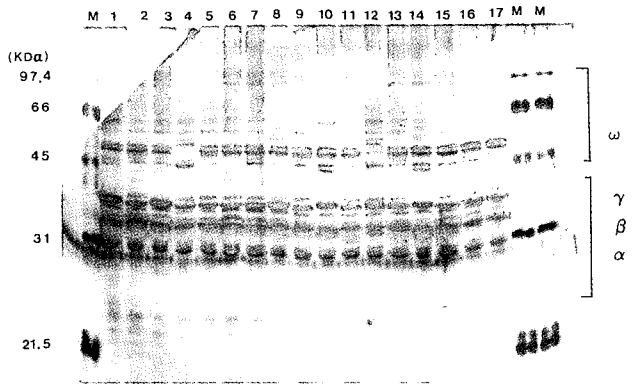

Fig 4A. One dimentional SDS-PAGE patterns of gliadin from Korean wheats. Lane 1: Chokwangmil; lane 2: Gobunmil; lane 3: Geurumil; lane 4: Urimil; lane 5: Alchanmil; lane 6: Olgeurumil; lane 7: Eunpamil; lane 8: Kumkangmil; lane 9: Tapdongmil; lane 10: Suwon 258; lane 11: Suwon 265, lane 12: Suwon 275; lane 13: Suwon 278; lane 14: Suwon 279; lane 15: Suwon 261; lane 16: Suwon 276; lane 17: Suwon 280. M: molecular size marker.

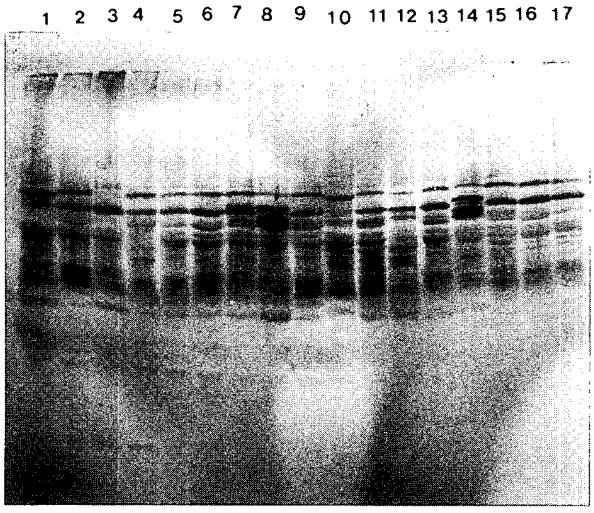

Fig 4B. Immunoblot of gliadin from Korean wheats. Lanes and sources are the same as shown in Fig. 4A.

Gliadins extracted from 17 Korean wheats and breeding lines were loaded in two gels in the same conditions as described above. The gliadin subunits from different wheat lines showed polymorphisms which could be used in cultivar identification and quality prediction. Gliadin subunit of 45 kDa which was known to be highly related to weak gluten and linked to white glume color was found in Urimil and Suwon 275 (Fig. 4A).

Immunoblotting results of 17 Korean wheat lines are shown in Figure 4B. AGPab reacted with gliadins from all wheat lines indicating that AGPab could be used in quantification of gliadins (Fig. 4B).

ELISA for Barley, Rye, and Wheat Lines

The results of ELISA for 18 Korean wheat lines, 11 rye cultivars and 3 barley lines are shown in Table 1. 'Olmil' nd 'Olgeurumil' showed higher optical density values about 0.9 among Korean wheats tested (Table 1). There were various reactions between AGPab and alcohol soluble proteins from rye and barley. The highest reaction was found for 'Geurumil' (O.D. 0.471) and the lowest reaction was found in 'Chankwang homil' (O.D. 0.19). Significant differences of optical densities among different crop species were found, as wheat showed the highest value (0.697), followed by rye (0.295), and barley (0.066). Therefore, the amount of alcohol soluble proteins could be quantified in wheat, rye, and barley.

DISCUSSION

There was decrease of reaction in the antigen dilution ranges higher than $1/5^4$ for antibody dilution of 1/100, and this result might be associated with unstable binding of antigen to microtiter plate well (Fig. 2). This result was often observed when antigen concentration was too high and it was the main objective to find proper range of antigen and antibody dilution by using titration curve. The high relationships from the reactions throughout most ranges for different antibody and antigen concentrations will give advantages of providing diverse experimental treatments with different antibody dilutions (Fig. 2).

Although we used total gliadins as an antigen, the produced antibody reacted mainly to gliadins except for ω-gliadins about 60 kDa (Fig. 3A and 3B). This might be due to the changes of salt and pH conditions for injected gliadin pellet which was differently prepared for SDS-PAGE. Other possibility might be structural change of ω-gliadins SDS -PAGE in which gliadin solution was boiled for 10 minutes. In spite of unusual thermal or salt treatments, α- , β-, and γ-gliadin subunits were stable because of tightly folded hydrophobic structure by their rich disulfied bonds. Vu and Popineau (1987) indicated that ω-gliadins contained low levels of or no sulphuric amino acids and were rod-shaped molecules with unusual secondary structures based predominantly on repetitive β-turn. It was reported that ω-gliadins gave no reactions with antibodies raised to total gliadin (Vu and Popineau, 1987). AGPab bindings in the immunoblot assay in triticale confirmed that both wheat gliadins and rye secalins were encoded by the genes located on wheat genome groups AABB and rye genome RR, respectively.

High polymorphisms were found, especially in high molecular weight fractions, among wheat lines by using 1D SDS-PAGE (Fig. 4A). Therefore, Korean wheats could be identified by simply analyzing gliadin proteins by 1D SDS-PAGE and this method could be used in cross breeding programs.

Although there was low reaction between barley alcohol soluble proteins and AGPab in ELISA, optical density showed almost zero value (Table 1). Negligible reaction was thought to be associated

Table 1. Enzyme-Linked Immunosorbent Assay (ELISA) of cultivars and breeding lines from different cereal species.

Crops	Entries	Mean absorbance
Wheat	Olmil (.901), Chokwang (.704), Chunggemil (.748), Urimil (.614), Geurumil (.794), Eunpamil (.606), Alchanmil (.624), Gobunmil (.772), Keumkamgmil (.733), Olgeurumil (.905), Tapdongmil (.711), Suwon 265 (.735), Suwon 272 (.579), Suwon 273 (.672), Suwon 274 (.622), Suwon 275 (.436), Suwon 277 (.8), Suwon 278 (.531)	.697 [a†]
Rye	Chilbohomil (.196), Chochunhomil (.279), Geruhomil (.471), Chankwanghomil (.19), Paldanghomil (.261), Homil 8 (.319), Homi 10 (.377), Homi 12 (.415) Homi 21 (.236), Homi 22 (.21), Homi 23 (.293)	.295[b]
Barly	Kangbori (.075), Kinssalbori (.109), Suwon 351(.014)	.066[c]

† Same letters following mean values indicate no significant different at the 5% level by Duncan's multiple range test.

with non-specific cross reaction or little structural homology between α- , β-, and γ-gliadin and hordein.

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