Immunochemical Characterization of Specific Albumins of Bread Wheat

S. E. PIAZZI, G. RIPARBELLI, and SANDRA SORDI, Department of Immunology, Istituto Sclavo, Siena, Italy; P. CANTAGALLI, Laboratorio Chimico Provinciale, Siena; and F. POCCHIARI and V. SILANO, Laboratori di Chimica Biologica, Istituto Superiore di Sanita, Roma

ABSTRACT

Preparation of an immuneserum containing antibodies reacting specifically with a bread wheat albumin is described. It was obtained by immunizing a goat with a partially purified fraction from a soluble protein extract of bread wheat flour (var. Mentana). After absorption with durum wheat proteins, purification, and concentration, the antibody solution showed, in agarose gel immunodiffusion, a positive reaction of precipitation with the extracts of all varieties analyzed of bread wheat (*Triticum aestivum*) and a negative one with the durum wheat (*Triticum durum*) varieties. The reaction of precipitation in immunodiffusion allows the detection of bread wheat in mixtures of *T. durum* down to a 5% concentration. The precipitating antibodies are directed towards a specific bread wheat protein, already identified as Mb 0.19 albumin. The analytical comparison carried out both by immunodiffusion and by immunoelectrophoresis, between this preparation of goat antiserum and a previous preparation of rabbit anti-bread-wheat immuneserum shows that the two antisera are directed towards two different antigens, both present in the soluble bread wheat extracts and absent in the durum wheat extracts. The antigen detected by goat antiserum shows an anodic migration, whereas that detected by rabbit antiserum shows a cathodic migration.

It has recently been shown (1,2) that in the albumin fractions of bread wheat seed endosperm an Mb 0.19 protein fraction is present which has never been found in durum wheat extracts. This protein, which can be considered "specific" for bread wheat, shows in the ultracentrifugation a molecular weight of about 23,700, and has an isoelectric point at pH 7.3 (3).

In recently published studies (4,5) we established the possibility of carrying out a specific immunochemical characterization of soluble bread wheat proteins, using a specific rabbit antiserum. In the light of those results, we attempted the preparation of a specific goat immuneserum: anti-Mb-0.19-albumin. However, because of the remarkable difficulty in obtaining a preparation of such degree of purity, a partially purified albumin fraction from bread wheat containing the Mb 0.19 protein (6) was used as an antigen for the preparation of the antiserum.

MATERIALS AND METHODS

Wheat

Durum and bread wheats of known variety and history were supplied by the Laboratory for the Application of Nuclear Energy in Agriculture (C.S.N., Casaccia, Roma).

Durum varieties used were: Appulo, Azizia, B-52, Camar 7, Canne, Capeiti, Cappelli, Casale 92, Casteldelmonte (Gr A 145), Castelfusano (Cp C 48), Castelporziano (Cp B 132), Ciclope 66, Dauno, Garigliano, Grifoni 235, Lakota, Maliani 1D, Maliani 8D, Patrizio 6, Russello, Sincape 9, Timilia SG 1, Tripolino, and Vera 63.

Bread varieties used were: Admonter, Bezostaja 1, Clédor, Combine, Diamante, Elia, Farnese, Flaminio, Fontarronco A, Funo, Gagliardo, Madif 21, Manitoba, Mara, Mentana, Novi Sad, Odesskaja, Probus, Rekord, Rex, S. Pastore, Strampelli, Turmalin, and Zenith.

Preparation of Antigen

The preliminary extraction of bread wheat soluble proteins was carried out according to the method of Piazzi and Cantagalli (4). Two hundred grams of bread wheat flour (var. Mentana) was suspended and kept at 4°C. for 24 hr. with intermittent agitation in 400 ml. of pH 6.6 phosphate buffer saline solution (PBS-6.6) of this composition: 0.025M $Na_2HPO_4 \cdot 2H_2O$, 0.041M KH_2PO_4, and 0.48M NaCl.

After centrifugation, 250 ml. of protein extract was obtained. It was purified according to Cantagalli et al. (6) by fractional precipitation with ammonium sulfite. The precipitate obtained between 0.93 and 1.33M was collected by centrifugation, washed with 1.33M ammonium sulfite, and dissolved in 20 ml. of pH 7.4 phosphate-buffer saline solution (PBS-7.4) of the following composition: 0.088M $Na_2HPO_4 \cdot 2H_2O$, 0.00147M KH_2PO_4, 0.1126M KCl, and 0.136M NaCl.

The solution obtained was then submitted to Sephadex G-100 gel filtration, and the contents of the tubes containing the Mb 0.19 albumin were pooled, dialyzed against water, sterilized by filtration, and freeze-dried.

When needed, the freeze-dried protein fraction was dissolved in sterile saline (NaCl, 0.85%) to a concentration of 5 mg. per ml.; this solution was used as antigen for goat immunization. The protein content was estimated by absorbance at 280 nm. on a Beckman DU spectrophotometer.

Preparation of Antiserum

Five milliliters of the antigen solution (corresponding to 25 mg. of protein), emulsified with the same volume of Freund's complete adjuvant, was injected subcutaneously into a goat of about 40 kg. weight. After 20 days a second subcutaneous injection of 10 ml. of the simple antigen solution was administered. Ten days after the second injection the animal was submitted to partial bleeding from the jugular vein. After coagulation, 200 ml. of serum was obtained from about 400 ml. of blood. This immuneserum showed multiple precipitation lines with durum and bread wheat protein extracts in agarose gel immunodiffusion analysis.

To eliminate aspecific antibodies, the antiserum was submitted to several absorptions with freeze-dried extracts of durum wheat.

The extracts of durum wheat were made according to the method used for the extraction of bread wheat: 200 g. of durum wheat flour was suspended and kept at +4°C. for 12 hr. with intermittent agitation in 400 ml. of pH 6.6 phosphate buffer saline solution and, after centrifugation, the supernatant was freeze-dried.

Durum wheat freeze-dried extract was added (0.1% w./v.) to the antiserum and the mixture was kept at 37°C. for 2 hr. with intermittent agitation and at +4°C. for 12 hr. After centrifugation at 2,700 X g for 30 min., the supernatant was checked by immunodiffusion and the absorption was repeated (five times) until the immunodiffusion analysis showed the total absence of antibodies directed towards durum wheat proteins.

To improve the clarity of the immunodiffusion analysis, the absorbed antiserum was then submitted to enzymatic treatment. The protein solution, containing 10 g. of protein, was diluted to a solution of 3% protein in 5% NaCl. This was adjusted to pH 4.2 with normalized HCl, and after pepsin (3% w./v.) was added, was kept at 40°C. for 7 hr. After a clarifying filtration, the protein solution (6.5 g) was brought back to neutrality (pH 6.8), dialyzed against water, sterilized by filtration, and freeze-dried.

When needed, a portion (about 200 mg.) of the freeze-dried product was dissolved with a 0.15M NaCl solution containing glycine (2.5%) and sodium azide (0.1%), so as to obtain a final protein concentration of 14%.

Agarose Gel Immunodiffusion and Immunoelectrophoretic Analyses

Immunodiffusion analyses were carried out according to a slight modification of Wieme's classic micromethod (7). Immunoelectrophoretic analyses were carried out by a slight modification of Scheidegger's micromethod (8). Additional immunodiffusion tests were carried out, at different levels of reaction, according to a method previously reported (9).

Twenty grams of agarose was dissolved in 1 liter of distilled water on a water bath, and when warm, was mixed with 1 liter of pH 8.2 and 0.1 ionic strength barbital buffer (Na barbital, 15.875 g.; 0.1N HCl, 230 ml.; and H_2O to 1,000 ml.). After addition of sodium azide (0.1%) and warm filtration, the solution was distributed in 50-ml. glass bottles, cooled at room temperature, and maintained at +4°C.

Microscope slides were kept in toluene for 3 days, washed carefully with detergent, and finally washed with distilled water. A very thin layer of melted 1% agarose solution was spread on the surface of the slides, which were then dried in a stove at 80°C.

When needed, the agarose solution was melted on a water bath, and 2 ml. was stratified on the prepared slides and left, at room temperature, to solidify.

The solidified agarose layer was cut according to the requirements of the different analytical conditions, and the holes were filled with the antiserum and with the samples under analysis.

For immunoelectrophoretic analysis, the antigen samples were put into the holes, and a migration was carried out at 40 V. per cm. for 50 min. in a pH 8.2, 0.05 ionic strength barbital buffer, followed by distribution of the antiserum in the longitudinal slit. In both cases (immunodiffusion and immunoelectrophoresis), the slides were then placed in a damp box for 48 to 72 hr. Positive reactions appeared as thin, but very evident, lines in the gel thickness when the slides were held over an oblique ray of light. The slides were then washed in saline buffer solution (pH 8.2; 0.05 ionic strength barbital buffer, 20 parts: 0.68% KCl, 0.1% sodium azide in distilled water, 80 parts), changing the washing solution three times a day, for 3 to 4 days. The slides were fixed by immersion in 1% tannic acid solution for 10 min. and, after blotted with paper, were dried at room temperature.

The agarose, reduced to a dehydrated film, was stained with a Blue Coomassie 0.1% solution (methanol, 50%; acetic acid, 10%; distilled water, 40%) for 10 min. The slides were then destained, until a colorless ground was obtained, with 3 to 4 washings in a mixture of methanol, 50%; acetic acid, 10%; glycerine, 10%; and

distilled water, 30%. Finally, the slides were blotted with absorbent paper and desiccated at room temperature.

RESULTS

The immunodiffusion reaction between the goat immuneserum before and after adsorption and purification, and the NaCl 0.48M (pH 6.6) protein extract of seeds of *Triticum aestivum* (var. Mentana) and *Triticum durum* (var. Cappelli) is reported in Fig. 1.

This figure shows that all specific antibodies present in the immuneserum before

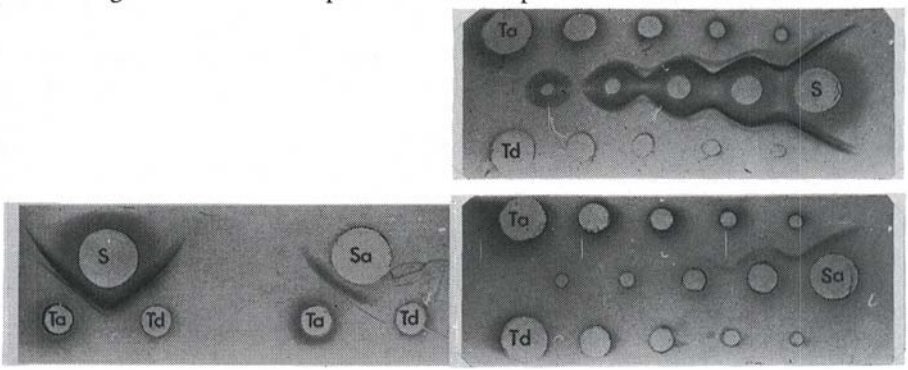


Fig. 1 (left). Immunodiffusion analysis of total extracts of bread wheat (Ta) and durum wheat (Td) soluble proteins with anti-bread-wheat goat immuneserum before (S) and after (Sa) absorption and purification.

Fig. 2 (right, top and bottom). Immunodiffusion analysis at different levels of reaction of total extracts of bread wheat (Ta) and durum wheat (Td) soluble proteins with anti-bread-wheat goat immuneserum before (S) and after (Sa) absorption and purification.

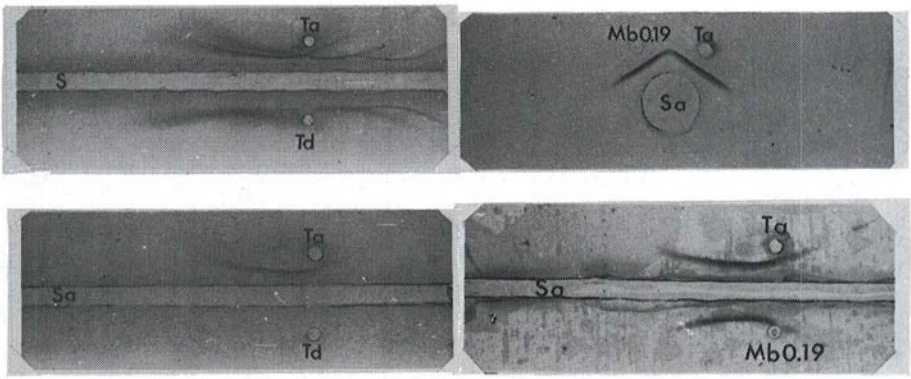


Fig. 3 (left). Immunoelectrophoretic analysis of total extracts of bread wheat (Ta) and durum wheat (Td) soluble proteins with anti-bread-wheat goat immuneserum before (S) and after (Sa) absorption and purification.

Fig. 4 (right). Immunodiffusion (top) and immunoelectrophoretic (bottom) analyses of the total extract of bread wheat (Ta) and of the purified albumin fraction (Mb 0.19) tested with anti-bread-wheat specific goat immuneserum (Sa).

absorption and directed toward the protein antigens common to *T. aestivum* and *T. durum* were eliminated during the absorption and purification process. The final preparation of the absorbed and purified antiserum reacted only with the bread wheat extract. This result was confirmed by the immunodiffusion analysis carried out at different levels of reaction according to Piazzi (9), as shown in Fig. 2, and by the immunoelectrophoretic analysis as in Fig. 3.

In Fig. 4, one can compare the reactivity of specific antiserum against an Mb 0.19 albumin fraction, purified by Sodini et al. (3), and against the total extract of *T. aestivum*. There is a reaction of identity in immunoprecipitation (upper photo) and a superimposable electrophoretic localization (lower) which enable one to believe that the antibodies are specifically directed towards this albumin fraction.

Therefore, the absorbed and purified goat immuneserum may be considered as a specific Mb 0.19 albumin antiserum.

This antiserum was used for the analysis of several varieties of bread and durum wheats. The results for 24 varieties of bread wheat and 24 varieties of durum wheat are reported in Fig. 5.

The antiserum gives a positive immuneprecipitation reaction with all the analyzed varieties of bread wheat, and a negative reaction with all the analyzed varieties of durum wheat.

In Fig. 6 is shown the reaction with extracts obtained from mixtures containing different percentages (40, 20, 10, and 5%) of bread wheat and durum wheat. A faint line is still visible at the 5% level.

Similar results were obtained with a rabbit antiserum previously prepared (4,5). Yet, when these analyses were carried out in parallel, it was detected that the two antisera were directed toward different protein antigens, as shown in Fig. 7.

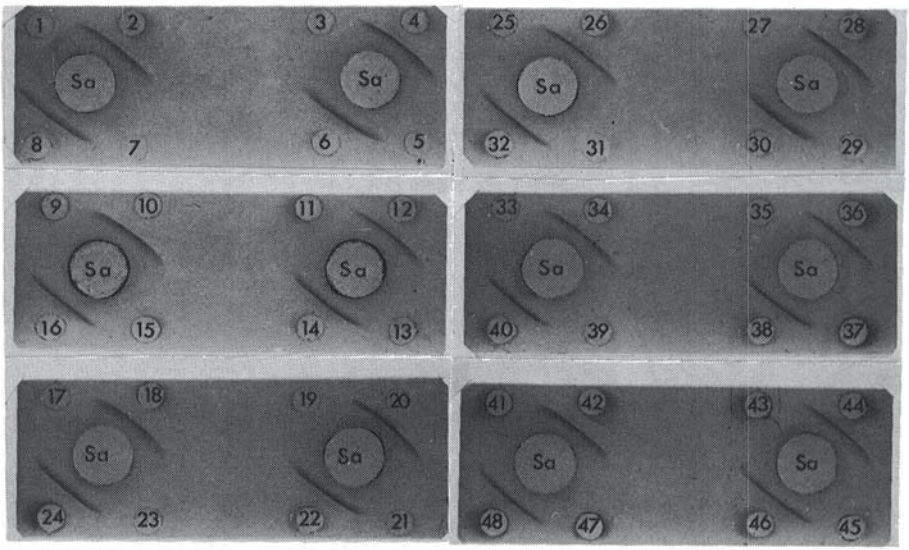
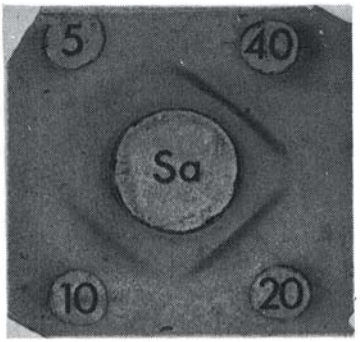


Fig. 5. Immunodiffusion analysis with anti-bread-wheat goat immuneserum (Sa) of 24 durum wheat varieties (odd numbers) and 24 bread wheat varieties (even numbers), as listed in Materials and Methods.



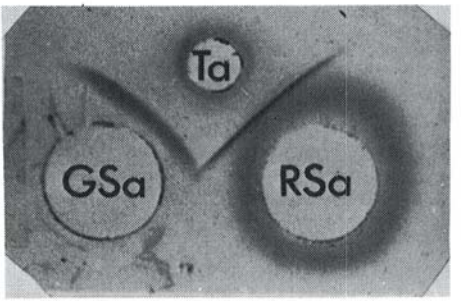


Fig. 6 (left). Immunodiffusion analysis of the soluble protein extracts prepared from flour containing different percentages of bread wheat and durum wheat. Sa = anti-bread-wheat specific goat immuneserum; numbers represent flour protein extract containing 40, 20, 10, and 5% bread wheat.

Fig. 7 (right). Immunodiffusion analysis of the total soluble protein extract of bread wheat (Ta) tested with anti-bread-wheat specific goat immuneserum (GSa) and a preparation of anti-bread-wheat specific rabbit immuneserum (RSa).

Furthermore, as shown in Fig. 8, the immunoelectrophoretic analysis carried out with the two antisera shows that two protein antigens, with different migration characteristics, are present in the extracts of *Triticum aestivum*.

The antibodies present in the goat antiserum, prepared according to the above-described method, react with a protein antigen identified as Mb 0.19 albumin, showing an anodic migration pattern (Fig. 8, left), whereas the previous preparation of rabbit antiserum reacts with a different protein antigen, showing a cathodic migration pattern (Fig. 8, right). This cathodic "soluble" protein has been named 'PCS". As a significant concentration of albumin Mb 0.19 was also present in the protein extract used to prepare the rabbit antiserum, we cannot explain why antibodies direct toward this albumin are not present in the rabbit antiserum.

The presence of these two different antigenic proteins might be of considerable interest from the technological and bromatological as well as genetic points of view. In fact, the biosynthesis of these two specific proteins might be regulated by specific genomes.

The above-described immunochemical analyses, requiring simple techniques, might prove very useful to integrate the more complicated biochemical analyses.

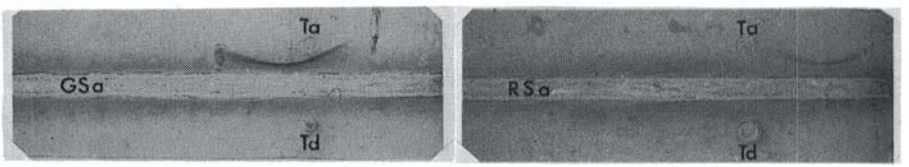


Fig. 8. Immunoelectrophoretic analysis of total soluble protein extracts of bread wheat (Ta) and durum wheat (Td) tested with anti-bread-wheat specific goat immuneserum (GSa) (left) and with anti-bread-wheat specific rabbit immuneserum (RSa) (right).

They could be used with advantage for large-scale screening procedures, which are often needed in routine analytical situations, such as detection of illegal mixtures (bread wheat in macaroni) and collection of statistical data for genetic studies.

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