Immunochemical Analysis on Soluble Proteins of Wheat

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ABSTRACT

A preparation of specific rabbit bread-wheat antiserum for immunological differentiation of bread wheat from macaroni wheat is reported. An extract of bread wheat showed on gel-filtration chromatography two fractions apparently not present in macaroni wheat. From an antiserum prepared by injection of these fractions, antibodies were absorbed by reaction with durum wheat proteins. The remaining antibody globulin was purified and concentrated by precipitation with 1.8M ammonium sulfate at pH 7.2, dialyzed against water and a saline solution, and sterilized by filtration. The antibody solution showed positive immunodiffusion precipitation tests with 16 bread-wheat extracts and negative tests with 16 durum-wheat extracts. Bread wheat was easily detected as 10% of a mixture with durum wheat.

The detection of bread wheat in durum wheat stocks to be used for the manufacture of macaroni and similar products is a problem in some countries. A variety of methods, such as chromatography (1,2), starch gel (3,4), and disc electrophoresis (5,6,7), have been used to analyze wheat flour proteins. Also, immunochemical methods such as immunodiffusion and immunoelectrophoresis, used extensively for animal protein analysis, have been applied in a small scale for barley (8), wheat (9), and soybean proteins (10), generally only for their characterization and on a very small scale, to find immunological differences between related species or genera (11,12).

A series of preliminary investigations performed by us on gluten proteins of durum wheat and bread wheat did not lead to the identification of specific proteins and therefore did not permit an immunological differentiation of these two wheat species. On the basis of other experimental results already reported (13), our attention was concentrated on the soluble protein fraction.

The aim of the present work is to report the preparation of a specific *Triticum aestivum* soluble-protein rabbit antiserum and comparative immunodiffusion analysis on varieties of *T. aestivum* and *T. durum* in order to study possible differences in precipitation patterns.

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, Rome), through A. Bozzini.

Durum wheats used were: Cappelli, Capeiti, Aziziah, Garigliano, Castelporziano, Castelfusano, Wells, Kyperounda, Yuma, SS-0/111, Maliani 1-D, Maliani 8-D, B-52, Lakota, GrA-145, and Sincape.

Bread wheats used were: Campodoro, Rex, Funo, Flaminio, Mentana, Mara, S. Pastore, Elia, Brescia, Combine, Gagliardo, Madif-21, Chianti, Diamante, Marzotto, and Fontarronco-A.

Extraction

Soluble proteins of *T. aestivum* and *T. durum* wheat were obtained by mixing flour with phosphate-buffered saline (PBS), pH 6.6, in the ratio 1:2 flour:buffer. The phosphate-buffered saline, pH 6.6, contained 0.025M $Na_2HPO_4 \cdot 2H_2O$, 0.041M KH_2PO_4, and 0.48M NaCl.

The mixture was stored at 5°C. for 24 hr. After centrifugation at 3,000 × g for 30 min., the supernatant was held 4 to 5 days at 5°C. and its clarification was completed by decantation.

Gel Filtration Analysis

Five milliliters of protein extract (50 to 60 mg. of proteins) of each bread and durum wheat variety was filtered through a column (72 by 3 cm.) of Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Uppsala) in phosphate-buffered saline, pH 7.2, containing 0.009M $Na_2HPO_4 \cdot 2H_2O$, 0.0015M KH_2PO_4, 0.0026M KCl, and 0.14M NaCl. The effluent was collected in 110 fractions of about 6 ml. each and the absorbance at 280 mμ was estimated on a Beckman DU spectrophotometer.

Preparation of Antigen

For preparing antigen of *T. aestivum* the soluble protein extract was obtained from bread wheat, "Rex" variety, one of the most-used French varieties for its high quality level.

After five gel filtrations (50 ml. each) of 250 ml. of the protein extract, the fractions from 310 to 410 ml. were pooled and freeze-dried.

The product was dissolved in 100 ml. of distilled water, sterilized by Seitz EKS filtration, and the solution (0.545% of protein) was used as antigen for preparing antiserum.

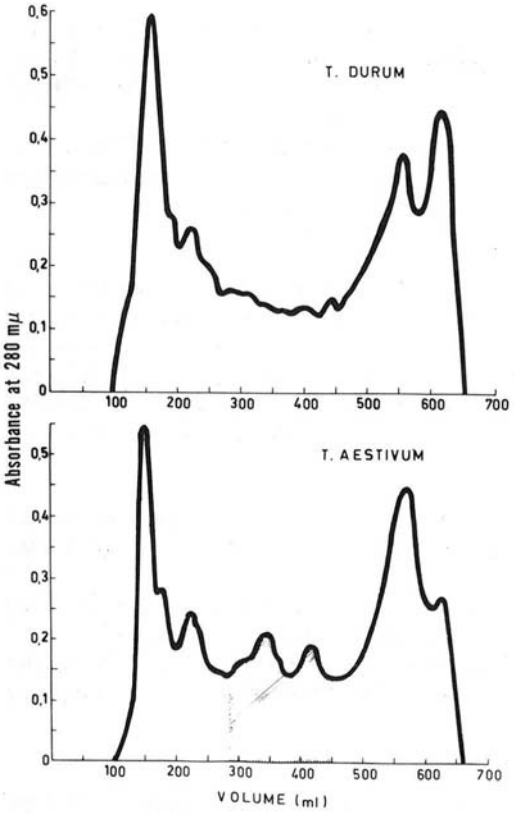
Preparation of Antiserum

Five rabbits, each about 3 kg. body weight, were individually injected with 2 ml. of antigen solution (10.9 mg. total protein) in Freund's complete adjuvant. The total antigen dose was given by multiple subcutaneous injection, distributed in several parts of the body. After 21 days a "booster" injection of 2 ml. of antigen solution was given subcutaneously. Seven days after, the rabbits were submitted to total bleeding and the sera collected and pooled.

For antibody absorption, 2.5 ml. of durum wheat protein extract (Cappelli variety) was first added to 100 ml. of serum. After storage for 2 hr. at 37°C. and a night at 4°C. the formed precipitate was discarded by centrifugation. This treatment was repeated until no reaction with *T. durum* extract (for each and every analyzed variety) was present in agar gel immunodiffusion analysis.

The obtained specific *T. aestivum* antiserum was purified by "Rivanol" and ammonium sulfate precipitation method (13). Three volumes of 0.4% "Rivanol" solution was added to 1 vol. of absorbed antiserum at pH 8.0. The sediment of inactive protein was discarded after centrifugation and the supernatant treated with "Carbex" (active charcoal) to eliminate "Rivanol."

After filtration, the gamma-globulin fraction was precipitated with ammonium sulfate (1.8M final concentration) at pH 7.2. The sediment, collected by centrifugation, was diluted in distilled water and dialyzed in Cellophane tubing, first against distilled water for 12 hr. at 4°C. and then against phosphate-buffered saline, pH 7.2, for 12 hr. additional at 4°C. After sterile filtration, the obtained globulin solution (7% protein) was used for the immunochemical tests.



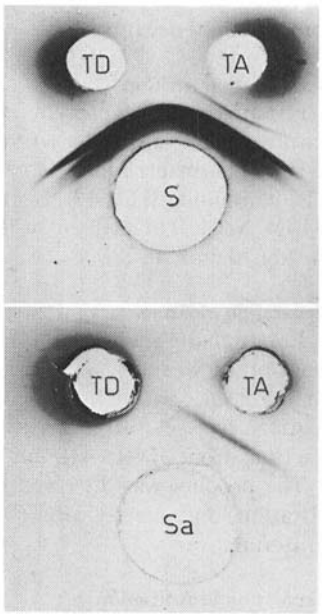


Fig. 1 (left). Sephadex G-100 gel filtration of *Triticum durum* and *Triticum aestivum* (subsp. *vulgare*) PBS extracts.

Fig. 2 (right). Ouchterlony plate showing precipitation patterns between *Triticum aestivum* (TA) and *Triticum durum* (TD) extracts, and *T. aestivum* rabbit antiserum, before (S) and after (Sa) absorption with *T. durum* extract.

Immunodiffusion Analysis

The immunodiffusion analyses were carred out in 1% agarose gel on microscopic slides as previously described (14).

Tests on the antigen solution and on antiserum samples during the preparation were carried out by a simple method of analysis at different antigen:antibody concentration ratios as already reported (15).

RESULTS AND DISCUSSION

Gel Filtration Analysis

In Fig. 1, typical patterns of soluble proteins of durum (Cappelli variety) and bread (Mentana variety) wheat after gel filtration are illustrated. As the diagrams

show, several peaks are present in both species, but peaks on 310 and 410 ml. were constantly present in all bread-wheat varieties and practically absent in all durum-wheat varieties analyzed.

Immunological Analysis

The results of the immunodiffusion analysis with *T. aestivum* rabbit antiserum before (S) and after (Sa) absorption are shown in Fig. 2, in which the antiserum is tested with soluble protein extracts of *T. durum* (TD) and *T. aestivum* (TA) wheat.

According to the precipitation patterns in the gel, it is evident that a clear difference is present in at least one specific protein of *T. aestivum*.

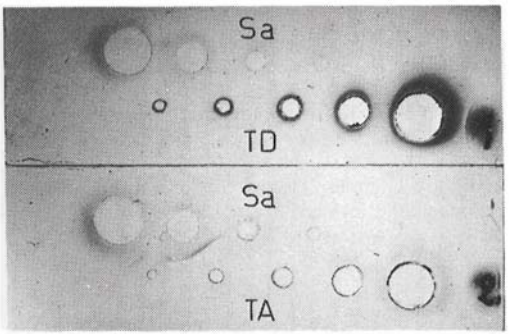
To obtain some information on specificity and on optimal conditions of reaction in gel, the *T. aestivum*-absorbed antiserum (Sa) was tested with *T. durum* (TD) and *T. aestivum* (TA) extracts at different antigen:antibody ratios by the simple immunodiffusion test (Fig. 3). Here, good patterns of precipitation in gel are evident only for definite ratios of *T. aestivum* (TA) antigen, whereas no reactions are present in all the different ratios of *T. durum* (TD) extract.

The specific antiserum was used for the testing of each durum- and bread-wheat variety mentioned above.

The results of the immunodiffusion analysis proved that all the *aestivum* wheat varieties showed specific positive reaction, whereas no reactions were present with durum varieties.

Mixtures prepared with 40, 20, and 10% of *T. aestivum* and durum wheat were analyzed with antiserum (Fig. 4). The analysis performed on mixtures showed that it is possible to obtain positive results up to 10% of *T. aestivum* present in *T. durum* wheat.

These results seem to open up a series of applications for identifying wheat flour or semolina products, leading to the possibility of analysis for identifying species and varieties. Also, implications in food analysis and prevention of illegal mixtures are clear.



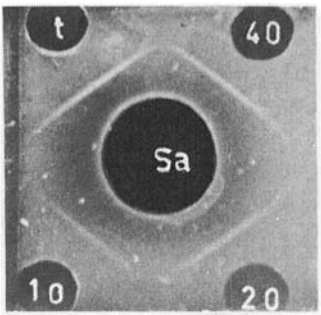


Fig. 3 (left). Immunodiffusion test at different antigen:antibody ratios of reaction: Sa, absorbed *T. aestivum* rabbit antiserum; TD, *T. durum* (Cappelli variety) soluble protein extract of wheat; TA, *T. aestivum* (Mentana variety) soluble protein extract of wheat.

Fig. 4 (right). Immunodiffusion patterns of soluble protein extracts of *T. aestivum*-*T. durum* wheat mixtures: t, total *T. aestivum* extract; 40, 40% of *T. aestivum* in *T. durum*; 20, 20% of *T. aestivum* in *T. durum*; 10, 10% of *T. aestivum* in *T. durum*; Sa, absorbed *T. aestivum* rabbit antiserum.

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