IMMUNOCHEMICAL CROSSREACTIVITY BETWEEN ZEIN, HORDEIN AND GLIADIN

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1. Introduction

The major seed proteins in cereals are the alcohol-soluble prolamins [1]. On SDS–PAGE these prolamins separate into individual polypeptides very different in number as well as in M_r depending on the type of cereal species from which they were extracted. Thus, zeins of maize consist of only two polypeptides of 19 000 and 22 500 M_r [2], hordeins from barley subclassified according to their M_r-values into B- and C-types, consist of at least 3 and 5 polypeptides, respectively [3] and gliadins of wheat separate into ~12 proteins [4]. These proteins share characteristic features: during seed maturation they are deposited in the endosperm inside protein bodies [5–7] and can be solubilized only in aqueous alcohol. This property may be related to their unusual amino acid composition. Prolamins contain high amounts of proline (up to 30 mol%) and glutamine (up to 40 mol%) – hence their name – with several of the essential amino acids originating from aspartate, notably lysine, in short supply [8,9]. This raised the question of whether the similarity in function and amino acid composition might be reflected in a certain homology of primary structure. If so, one might expect to demonstrate antigenic crossreactions between these prolamins using appropriate antisera. We investigated this aspect using rabbit antibodies directed against zein and hordein, respectively. We report here that purified anti-zein IgG crossreacted with hordeins, but that anti-hordein IgG did not crossreact with zeins and that both crossreacted with a few of the proteins of the gliadin family.

Abbreviations: SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; M_r, relative molecular mass

2. Materials and methods

2.1. *Materials*

Zeins from *Zea mays* was obtained from ICN Pharmaceuticals (Cleveland OH). Its purity was checked on SDS–PAGE and found to produce only the 2 polypeptide bands expected [6,10]. Wheat (*Triticum aestivum* cv. Diplomat) seeds were ground to a flour and gliadins were extracted from 1 g flour using the same conditions as for zeins [11]; the pooled ethanol extracts were lyophilized to dryness. Hordein from barley (*Hordeum vulgare* cv. Bomi) was a gift from S. K. Rasmussen (Dept. Physiology, Carlsberg Laboratory, Copenhagen). These zein and hordein preparations were used for the production of antibodies in rabbits as described below. Normal rabbit IgG and swine anti-rabbit IgG conjugated to horseradish peroxidase were obtained from Dako (Copenhagen); Freund's adjuvants and Bacto-Bordetella Pertussis antigen were from Difco Labs. (Detroit MI). Protein A crosslinked to Sepharose 4B was from Pharmacia Fine Chemicals (Uppsala). Bovine serum albumin (fraction IV) was from Sigma (St Louis MO) and normal horse serum from Gibco (Glasgow). 4-Chloro-1-naphtol was from Merck.

2.2. *Preparation of zein and hordein antibodies*

Zein or hordein were dissolved in 6 M urea–10 mM 2-mercaptoethanol at 5 mg/ml, emulsified with an equal volume of Freund's incomplete adjuvant and injected intracutaneously into 2 rabbits each distributed over several sites. Each rabbit received 5 mg protein the first time and 2.5 mg each time thereafter. With the first antigen injection, 0.2 ml Pertussis antigen was given subcutaneously. Booster injections of the antigen in the above formula (replacing Freund's incomplete by complete adjuvant) were given 21 and 31 days after the first injection and thereafter regularly

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once a month. Rabbits were bled from the ear 8 days after each injection. After 1 month all sera were positive by the immunoblotting assay (see blow). They were pooled and the IgG fraction was purified by the one-step method [12] using protein A–Sepharose 4B. The lyophilized IgG fractions of anti-zein or anti-hordein were redissolved at 10 mg/ml in 10 mM potassium-phosphate buffer (pH 7.2) containing 0.15 M NaCl. To this stock solution, 10 mg/ml of bovine serum albumin was added to protect the antibodies against loss of activity when kept at –20°C for several months.

2.3. *SDS–PAGE of prolamins*

Zeins, hordeins and gliadins were dissolved at 2 μg/μl in Laemmli's sample buffer [13] containing 6 M urea and electrophoresed in 15% polyacrylamide–1% SDS gels as in [11]. Zeins (50 μg protein), hordeins and gliadins (100 μg protein) were loaded onto each track. Fixing and staining was as in [11].

2.4. *Immunological reactions of proteins immobilized on nitrocellulose*

We prepared 1.5 mm thick gels containing 15% polyacrylamide–1% SDS and evenly distributed, omitting the comb, up to 1 mg protein (zein, hordein or gliadin) dissolved in 500 μl sample buffer across the entire gel. Electrophoresis was then performed as in [11]. Immediately thereafter the separated proteins were electrophoretically transferred to nitrocellulose sheets cut to the size of the gels according to [14]. The transfer was done using 0.1 M Tris adjusted to pH 9.3 with solid glycine and containing 20% methanol to prevent the gel from expanding during the protein transfer. The transfer was usually completed in 45 min using 60 V and 1.6 A maximum. The sheets were dried between filter papers. For the immunoassay, strips of <5 mm width were cut from the sheet and placed into individual trays holding 1 ml solution. The strips were incubated with 2 μl anti-zein IgG or anti-hordein IgG stock solutions in 1 ml immunobuffer (consisting of 20 mM Tris-buffer (pH 7.2), 0.9% NaCl, 2.5% bovine albumin and 5% normal inactivated horse serum). As controls the IgG fraction from non-immunized rabbits was used. Each strip was then incubated with 1 μl swine anti-rabbit IgG conjugated to peroxidase in 1 ml immunobuffer as in [14]. As substrate for the peroxidase we used instead of the recommended *o*-dianisidine, which is reputedly carcinogenic, 4-chloro-1-naphtol giving a blue reaction without background staining, as a 10 mg% solution in 16% methanol–0.01% H_2O_2.

3. Results and discussion

Standard antigen–antibody reaction assays, such as immunotitration, Ouchterlony immuno-diffusion and cross-adsorption tests cannot be carried out when dealing with hydrophobic antigens such as the cereal prolamins which are soluble in aqueous alcohol, 6 M urea or detergents only. Instead, our antigens had to be tested for immunochemical reactivity after immobilization on nitrocellulose sheets [14]. This method is based on the following principle: proteins from gels, electrophoretically transferred and immobilized on nitrocellulose sheets, are reacted with a specific antibody. A second antibody conjugated to peroxidase is directed against the first antibody. The antigen–antibody complex is then visualized by the color produced by the peroxidase reaction. This method, although qualitative, is very sensitive and allows to detect μg levels of antibodies. If the electrophoretic

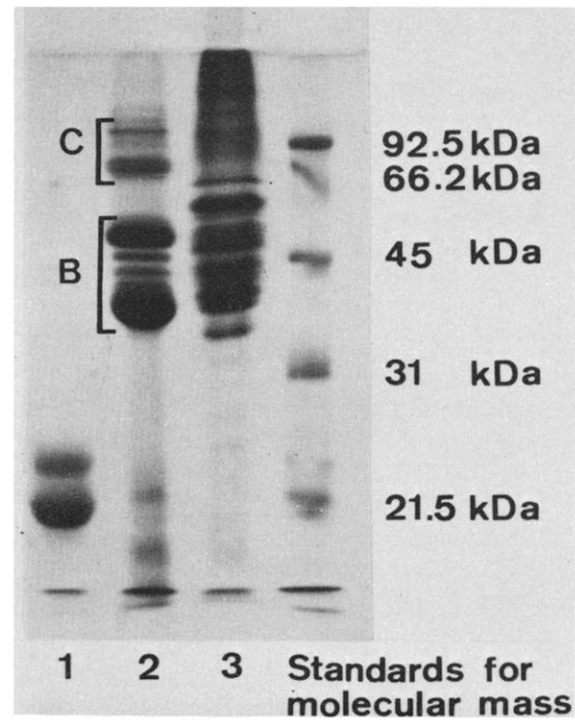

Fig.1. SDS–PAGE of zein, hordein and gliadin. The electrophoresis in 15% polyacrylamide–1% SDS gels was performed as in section 2. Track 1 contained 50 μg zein, tracks 2 and 3 contained 100 μg hordein and gliadin, respectively.

transfer of proteins from gel to nitrocellulose is complete (this can be checked by staining the nitrocellulose sheet with Amido-schwarz) then the bands which have reacted with the anti-sera or with specific IgG fractions can be directly compared with the appropriate bands stained on the gel. The typical SDS–PAGE pattern of our cereal antigens is represented on fig.1. It shows, prior to transfer, the respective positions of the polypeptides pertaining to zeins, hordeins or gliadins (tracks 1–3, respectively). Zeins are represented by the characteristic two polypeptides and hordeins are of the B- and C-type [3]. Gliadins separated into ⩾15 polypeptides in the same M_r-range as the hordeins. These proteins could be easily and completely blotted on nitrocellulose sheets (not shown);

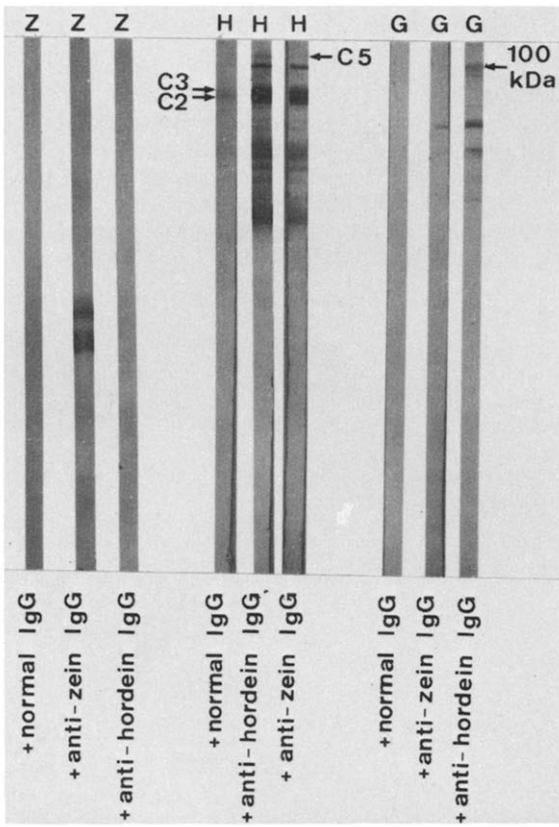

Fig.2. Immunochemical crossreactions between zein, hordein and gliadin. Zein, hordein and gliadin separated into individual proteins on SDS–PAGE were blotted to nitrocellulose sheets as in section 2. For each antigen a separate gel and blot was performed. Strips cut from blots and containing zein (Z), hordein (H) and gliadins (G) as antigens were incubated with normal rabbit IgG, with anti-zein IgG, and with anti-hordein IgG.

the resulting picture after Amido-schwarz staining was identical with that of the stained gel. The even distribution of one protein preparation across the entire gel prior to electrophoresis produces an even banding pattern of polypeptides on the gel which after blotting to nitrocellulose allows one to carry out many immunochemical reactions by using strips cut from the sheet. These contain identical amounts of the antigen. The strips can be used to 'titrate' a specific antibody by using serial dilutions of serum and/or to test crossreactivity with different antibodies. Thus, the cereal prolamins we used as antigens produced sera containing the specific antibodies which were normally used in a 1:200 dilution. Strips containing zein as antigen, when reacted with anti-zein IgG produced 2 colored bands as expected coinciding with the 2 zein polypeptides (fig.2). Similar controls were run with anti-hordein IgG reacting with all the B- and C-type hordeins. Immunochemical crossreaction was detected when anti-zein IgG reacted with hordeins; all hordeins except C5 reacted. However, the reverse reaction, e.g., that of anti-hordein IgG and zein, did not occur. Anti-zein IgG reacted with 2 of the main proteins of the gliadin family; anti-hordein IgG reacted with the same 2 proteins, but in addition with 1 protein of ~100 000 M_r and also with a smaller one in the M_r-range of the B-type hordeins. Controls with normal rabbit IgG from non-immunized animals were negative except in the case of hordeins, when a weak reaction with C2 and C3 hordein was seen. All these reactions were carried out many times with the same result.

It thus would appear that the different IgG fractions recognize different antigenic determinants. IgG fractions in sera prepared against zeins recognize cross-reacting determinants on hordeins. These are probably not the ones recognized by the anti-hordein IgG as this IgG does not react with zein. One explanation could be that the hordein antigens recognized by both IgG fractions are not immunogenic in vivo, at least not under our immunization conditions. It is noteworthy that both anti-zein and anti-hordein sera contained antibodies reacting with a limited number of proteins from the family of wheat gliadins.

It is tempting to speculate that antigenic recognition by the antibodies is possibly based on structural similarity. However, the structures of prolamines are not yet known, with the exception of one zein protein [15] of which the first complete amino acid sequence was recently completed. It showed 7 most

unusual tandem repetitions of a highly conserved 20 amino acid repeating unit containing dipeptide repeats such as Ala–Ala, Leu–Leu and especially Gln–Gln present up to 14 times. It would be most interesting to compare to this primary structure that of other cereal prolamins and thus to be able to draw more conclusions concerning the immunochemical crossreactions described. These findings establish an immunochemical relatedness of zein, hordein and gliadin, most likely reflecting their similar function in developing cereal endosperms. Whether these similarities reflect convergent evolution dictated by similar functional requirements or divergent evolution from the same ancestral genes remains to be investigated.

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