

Antiserum Against the 33-kDa Herbicide-Binding Protein

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A rabbit antiserum was prepared against the purified 33-kDa herbicide-binding protein (HBP) from the alga *Bumilleriopsis filiformis*. Specificity at 1:10,000 dilution (v/v) of the unpurified serum is detectable in the immunoblotting assay with both the 33-kDa protein from the alga *Bumilleriopsis* as well as that from *Spinacia*. Agglutination can be observed with photosystem-II particles only, not with intact thylakoids, indicative of a hidden location of the protein determinants in the membrane. Neither herbicide binding nor electron transport is influenced by the antibody. Apparently, the antigenic site of the HBP for the antibody used here is different from the herbicide-binding region.

Introduction

During the last few years many attempts have been made to obtain more details of the molecular structure of the herbicide-binding protein (HBP). Most of these studies dealt with cloning and sequencing of the gene of this protein, and it is known so far that resistance against many electron-transport inhibitor herbicides is caused by only one base substitution resulting in one amino-acid change in the HBP of the resistant biotype [1].

Another approach to characterize the HBP is the use of monospecific antibodies against this protein. These should be a tool to possibly obtain information on relationship between HBP's from different plant species. Furthermore, surface parts of the HBP outside the thylakoid membrane may be determined by the use of antibodies. Additionally, antibody binding may influence the function of the HBP, *i.e.* herbicide binding and electron-transport activity, leading to a better understanding of the molecular mechanism of this membrane peptide.

The HBP from higher plants has not yet been purified for antibody induction using preparative sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), partly due to difficult identification [2] and lack of a functional assay for the isolated protein. The HBP of the alga *Bumilleriopsis filiformis*, however, migrates on SDS-gel electrophoresis as a single, well-stainable band and, therefore, preparative gel electrophoresis has been possi-

ble to obtain the purified protein. Specific antibodies (present in a rabbit serum) became available against this purified HBP and some characteristics are presented in this communication.

Materials and Methods

Thylakoid membranes from *Spinacia oleracea*, strain Atlanta, and *Bumilleriopsis filiformis* were prepared as previously described [3] with modifications: Spinach leaves were homogenized in a medium containing 5 mM sodium-phosphate buffer, pH 7.8; 0.3 M sucrose; 2.5 mM NaCl, and 2 mM MgCl₂. The homogenate was filtered through nylon cloth and centrifuged for 10 min at 2000 × g. The resulting pellet was resuspended, washed once with the isolation medium from which sucrose was omitted, and then resuspended in the same medium adding 0.2 M sucrose. Thylakoids from *B. filiformis* were prepared according to [3] in an isolation medium containing 5 mM sodium phosphate instead of TRIS-HCl [tris(hydroxymethyl)-aminomethane] and pyrophosphate. Isolated thylakoids were washed and resuspended in 10 mM sodium-phosphate buffer, pH 7.8; 70 mM sucrose; 10 mM NaCl, and 5 mM MgCl₂. Photosystem (PS)-II particles were prepared according to [4] with modifications: Triton X-100 treatment was performed in an incubation medium consisting of 0.4 M sucrose, 15 mM NaCl, 5 mM MgCl₂, 20 mM MES-NaOH (morpholinoethanesulfonic acid), pH 6.5. Then, washing and centrifugation at 35,000 × g was carried out for 20 min, omitting Triton X-100. The particles obtained were resuspended in the washing medium and stored at –120 °C.

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Photoaffinity labeling with [^{14}C]azidoatrazine (2-azido-4-ethylamino-6-isopropylamino-s-triazine; 49.4 mCi/mmol, Pathfinder Lab. Inc., St. Louis, Missouri/USA) was performed according to ref. [5].

For sodium dodecyl sulfate polyacrylamide gel electrophoresis (see ref. [6]), the polyacrylamide concentration was 17.5% (w/w) for the separating gel and 5% for the stacking gel. The samples were solubilized for 30 min at room temperature. Gels were stained with Coomassie brilliant blue R 250 or treated with enhancer solution (Enlightning, New England Nuclear, Boston/USA) for fluorographic detection of radiolabeled proteins. Marker proteins were purchased from Sigma, Munich.

Electrophoretic transfer of proteins to nitrocellulose (of 0.45 μm pore size, Schleicher und Schüll, Dassel/W. Germany) and subsequent antibody detection with peroxidase-conjugated goat anti-rabbit IgG (immunoblotting) was done according to [7]. For the (primary) antibody reaction, incubation with a 1:10,000 dilution (v/v) of the antiserum was carried out overnight.

Results and Discussion

Peptide patterns of thylakoids from spinach and *Bumilleriopsis* are considerably different (a detailed pattern will be published elsewhere). Such a peptide pattern, Coomassie-stained, is advantageously resolved, when proteins are separated by SDS-PAGE using a linear urea gradient (from 0 to 6 M) which leads to modified mobilities of the proteins.

For spinach the HBP of 33-kDa exhibited a single diffuse band at zero urea concentration (left side of Fig. 1A), but split into four bands with increasing urea concentrations (as seen best at 3 M urea). Two minor bands are visible, located above a (third) intensely stained band (indicated by symbol ●, right part of the figure) and one below the third band, indicative of a somewhat decreased apparent molecular weight of this 33-kDa peptide at higher urea concentrations. [^{14}C]azidoatrazine labeling of thylakoids and subsequent urea-gradient gel electrophoresis exhibited only one labeled band identical with the resolved 33-kDa band as shown in Fig. 1A and indicated by arrows. Azidoatrazine labeling is not documented, since the ^{14}C -labeled 33-kDa band on the fluorogram coincided exactly with that shown by Coomassie staining. Also Hirschberg *et al.* [8] demonstrated that in the presence of 4 M urea a pro-

tein labeled with [^{14}C]azidoatrazine migrates on SDS-PAGE as a band of apparent molecular weight lower than 33-kDa as found at zero urea concentration.

For the 33-kDa band in the gel of *Bumilleriopsis*, however, no splitting was observed at any concentration from 0 M to 6 M urea. Only *one* band was evident (Fig. 1B, arrows), which was well-stained by Coomassie brilliant blue R 250 (as shown here), again labeled by [^{14}C]azidoatrazine (data not shown) and sufficiently separated from other proteins. Therefore, the HBP from *Bumilleriopsis* was chosen for the purification of the protein.

Purification was performed by cutting the stained protein from SDS slab gels. The slices from several gels were pooled and eluted by electro dialysis, then pooled again, thoroughly dialyzed against 10 mM sodium-phosphate buffer, pH 7.0, lyophilized and resuspended in a medium containing 70 mM sodium-phosphate buffer, pH 7.0, including 1% SDS. Re-electrophoresis of aliquots of the purified protein did not show impurities, as assessed by staining intensity with Coomassie. 1 ml containing 300 μg of the purified 33-kDa HBP complexed with SDS was emulsified with 1 ml of complete Freund's adjuvant (Difco) and subcutaneously injected into a rabbit. After fifteen days, the rabbit was boosted with 120 μg protein (0.4 ml mixed with an aliquot of incomplete Freund's adjuvant). After another 14 days, 120 μg of HBP was injected (0.4 ml mixed with an aliquot of 0.9% NaCl solution). The rabbit was bled on the 52nd day. After having been kept at room temperature for 2 hours, the serum with the antibodies was obtained by simple centrifugation.

Specificity of the antibodies was assayed by immunoblotting. As indicated in Fig. 2, antibodies against the purified 33-kDa protein not only reacted with the isolated HBP, but also specifically with the HBP present in a crude extract from thylakoid membranes. No reaction was obtained with the pre-immune serum, not even at 10-fold higher serum concentrations than used in the immunoassay. Noteworthy, there is no significant cross-reaction with other thylakoid-membrane proteins except for a very faint reaction with peptides of high molecular weight (Fig. 2, lane 5, and Fig. 3, lane 4). The antiserum did not only react with the solubilized HBP from *Bumilleriopsis*, wild-type (Fig. 3, lane 4), but also with HBP's from two herbicide-resistant mutants (lanes 2, 3) and spinach (lane 1).

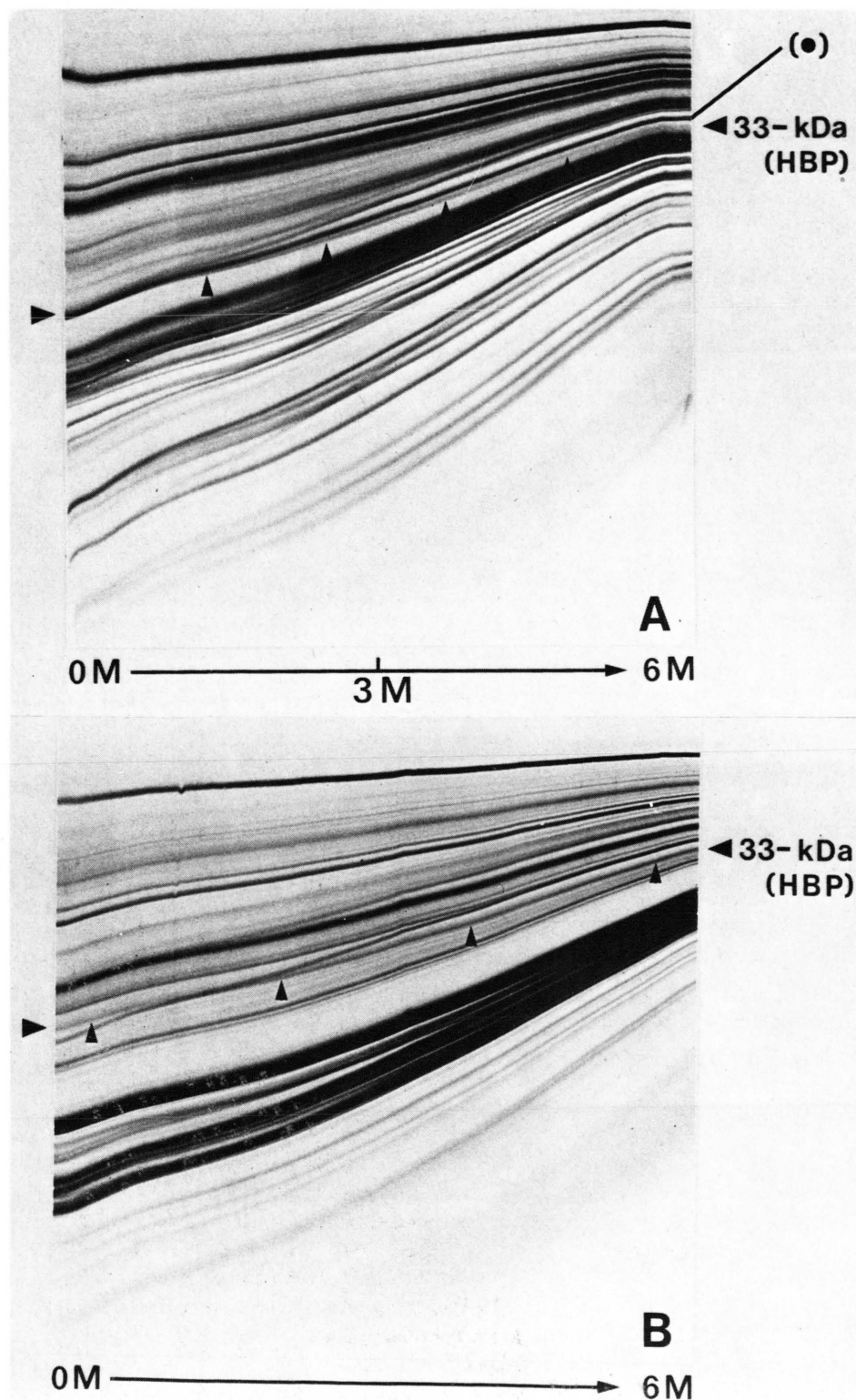


Fig. 1. SDS polyacrylamide gel electrophoresis of thylakoid-membrane peptides from spinach (A) and *Bumilleriopsis* (B). For explanation see text. After solubilization, proteins were separated on 17.5% polyacrylamide gels containing a linear urea gradient (ranging from 0 to 6 M); staining was achieved with Coomassie brilliant blue R-250. 300 μ g of chlorophyll per gel was used. The HBP is indicated by arrows. A molecular weight of 35-kDa for the HBP from *Bumilleriopsis* was reported previously [6], because molecular-weight determination had been carried out with marker proteins purchased from Biorad, Munich (mixture Cat. No. 161-0304).

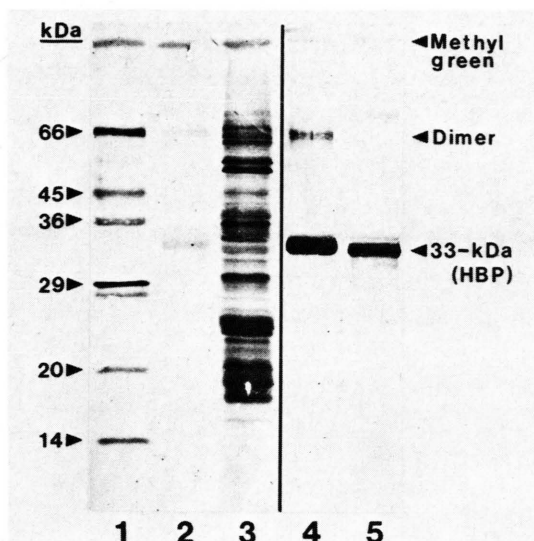


Fig. 2. Immunoblotting of thylakoid-membrane peptides from *Bumilleriopsis*. After electrophoretic transfer to nitrocellulose, proteins were stained with Amido black (lanes 1–3) and antibody complexes were visualized by staining for peroxidase activity (lanes 4 and 5). Lane 1: marker proteins (from top to bottom: bovine albumin, egg albumin, glyceraldehyde-3-phosphate dehydrogenase from rabbit muscle, carbonic anhydrase from bovine erythrocytes, trypsin inhibitor from soybean and α -lactalbumin, all combined in the Sigma mixture No. SDS-7); lanes 2 and 4: 4 μ g of the purified 33-kDa herbicide-binding protein (HBP) was used per lane; lanes 3 and 5: thylakoid membrane proteins (equivalent to 25 μ g of chlorophyll/lane).

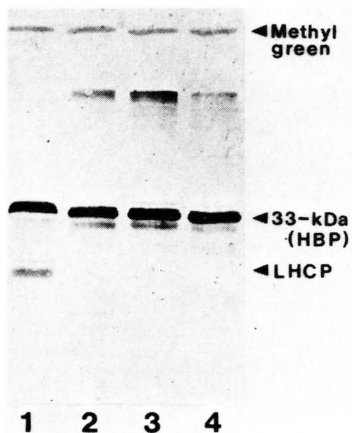


Fig. 3. Immunoblotting of thylakoid-membrane peptides from spinach and *Bumilleriopsis*. After electrophoretic transfer to nitrocellulose, protein-antibody complexes were identified by a secondary antibody reaction with peroxidase-conjugated goat anti-rabbit IgG. Lane 1: spinach; lane 2: *Bumilleriopsis filiformis*, mutant BV; lane 3: *Bumilleriopsis*, mutant KNJ-724; lane 4: *Bumilleriopsis*, wild-type. The band of the LHCP (light-harvesting chlorophyll protein) is due to chlorophyll, not to protein-antibody interaction. All lanes had been loaded with thylakoid material equivalent to 20 μ g of chlorophyll.

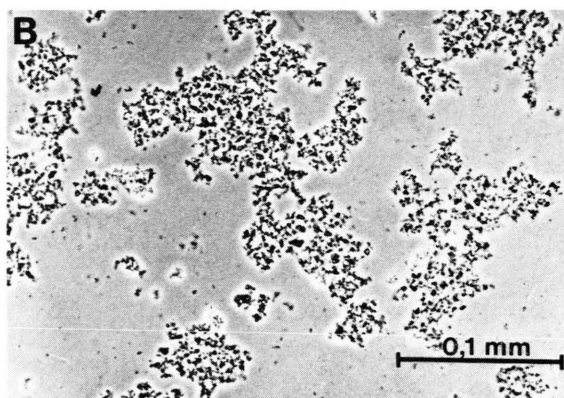
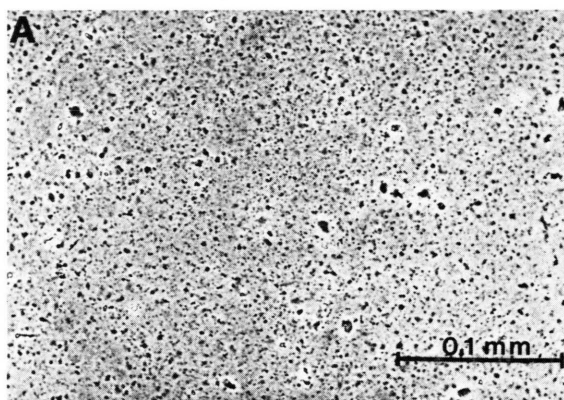


Fig. 4. Agglutination reaction of antiserum with photosystem-II particles from spinach. 5 μ l of particles (1 mg chlorophyll/ml) were mixed with an aliquot of either pre-immune serum (A) or antiserum (B) against the 33-kDa HBP from *Bumilleriopsis*. After gentle shaking, photographs were taken with a phase-contrast microscope (Zeiss Photomikroskop III, magnification 350-fold).

Since antibodies were produced against SDS-peptide complexes, it was important to ascertain whether they would recognize the native HBP. A simple test is the agglutination reaction with isolated membranes. There was no agglutination observed with thylakoids from either *Bumilleriopsis* or spinach. However, when PS-II particles derived from spinach were used, strong agglutination occurred (Fig. 4). Apparently, parts of the HBP have to be exposed in the membrane to react with an antibody. Presumably, the intrinsic HBP is folded, spanning the lipid bilayer for several times [8], and only small pieces of the protein are accessible from the water phase. Furthermore, stacking of the grana membranes diminishes the number of sites attainable for antibodies. Particles are flatly appressed double-membrane sheets with free (open) ends and (as shown by freeze-fracture electron microscopy) the

exposed surface of these fragments corresponds to the luminal surface of the grana thylakoids [9]. PS-II particles from *Bumilleriopsis* comparable to those from spinach are not available until now.

An important question is the influence of antibody binding on electron transport and herbicide activity. O₂-evolution of PS-II particles suspended in the washing medium was unaffected by the antiserum or preimmune serum using 0.5 mM 2,6-dichloro-*p*-benzoquinone as electron acceptor, yielding 600 $\mu\text{mol O}_2 \times \text{mg chlorophyll}^{-1} \times \text{h}^{-1}$ (identical to the rates as reported in ref. [10]). I₅₀-values were 5×10^{-8} M for diuron [3-(3,4-dichlorophenyl)-1,1-dimethylurea] and 3×10^{-7} M for atrazine (2-chloro-4-ethylamino-6-isopropylamino-s-triazine), respectively. k_b 's were found as 2.8×10^{-8} M for diuron and 1.2×10^{-7} M for atrazine, no k_b -values can be given for ioxynil (4-cyano-2,6-diiodophenol) and other phenolic herbicides, since these compounds were found to be strongly bound to albumin present in the rabbit serum.

Lack of a functional response of antibody binding to PS-II particles documents that this binding does not influence the activity of the HBP *in situ*. Pro-

teins like the HBP which are only partially surface-exposed probably have many antigenic sites inaccessible to antibodies due to conformational folding and (steric) hindrance by lipids and other proteins (comp. [11]). As noted above, most of the *inner* thylakoid surface is exposed in PS-II particles [9], so that the antibody will react with luminal antigenic sites of the 33-kDa peptide, which are different from its herbicide-binding region (located at the outer thylakoid surface).

Additional experiments are under way to expose other antigenic sites of the HBP by further peptide "stripping" or modification of the thylakoid. This report is meant to introduce the antibody as a possible tool for access to the function of the HBP.

Acknowledgement

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Note added in proof:

During proofreading a report of J. Ohad *et al.* (EMBO J. **4**, 1655–1659 (1985) appeared on an antibody against a 22-KDa piece of HPB used in an immunoblotting assay.

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