Immunochemical Methods in Cereal Research and Technology[1]

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ABSTRACT

Various applications of immunochemical techniques in basic and applied studies of cereals, here exemplified by studies on barley, malt, and beer, are reviewed. After describing the production of antibodies and the immunochemical methods (precipitation-in-gel techniques, nephelometry, radioactive immunoassay, enzyme-linked immunosorbent assay, immuneblot, immunoprecipitation), the paper reviews investigations where immunotechniques have been used to trace enzymes and other proteins from barley and malt during grain filling and germination and used to follow these proteins through the technological processes of brewing. Detection of proteins of nonbarley origin in barley and beer, i.e., contaminants, yeast proteins, and additives, is also discussed.

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The basic antigen-antibody reaction provides a means for very sensitive and specific analytical procedures. Since the first reports about 50 years ago on the use of antibodies for quantitative analytical purposes (Heidelberger and Kendall 1932), immunology has been increasingly recognized as a valuable analytical tool. The origin and also the main application of immunomethods has been in the field of medicine, but their use has now spread to many other disciplines, including cereal chemistry and biotechnology. An important factor in the wide acceptance of immunochemical methods has been their versatility. A multitude of techniques has evolved, using either antibody-antigen recognition per se or a combination of this with other well-established techniques such as electrophoresis and chromatography.

Various reviews have focused on topics such as immunochemistry applied to seed proteins in general (Daussant and Skakoun 1983), to barley proteins in particular (Daussant 1977), to plant biology in general (Knox 1982), to enzyme regulation in plants (Daussant and Skakoun 1981), to plant growth regulators (Weiler 1984), and to food products (Daussant 1982, Daussant and Bureau 1984). This paper aims at reviewing recent applications of immunochemical techniques in basic as well as applied cereal research, with examples taken from our work in the field of brewing. After descriptions of basic principles in immunology and of analytical immunochemical techniques, a number of applications are cited, ranging from investigations into the physiology of the barley seed to problems concerned with quality control of finished beer.

DEFINITIONS AND BASIC IMMUNOLOGICAL PRINCIPLES

Unfortunately, quite a large number of different definitions have been used for the basic terms in immunology. It is therefore necessary to define the terms used in this paper more precisely. These are generally in accordance with the definitions compiled by Morris (1985) and may on some points vary from definitions stated elsewhere (for instance de Weck 1974, Kabat 1980).

When higher vertebrate animals are exposed to material of foreign origin, *antibodies* (also termed immunoglobulins) will be elicited as one part of the immune response of the animal. Antibodies are high-molecular-weight proteins that are able to recognize and bind that substance which gave rise to their formation, the so-called immunogen. Antibodies may be divided into various classes, designated immunoglobulin G, A, D, E, or M. Immunoglobulin G (IgG) is the type found in largest quantities in serum, and thus of main interest when antibodies are to be used for analytical purposes. IgG has a molecular weight of about 160,000, and the structure of the molecules resembles the letter Y, with one recognition site residing on each of the two upper branches. The two recognition sites on one antibody molecule are identical. The basic structure of the other immunoglobulins is similar to IgG, but IgA may form a dimer of the Y-unit, and IgM, which also appears in serum, forms a pentamer with a total of ten recognition sites.

Complementary to the antibody definition, an *immunogen* may be defined as any substance that, in a suitable animal, stimulates the production of antibodies recognizing the immunogen itself. An *antigen*, however, is defined by its ability to be recognized and bound by preformed antibodies. An immunogen will always be able to react as antigen, that is, to combine with antibodies, but not all antigens can elicit antibody formation, as a certain size is required for a substance to be immunogenic. This size is usually estimated to be equivalent to a molecular weight of around 4,000, although some examples of even smaller immunogens are known (de Weck 1974). Proteins are in general good immunogens and antigens. Antibodies may also be raised to nucleic acids or carbohydrates, although these are generally poor immunogens in purified state, despite their size. However, antibodies recognizing the native substances may be obtained after immunization with nucleic acids or carbohydrates coupled to carrier proteins (Stollar 1980, Schiffman 1966, Vreeland and Chapman 1978).

Small molecules that are not immunogenic on their own may act as antigens in the sense that they will react with antibodies. The appropriate antibodies may be elicited by injection of complexes between the small molecule, termed the *hapten*, and a larger carrier, for instance a protein (Erlanger 1980). Examples of this type of substance are hormones and mycotoxins.

The ability of antibodies to combine with molecules, which are not large enough to induce their production, is caused by the nature of the two recognition sites on the antibodies. These sites are directed not against the antigen as a whole, but against a minor structure on the surface of the antigen, a so-called *epitope* or *determinant*. An epitope on a protein antigen is composed of a few amino acids, which are situated near each other in the native molecule, resulting from either the primary structure of the protein (*sequential epitopes*) or the folding of the peptide chain(s) (*conformational epitopes*) (Sela 1969). Similarly, epitopes on carbohydrates and nucleic acids consist of a few, closely spaced sugar or nucleotide units.

In general, 4–6 units seem to be required to form an epitope (de Weck 1974), and sizes from about 4 to 34 Å have been estimated (Kabat 1980). Many antigens contain several epitopes. Although it may be difficult to assess the actual number of these, it can be taken as a general rule that proteins contain one determinant per 5,000 mol wt (Crumpton 1974).

It is evident that the nature of the antibody recognition site provides a base for high specificity. However, different antigens may contain one or more identical epitopes. If so, antibodies raised against one antigen as an immunogen may also recognize the other antigen, leading to so-called *cross-reactions*. This has for instance

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been observed between plant proteases from various related plants (Vaag 1985b), or between storage proteins from various cereals (Dierks-Ventling and Cozens 1982).

Antibodies are produced by the B-lymphocyte cells of animals. Each cell synthesizes a unique type of antibody, different from the types produced by other cells in the same individual. Therefore, the population of antibodies found in the serum of immunized animals is a very heterogeneous mixture of molecules (at times termed *polyclonal antibodies*), which varies from one animal to the other and also from one bleeding of the same animal to the next. Despite this heterogeneity of actual epitope specificity, it is possible to obtain antibody preparations from serum that are monospecific in the sense that they react only with epitopes on one particular antigen.

The polyclonal antibodies may, when combining with appropriate amounts of an antigen carrying at least two epitopes, form very large insoluble networks. One antibody molecule will recognize identical epitopes on two separate antigen molecules, leaving the rest of the epitopes free to react with one recognition site on other antibody molecules, which may combine with yet other antigen molecules by means of the residual recognition site. This possibility is employed in several of the analytical techniques described in following sections of this paper.

By special techniques, individual antibody-producing cells from immunized animals can be proliferated in tissue culture. It is thus possible to obtain preparations of antibodies possessing exactly the same specificity, the so-called *monoclonal antibodies*.

In contrast to the polyclonal antibodies, monoclonal antibodies from one clone can only precipitate the corresponding antigen if this antigen possesses at least two copies of the particular epitope that is recognized by the monoclonal antibodies. If only one copy is found per antigen molecule, only soluble complexes consisting of one antibody molecule combining with identical epitopes on two separate antigen molecules can be formed. This is mostly the case with protein antigens, but exceptions may be found, for instance when a protein is composed of identical subunits. Also antigens containing sugar residues such as glycoproteins or pure carbohydrates may possess several copies of identical carbohydrate epitopes. These antigen types may thus be able to form large insoluble complexes, composed of several antibody and antigen molecules, with monoclonal as well as polyclonal antibodies. But apart from such exceptions, monoclonal antibodies from one clone alone cannot be used in immunochemical techniques based on formation of large antigen-antibody complexes. They are, however, well suited for other analytical techniques, where detection of antibody-antigen recognition is based on other means. Such methods include the immunofluorescence method and the enzyme-linked immunosorbent assay (ELISA), which will be described in following sections. Because of the very well-defined structural specificity of monoclonal antibodies, they may be used in studies of the configuration of native or denatured antigens.

METHODS

Production of Antibodies

Polyclonal antibodies are obtained from the serum of laboratory animals after they have been injected with a small amount of purified immunogen (or a mixture of immunogens). The amount of a particular immunogen necessary for production of antibodies may vary—antibodies to purified immunogens have been obtained with as little as 20–100 μg of purified sample (Vaitukaitis 1981) or, in special cases, with samples of only 500 ng (Krøll 1981). Certain substances that stimulate the immune response of the animal, the so-called *adjuvants*, may be included in the preparation used for immunization. Mineral oils are widely used as adjuvants, either alone (Freunds incomplete adjuvant) or together with dried, heat-killed bacteria (Freunds complete adjuvant) (Herbert 1978). The specific layout of an immunization schedule as concerns dosage, period(s) between immunization and bleeding, adjuvant, and other factors may vary widely. Examples may be found in the literature on the use of antibodies, or the general procedures suggested by Harboe and Ingild (1973), for instance, or Herbert (1978) may be followed. The major part of the immunoglobulins can be separated from other serum proteins by precipitation with ammonium sulfate (Harboe and Ingild 1973), or by affinity chromatography according to the directions of the manufacturer on matrices containing protein A from *Staphylococcus aureus*, which specifically binds certain immunoglobulin classes (Kronvall et al 1970).

If facilities for antibody production are not available, immunization of animals and the subsequent bleedings may be performed on a contract basis by companies that prepare antisera to immunogens supplied by the customer.

Monoclonal antibodies are obtained by fusing myeloma cells with normal spleen cells from an animal immunized with the immunogen of interest to form hybridomas (Köhler and Milstein 1975). Individual hybridoma cells can be propagated in tissue cultures, and antibodies that are all derived from one particular spleen cell and thus all alike can be obtained from the spent culture medium (Shulman et al 1978, Kearney et al 1979).

General protocols for production of monoclonal antibodies have been published (Galfré and Milstein 1981), as well as books dealing with various aspects of the hybridoma techniques (Kennett et al 1980, Goding 1983, Langone and Van Vunakis 1986). Production of monoclonal antibodies requires more specialized equipment than production of polyclonal antibodies, but contract production of specific, monoclonal antibodies is now also offered by various companies.

Precipitation-in-Gel Techniques

The ability of antibodies and antigens to form large, insoluble precipitates in agarose gels has been employed in a number of different ways. Antigens and antibodies may simply be allowed to diffuse towards one another in gels, giving rise to precipitate lines in the merging zones ("double diffusion in two dimensions," Ouchterlony 1949), or the antigens may be separated by electrophoresis prior to diffusion towards the antibodies ("electroimmunodiffusion," Grabar and Williams 1953). Alternatively, only one of the reactants may diffuse into a gel containing an even concentration of the other reactant, leading to circular precipitates ("single radial immunodiffusion," Mancini et al 1965). If the latter technique is combined with electrophoresis of the proteins in one dimension ("rocket immunoelectrophoresis," Laurell 1966) or two dimensions ("crossed immunoelectrophoresis," Ressler 1960, Laurell 1965), rocket- or arc-shaped precipitates result. The precipitates are often directly visible as white lines in the greyish gel, or they may be visible after application of a protein stain. Many enzymes retain their activity after complex formation with antibodies and may be localized in gels by incubation with appropriate substrates (Hejgaard 1976).

These basic techniques have been very widely applied since their introduction, and a multitude of variations has been developed (Axelsen et al 1973; Axelsen 1975, 1983).

In a special precipitation-in-gel technique, immunoabsorption, antibodies against one antigen are applied to the sample well before applying the sample itself. By complex formation, the corresponding antigen is retained close to the site of application, whereas other antigens in the sample are not, facilitating study of the antigens that diffuse into the gel (Daussant and MacGregor 1979b). A similar approach may be used in combination with isoelectric focusing (Daussant and MacGregor 1979a).

Nephelometry

Nephelometric methods are based on measurement of the light-scattering ability of soluble complexes formed between antigens and antibodies in solution. The technique was introduced quite early (Libby 1938a, 1938b) and has been of value in diagnosing certain diseases, but seems not to be widely used outside the field of medicine. Within the scope of this review, however, it has been used for quantitation of hordeins in barley samples (Festenstein and Hay 1982, Festenstein et al 1984). The method requires the use of special nephelometers, often employing a laser beam as the light source (Höffken and Schmidt 1981).

Radioactive Immunoassay and ELISA

In the techniques known as radioactive immunoassay (RIA) and ELISA, antibodies labeled with either a radioactive isotope (Miles and Hales 1968 a,b) or an enzyme (Engvall and Perlmann 1972) are used to detect the relevant antigens. Alternatively, standard antigens may be labeled either radioactively (Yalow and Berson 1960) or enzymatically (Engvall and Perlmann 1971, Engvall et al 1971, van Weemen and Schuurs 1971). Apart from the nature of the labeling substance, the principles of the two methods are quite similar. A large number of variants of each method has been described (reviewed by Ekins 1974, Engvall and Pesce 1978, Hunter 1978, Voller et al 1979, Blake and Gould 1984), and only one commonly used ELISA technique will be described here.

The so-called "double antibody sandwich technique," a noncompetitive type of ELISA, involves four principal steps, as outlined in Figure 1. In the first step, unlabeled antibodies specific for the antigen of interest are attached to a solid surface, such as the inside walls of polystyrene cuvettes. Unbound substances are removed by washing before the next step, where solutions to be analyzed for their antigen contents are incubated. In the second step, the antigens combine with the immobilized antibodies, and after another washing to remove other substances, a solution of conjugate (i.e., a preparation of enzyme-labeled antibodies) is incubated. In this procedure, the antibodies used to prepare the conjugate are identical with the antibodies used in the first step. The antibody part of the conjugate thus recognizes the antigens in the complexes already formed, and this reagent is therefore also retained in step 3. Excess conjugate is washed away after incubation, and a colorless substrate, which can be converted to a colored product by the enzyme part of the conjugate, is finally incubated in step 4. The intensity of the developed color can be read in a spectrophotometer and is directly proportional to the amount of antigen in the samples.

Nonfluorescent substrates, which are converted to fluorescent products, are available for some enzymes and can additionally increase the sensitivity. Examples of such substrates are 4-methylumbelliferyl-β-galactoside and 4-methylumbelliferylphosphate. These are degraded by β-galactosidase and phosphatase, respectively, yielding free fluorescent methylumbelliferol as the product.

A faster and more sensitive variant of the original ELISA technique was introduced by Guesdon et al (1979). In this method, the antibodies are not directly labeled with enzyme. Instead, antibodies as well as enzymes are labeled with the low-molecular-weight molecule biotin. Avidin, a protein from egg white (Green 1963, 1964), and streptavidin, a protein secreted by *Streptomyces avidinii* (Chaiet and Wolf 1964), both possess four high-affinity binding sites for biotin. Avidin or streptavidin can therefore be inserted as a bridge between biotin-labeled antibodies and enzyme. As a very large number of biotin molecules can be attached to each antibody and enzyme molecule (Guesdon et al 1979, Habeeb 1966), a very high ratio of labeling enzymes to antibody molecules can be obtained. This is in contrast to the original ELISA technique, where conjugates containing only one or a few enzyme molecules per antibody molecule are normally produced. This variant is sometimes tentatively termed BRAB-ELISA (Guesdon et al 1979) or EBStrALISA (Vaag 1985b), depending on whether avidin or streptavidin functions as the bridging molecule.

One way of performing the assay is outlined in Figure 2. Apart from the greater number of steps in the EBStrALISA procedure, the actual performance of this method is similar to the way in which an ELISA is done, with sequential incubation of unlabeled antibodies, antigen samples, biotinylated antibodies, avidin/streptavidin, biotinylated enzymes, and substrate.

Immunofluorescence and Related Techniques

Immunofluorescence (Coons et al 1942) denotes a technique in which antigens are localized by means of antibodies labeled with a fluorescent molecule such as rhodamine or fluoresceinisothiocyanate (FITC) (Fig. 3). The antigens are first fixed in the tissue, for instance by means of cross-linking the proteins with paraformaldehyde or glutaraldehyde. For each antigen, a fixation procedure that does not destroy the immunological reactivity of the antigen must be established (Wachsmut 1976, Knox and Clarke 1978). The labeled antibodies are applied and react with the immobilized antigens (Fig. 3, step 2). Excess antibodies are removed by washing, and the antibody-antigen complexes are

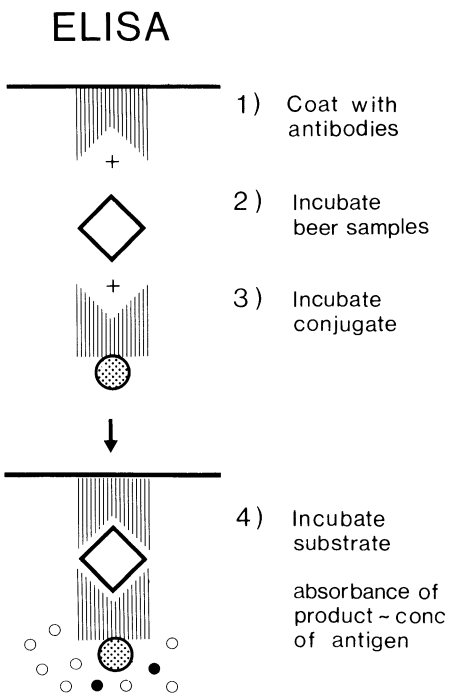

Fig. 1. Performance of an enzyme-linked immunosorbent assay (ELISA) as described in the text. (Reprinted with permission from Vaag 1985b.)

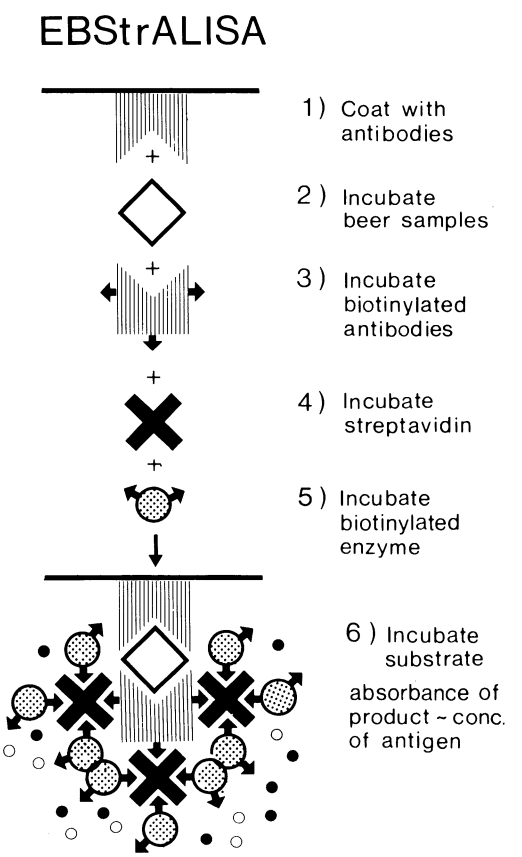

Fig. 2. Performance of an enzyme biotin streptavidin linked immunosorbent assay (EBStrALISA) as described in the text. (Reprinted with permission from Vaag 1985b.)

finally visualized in a fluorescence microscope by excitation of the fluorochrome coupled to the antibody (Fig. 3, step 3).

Alternatively, unlabeled antibodies raised in one animal species are first reacted with the antigens, after which these antibodies react with fluorochrome-labeled, species-specific antibodies raised in another animal species. This indirect technique is at times referred to as the double antibody technique.

Descriptions of the basic methodology of immunofluorescence can be found in various reviews (DeLuca 1982). As in ELISA, the biotin/avidin system has been used to enhance sensitivity (Heggeness and Ash 1977). It should also be noted that enzyme-labeled antibodies have been used to localize antigens fixed in tissue by methods similar to immunofluorescence (Avrameas 1970), in some descriptions combined with biotin and avidin to enhance sensitivity (Guesdon et al 1979, Hsu et al 1981).

Immuneblot

The immuneblot technique (Towbin et al 1979) combines electrophoretic separation of protein mixtures with identification of the antigens by procedures derived from ELISA, RIA, and immunofluorescence (Fig. 4).

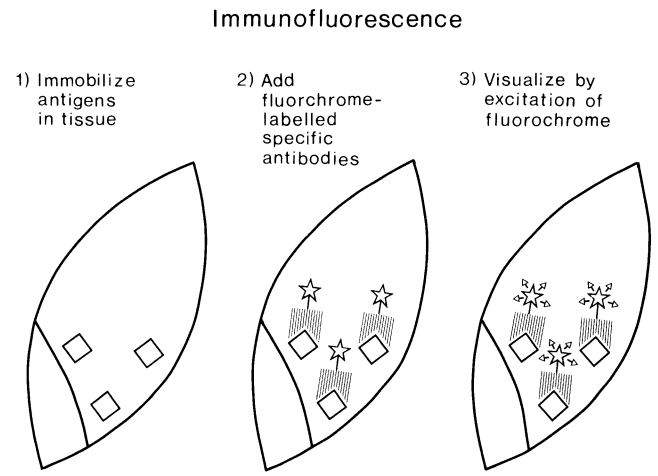

Fig. 3. Direct immunofluorescence localization of antigens in tissues as described in the text.

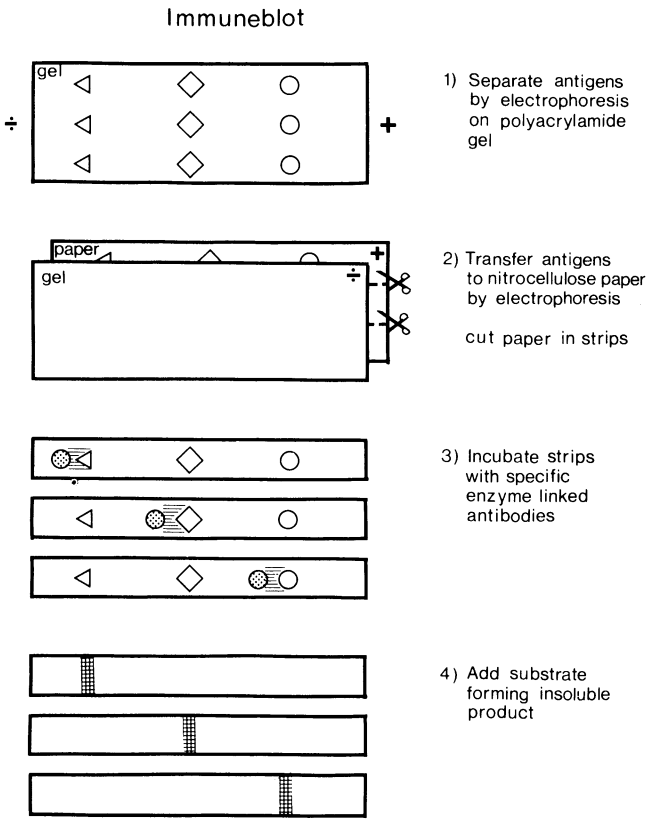

Fig. 4. Performance of the immuneblotting procedure described in the text.

After an initial separation of the proteins in one dimension by electrophoresis in polyacrylamide gels (Fig. 4, step 1), the protein bands are transferred to sheets of nitrocellulose paper by electrophoresis in a direction perpendicular to the surface of the gel (Fig. 4, step 2). The paper can then be cut into strips, which are either stained for total proteins with a protein stain or incubated with specific antibodies for the substance(s) of interest. The antibodies can either be directly labeled with an enzyme (Fig. 4, step 3), which is then detected by incubation with a substrate forming an insoluble, colored product (Fig. 4, step 4), or unlabeled antibodies are first reacted with a second, enzyme-labeled, species-specific antibody preparation, after which substrate is added.

Alternatively, the antibodies can be marked with a radioactive isotope or a fluorescent molecule. Use of avidin and biotin as a bridge between antibody and marker can also enhance the sensitivity of this method (Ogata et al 1983).

Immunoprecipitation

Immunoprecipitation techniques were originally employed to isolate polysomes involved in synthesizing specific proteins from total polysome preparations (Palmiter et al 1972). The various procedures are all based on the ability of nascent peptides, still attached to the polysomes, to be recognized and bound by antibodies directed against the native protein (Palacios et al 1972). The soluble antibody-nascent peptide-polysome complexes can then be precipitated, either by sequential addition of purified antigen and extra antibodies, allowing large insoluble complexes to be formed (Palacios et al 1972, Palmiter et al 1972), by addition of large cross-linked complexes of purified antigen (Palacios et al 1973), or by addition of a second antibody, directed against the first, specific antibody (Shapiro et al 1974). In each case, the precipitates are collected by centrifugation over sucrose gradients. Alternatively, a cell wall protein from *Staphylococcus aureus*, protein A, can be applied for the selective removal of the immune complexes. Protein A possesses binding sites for various classes of immunoglobulins (Kronvall et al 1970). Inactivated, formaldehyde-fixed cells of *S. aureus* may be used directly as an adsorbant (Mueller-Lantzsch and Fan 1976, Gough and Adams 1978). The cells with the attached polysomes are then easily collected by centrifugation. In another technique, the immunocomplexes are adsorbed to columns of protein A coupled to Sepharose (Shapiro and Young 1981).

Individual proteins or peptides, for example, synthesized in vivo or translated in vitro from isolated mRNA, have also been selectively removed from protein mixtures by similar approaches. The peptides are reacted with specific antibodies, and the resulting precipitates are either collected directly by centrifugation over a

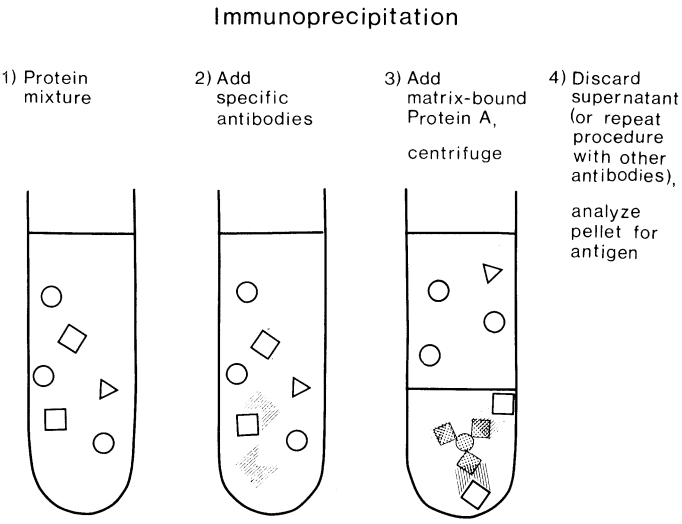

Fig. 5. Performance of immunoprecipitation as described in the text.

sucrose gradient (Rhoads et al 1973) or after addition of a second antibody (Higgins et al 1976). Protein A has also been used, either as inactived, fixed *S. aureus* cells (Kessler 1975) or by adding small aliquots of protein A-Sepharose (Jonassen et al 1981). The latter technique is sketched in Figure 5.

EXAMPLES OF IMMUNOTECHNIQUES APPLIED IN STUDIES OF BARLEY, MALT, AND BEER

Proteins in Mature Barley and Their Synthesis During Grain Filling

Various barley proteins of either nutritional or technological importance have been investigated.

As lysine is the first limiting amino acid in barley, it is of interest in plant breeding to be able to identify high-lysine varieties in order to improve the nutritional quality of barley. One group of interesting proteins is the lysine-rich albumins. Four albumins in barley have been found to be present in increased amounts in the high-protein, high-lysine barley variety Hiproly (reviewed by Hejgaard and Boisen 1980), and as these albumins were found to contain quite large amounts of lysine (5.0–11.5% lysine in total protein), they have been studied quite intensely and to a large extent with immunochemical methods. Of these four proteins, one was originally purified from Hiproly barley and termed SP-II (Jonassen 1980a). Because of the high lysine content of this protein (11.5% lysine), a screening procedure was established based on single radial immunodiffusion (Jonassen 1980b). This method has been used in studies of the inheritance of the Hiproly trait (Jonassen and Munck 1981, Jonassen 1982) and is at present used in a plant breeding program for high-lysine barley mutants executed at Carlsberg. Protein SP-II was later found to be identical to a chymotrypsin inhibitor (Boisen et al 1981) and thus renamed CI-2 (Jonassen and Svendsen 1982). Recently, an immunofluorescence method for detection of specific proteins such as CI-2 was developed (Rasmussen 1985). By this method, synthesis of equal amounts of CI-2 during grain filling was demonstrated to require about 12 days less in Hiproly than in its low-lysine sister line. The immunofluorescence technique has been modified to half kernels as well (allowing the germ part to be kept for sowing) and is being investigated as a possible substitute for the more laborious immunodiffusion technique as a screening method for specific proteins in cereal breeding programs (Fig. 6). Monoclonal antibodies towards CI-2 have been produced and are at present under investigation.

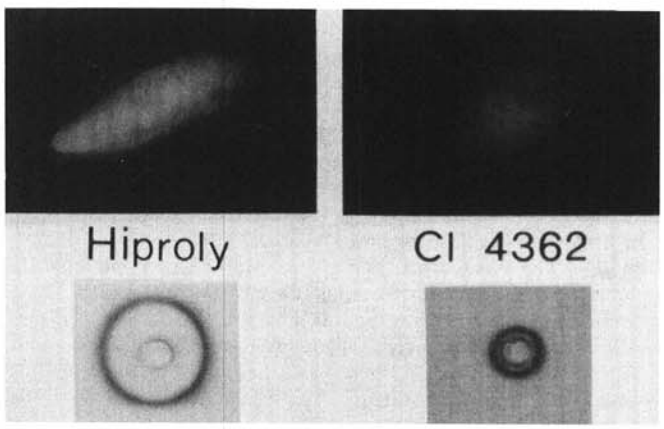

Fig. 6. Evaluation of content of CI-2 in kernels of the barley varieties Hiproly and CI 4362 by a direct immunofluorescence method and by single radial immunodiffusion. Individual kernels were sectioned in halves. One half of each was fixed with paraformaldehyde and reacted with FITC-labeled antibodies directed against CI-2. The intensity of the fluorescence is proportional to the level of CI-2 in the kernels. The proteins in the residual half were extracted and allowed to diffuse into an agarose gel containing anti-CI-2, thus forming circular precipitates. The areas of the circles are proportional to the level of CI-2 in the samples. (Reprinted with permission from Rasmussen 1985.)

Another of the lysine-rich albumins is also a chymotrypsin inhibitor, termed CI-1. It has been shown to be immunologically distinct from CI-2 (Boisen et al 1981), although a quite high degree of amino acid sequence homology was also determined (Svendsen et al 1982).

The other two major lysine-rich albumins in Hiproly barley are β-amylase and the so-called protein Z (Hejgaard 1982). By crossed immunoelectrophoresis, β-amylase was demonstrated to exist in barley either in a "free" state or as a "latent" or "bound" form, which is aggregated with protein Z and can be extracted by including mercaptoethanol or the proteolytic enzyme papain in extraction buffers (Hejgaard 1976, Hejgaard and Carlsen 1977).

One study traced the synthesis of β-amylase during ripening of barley grain (Laurière et al 1985). Enzymatic activity was estimated by diffusion in amylase-substrate gels after immunoabsorption of α-amylase, and the quantity of β-amylase on a protein basis was measured by means of rocket immunoelectrophoresis. Free β-amylase could be detected as soon as seven days post anthesis, whereas the latent form was not measurable until about ten days later. Two winter barley varieties generally had a higher content of total β-amylase (especially of the free form) than three spring varieties.

Recently, four other major basic albumins from barley have been purified and partly characterized. Various precipitation-in-gel techniques were used throughout the purification procedures to follow the four proteins (Hejgaard and Bjørn 1985). One of these is, like the four proteins just described, relatively rich in lysine (7.9 mol%). However, the detailed composition and biological activity of these proteins is unknown at present.

A recently described inhibitor of barley α-amylase II and bacterial subtilisin (Mundy et al 1983; Weselake et al 1983a,b) also belongs to the albumins. The synthesis of this protein, BASI (barley amylase/subtilisin inhibitor), in vivo during grain filling was studied by means of the ELISA technique (Munck et al 1985). The logarithmic phase of BASI synthesis occurred rather late, and the synthesis was terminated about 30 days after pollination. The level of BASI then remained constant until harvest (Fig. 7). In another study, a linear increase in the content of BASI was found from approximately day 15 until harvest at day 38 after pollination, where radial immunodiffusion was used to quantify BASI levels (Laurière et al 1985). Because of the inhibitory activity of BASI on α-amylase II, the existence of possible relations between the level of BASI and pregermination in the field or dormancy was also investigated with the ELISA method by Munck et al (1985). However, such relations could not be established, either for barley varieties as a whole or for populations of individual kernels.

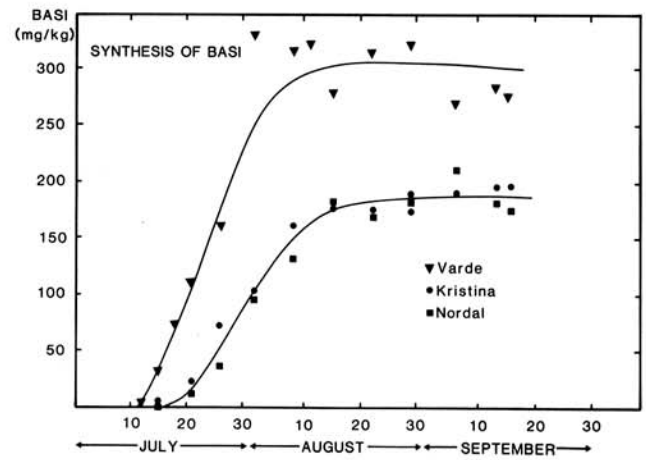

Fig. 7. Synthesis of barley amylase/subtilisin inhibitor (BASI) during grain filling. An ELISA method was used for determining the content of BASI during grain filling in three varieties, one (Varde) with a high level of BASI at harvest and two with intermediate levels (Kristina and Nordal). The Kristina variety tends to germinate in the field prior to harvest, in opposition to Nordal and Varde. (Reprinted with permission from Munck et al 1985.)

Other aspects of BASI are discussed in the sections on localization of enzyme synthesis during germination, hormonal control of protein synthesis, and impact of brewing processes on proteins.

Immunoprecipitation has been used to investigate the synthesis of various albumins. In one study, polysomes were extracted from developing endosperm and translated in vitro with a wheat germ translation system (Jonassen et al 1981). By sequential immunoprecipitation with anti-CI-2, anti-β-amylase, anti-Z, and anti-CI-1, the rough endoplasmic reticulum of the endosperm was found to be the origin of the synthesis of CI-1, CI-2, and β-amylase, but no translated protein Z from rough endoplasmic reticulum could be demonstrated in this study. Protein Z was immunoprecipitated in the same way as CI-1, CI-2, and β-amylase in a recent study, where poly(A) mRNA from developing endosperm was translated in vitro with a rabbit reticulocyte translation system (Mundy et al 1986). In the same study, developing starchy endosperm was demonstrated to contain mRNA encoding for BASI and an inhibitor of animal cell-free protein synthesis inhibitor, termed PSI. mRNA from developing aleurone also encoded for β-amylase, protein Z, CI-1, CI-2, and a probable amylase/protease inhibitor, PAPI, but not for BASI and PSI. When mRNA from organ-cultured, mature aleurone layers was translated in vitro, only BASI, CI-1, and PAPI could be immunoprecipitated.

Concerning experiments involving in vitro translations of mRNA, it should be noted that some dissimilarities in translation patterns have been observed between peptides translated from the same preparation of barley aleurone poly(A) mRNA with both the wheat germ and rabbit reticulocyte systems (Mozer 1980a). A closer comparison of the two translation systems might be of use in evaluating results obtained by in vitro translation methods.

The main storage proteins in barley, the alcohol-soluble hordeins, have also to some extent been studied immunologically. The hordeins are in general low in lysine and contribute to the low lysine level of barley. Based on their molecular weight and relative content of sulphur, they can be classified as B- (S-rich-), C- (S-poor-), or D- (high-molecular-weight-) hordeins. A low-molecular-weight prolamin group is also present in barley. The "A"-hordeins, also of low molecular weight, differ somewhat from the true prolamins regarding solubility and amino acid composition, and they are now mostly referred to as CM-proteins due to their solubility in chloroform/methanol mixtures (Shewry and Miflin 1985, Paz-Ares et al 1983a). Hordeins migrate slowly in electrophoresis at acidic pH and, based on this, are at times classified as α-, β-, γ-, δ-, or ε-hordeins by decreasing mobility (Laurière and Mossé 1982). A possible impact of B-hordeins on the filterability of wort and on haze and foam properties of finished beer has been suggested (Baxter 1980, 1981), as has a correlation with malting quality (Baxter and Wainwright 1979); however, this last assumption was not verified in other studies (Shewry et al 1980a).

The low solubility of hordeins in aqueous buffers and their poor antigenicity (Daussant 1977) gives rise to technical difficulties both in the production of antibodies and in the performance of various immunochemical techniques. However, immunological methods have been employed in hordein research, mainly in studies of the relationships between different hordein groups and between hordeins and prolamins from other cereals.

Double diffusion in agarose gels demonstrated that B-hordeins share some antigenic determinants with both A-hordeins (CM-proteins) and C-hordeins, but furthermore contain extra specific determinants not present in either A- or C-hordeins (Festenstein and Hay 1982). Attempts have been made to establish radioimmunoassays for A- and total hordeins, but the assays are of poor sensitivity. However, a quantitative nephelometric assay has been developed (Festenstein and Hay 1982) and applied in another study of the relations between hordeins and other prolamins. In this work (Festenstein et al 1984), antibodies raised against C-hordeins cross-reacted with other S-poor prolamins from wheat and thus confirmed previously established homology between these proteins (Shewry et al 1980b). S-rich wheat gliadins and rye secalins of the γ-type, known to possess some degree of homology in their N-terminus amino acid sequences, but differing from the N-terminus of C-hordein (Shewry and Miflin 1985) also reacted with the antibodies towards C-hordein, thus suggesting homology not yet established by other means. When tested against prolamins from maize, millet, and sorghum, no reaction with anti-C-hordein was detected.

Correspondingly, antibodies raised against total hordeins reacted with four unclassified wheat gliadins but with no maize zeins when investigated with the immuneblotting technique (Dierks-Ventling and Cozens 1982). However, antibodies raised against zeins did react with several hordeins and two gliadins.

The δ- and ε-hordeins, which seem to constitute mainly hordeins otherwise classified as B-, but also to a minor extent C- and D-hordeins, have been studied with various immunodiffusion techniques (Laurière et al 1983). Based on the formation of two distinct types of precipitates, it was suggested that these hordeins consisted of minor subunits.

In accordance with the findings for the "true" prolamins, immunological relationships between CM-proteins from barley and wheat and between individual CM-proteins have been demonstrated. Double diffusion tests and immunoprecipitation of in vivo synthesized, ^{35}S-labeled barley and wheat endosperm proteins were used to demonstrate immunological identity or partial identity of various CM-proteins from barley and wheat, and also relations to rye CM-proteins (Paz-Ares et al 1983b). Immunoprecipitation was also used in studies of the in vivo and in vitro synthesis of barley CM-proteins. In vivo, the synthesis was maximal between 15 and 20 days after anthesis, and a slight asynchrony between individual CM-peptides could be observed. CM-proteins were reproducibly precipitated from the soluble fraction of the endosperm cells, irrespective of the homogenization and fractionation procedures employed, although CM-proteins could only be precipitated from the in vitro translation products of membrane-bound polysomes and not from free polysomes (Paz-Ares et al 1983a).

Localization of the Sites of Enzyme Synthesis During Germination

Hydrolytic enzymes synthesized during germination of barley are of great interest to both the plant physiologist and the maltster. The excised barley aleurone and embryo are ideal systems for studies of enzyme regulation and synthesis effected by plant hormones of both theoretical and practical significance. Much research has therefore centered on the synthesis and regulation of these hydrolases, especially on α-amylase, but also to some extent on β-glucanases degrading cell walls and on peptidases/proteases attacking stored proteins.

Two isozyme groups of α-amylase are found in barley. The major form, α-amylase II, has a pI of about 5.9–6.1 and is only synthesized during germination, while another form, α-amylase I, or the "green" α-amylase, has a pI around 4.6–5.2 and may also be synthesized during development of the seeds (MacGregor and Ballance 1980). The two isozymes are immunologically distinct (Bøg-Hansen and Daussant 1974), and α-amylase I constitutes only about 2% of the total α-amylase activity synthesized during germination (MacGregor and Daussant 1981). A third form, "α-amylase III," was recently demonstrated to be a complex between α-amylase II and an endogenous inhibitor of the enzyme, BASI, mentioned in the previous section. This inhibitor also possesses an inhibitory site for the bacterial protease subtilisin but does not bind α-amylase I (Mundy et al 1983; Weselake et al 1983a,b). α-Amylase I and II are identical to the α-amylases termed A and B by other workers (Jacobsen and Higgins 1982).

For the last 100 years, whether the scutellum or aleurone layer is the prime synthesis site for α-amylase synthesized de novo during germination has been intensely discussed. Reviews of this debate can be found elsewhere (Gibbons and Nielsen 1983, Palmer 1982). Immunochemical approaches to this problem have also been employed in order to provide new arguments.

In one study, α-amylase II synthesized during germination was localized by immunofluorescence on thin sections of grains (Gibbons 1979, 1980, 1981). This demonstrated (Fig. 8), that

during the first three days of germination, α-amylase II seemed to spread out from the scutellum into the endosperm in a broad, symmetrical front. The immunofluorescent staining for α-amylase II was most intense in the loose cell layer forming the interface between the scutellum and the endosperm, the so-called crushed cell layer. Only after the first three days could α-amylase II be visualized in the aleurone and in the endosperm situated below this tissue. Based on these findings, it was suggested that as much as 50% of the total α-amylase activity in barley malt originated from the scutellum.

In another study, germinating barley (1–4 days) was dissected into various tissue fractions, and the amount of α-amylase I and II in each fraction was evaluated by rocket immunoelectrophoresis (MacGregor et al 1984). Only very small quantities of either enzyme were found in the scutellum at any time, whereas aleurone contained considerable amounts at days 3 and 4. However, endosperm fractions located adjacent to either scutellum or aleurone contained roughly equal and quite large amounts of both α-I and α-II at days 3 and 4, indicating a larger secretion to the endosperm from scutellum than from aleurone.

However, recent research on the mRNA level including immunological methods seems to indicate that the role of the scutellum in synthesis of α-amylase II may be overestimated in the previous immunological studies. Poly(A) mRNA from dissected scutellum and aleurone layers from barley germinated two, five, and eight days was translated in vitro, and the peptides recognized by antibodies towards α-amylase II were immunoprecipitated from the resulting mixtures. α-Amylase precursors were found among the translation products of scutellar mRNA but in substantially smaller amounts than among translation products from aleurone (Mundy et al 1985). The latter study also included immunoprecipitation of β-glucanases and a carboxypeptidase from scutellar and aleurone mRNA. As for α-amylase, mRNA for β-glucanases was most abundant in aleurone, whereas mRNA for the carboxypeptidase was predominantly found in scutellum (embryo).

The synthesis of two immunologically cross-reacting (1→3, 1→4)-β-D-glucanases, "I" and "II" (Woodward and Fincher 1982) was also studied by immunochemical means. Immuneblots performed on extracts of whole germinated barley grains showed that the two enzymes were synthesized in approximately equimolar amounts when grains were germinated up to six days (Stuart and Fincher 1983). Very recently, immuneblots and immunoprecipitations of in vitro translations of mRNA from scutellum and aleurone indicated that β-glucanase II is dominant among the aleurone-synthesized enzymes, whereas scutellum synthesizes β-glucanase I and possibly also a third form, but not β-glucanase II (G. B. Fincher, La Trobe University, Bundoora, Australia, *personal communication*).

It thus seems verified that although the scutellum may play a minor role in production of α-amylase during germination, it is indeed capable of synthesizing other hydrolytic enzymes, which also play important roles in the beginning of the germination process. In support of this, ultrastructural studies have demonstrated that during the first three days of germination, only the scutellum contains large amounts of the cellular structures involved in protein synthesis, i.e., rough endoplasmic reticulum, mitochondria, and Golgi apparatii, whereas the aleurone structure resembles that in dormant grains (Gram 1982). Tentatively, the loose crushed-cell layer situated at the interface of scutellum and endosperm may be able to serve as a transportation organ, mixing enzymes produced by the aleurone and scutellum and allowing them rapid access to a large area of endosperm (Mundy and Munck 1985).

Hormonal Control of Protein Synthesis

In the 1960s, it was established that the plant hormone gibberellic acid (GA_3) is able to enhance the synthesis of α-amylase in barley during germination (Paleg 1960, Chrispeels and Varner 1967). The hormone abscisic acid (ABA) was proved to block the stimulating effect of GA_3 on α-amylase (Chrispeels and Varner 1966), and various recent studies deal with the hormonal regulation of this enzyme and other proteins on a molecular level.

By immunoprecipitating peptides translated in vitro from mRNA extracted from GA_3-treated or untreated aleurone layers with anti-α-amylase, GA_3 was demonstrated to increase the amount of translatable mRNA encoding for this enzyme (Higgins et al 1976). Further studies on the combined effects of GA_3 and ABA, by immunochemical (Higgins et al 1982) and other means (Muthukrishnan et al 1983, Chandler et al 1984), indicate that ABA prevents the accumulation of mRNA for α-amylase and thus works on a transcriptional level, whereas other studies point to an effect of ABA at the level of translation of the α-amylase mRNA (Mozer 1980b).

The two immunologically distinct isozyme groups of α-amylase have been demonstrated to be regulated differently by GA_3. α-Amylase I appears earlier after addition of GA_3 than the II isozyme, and less GA_3 is required to stimulate the synthesis of isozyme I than of isozyme II (Jacobsen and Higgins 1982). Later experiments using cloned α-amylase cDNA confirm the differential effect on α-amylase I and II (Rogers 1985).

Several of the studies mentioned above also demonstrated a stimulating effect of GA_3 and ABA on other, unidentified proteins (Mozer 1980b, Higgins et al 1976). One such ABA-stimulated peptide was identified by immunoprecipitation of in vivo synthesized proteins as the endogenous inhibitor of α-amylase II and subtilisin, BASI (Mundy 1984). The effect of GA_3 and ABA on the production of BASI by embryoless half kernels appears to be the opposite of the effect on α-amylase: GA_3 eliminates the synthesis of BASI, whereas ABA stimulates it. Treatment of embryoless kernels with both hormones also induces the synthesis of BASI. (Fig. 9).

In a recent study, these findings were confirmed by immunoprecipitation of the products obtained by in vitro translation of mRNA from organ-cultured aleurone layers, treated with hormones or not (Mundy et al 1986).

Recent immunochemical studies also seem to indicate an effect of GA_3 on β-glucanase II (J. Mundy, Rockefeller University, New

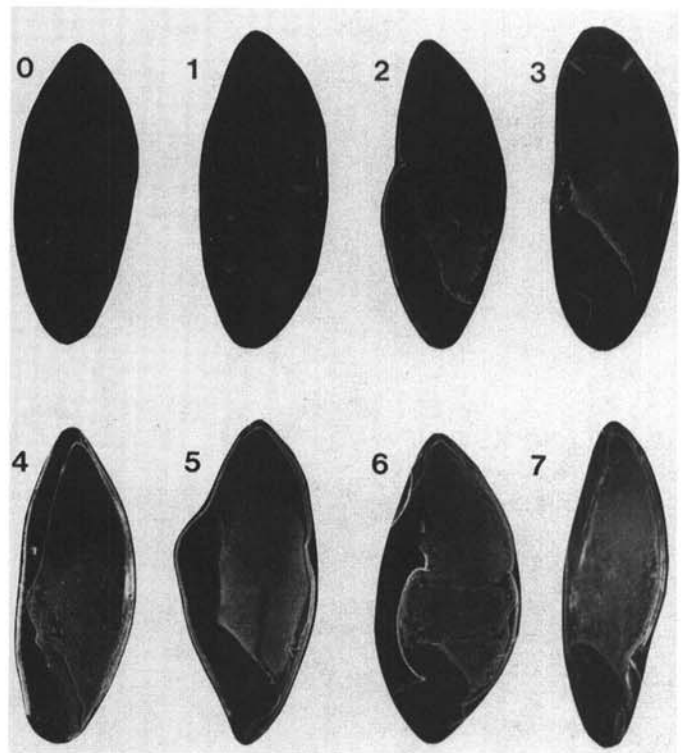

Fig. 8. Localization of α-amylase II during germination and early seedling growth by an indirect immunofluorescence technique. Antigens in the kernels were immobilized by cross-linking with formaldehyde and sections (8 μm) were prepared. The sections were first reacted with antibodies raised in rabbits against α-amylase II, then with FITC-labeled swine-antirabbit globulins. (Reprinted with permission from Gibbons et al 1981.)

York, and G. B. Fincher, La Trobe University, Bundoora, Australia, *personal communication*), thus adding hormonal regulation to the tissue specificity of the I and II isozymes.

Impact of Various Brewing Processes on Barley and Malt Proteins

The influence of steeping on the synthesis of α- and β-amylases during malting has been investigated by immunochemical methods (MacGregor and Daussant 1979). Barley kernels were either steeped for two days and then germinated for three or four days under humid conditions, or the kernels were germinated five or six days in all without prior steeping. Immunoelectrophoresis revealed large differences in the amounts of α-amylase I, II, and "III" (α-amylase II + BASI) between steeped and unsteeped kernels. Unsteeped, germinated kernels contained considerably more of all α-amylase isozymes, and in particular of the α-I isozyme, than the steeped kernels. Also, the ratio between α-II and α-"III", that is, α-II free or complexed with BASI, was influenced by the germination conditions. Steeping gave rise to a lower ratio of α-II to α-"III" compared with unsteeped samples (MacGregor and Daussant 1979). Rocket immunoelectrophoresis was used to demonstrate a similar difference in the content of β-amylases between steeped and unsteeped malts (Daussant and MacGregor 1979b).

The fate of a few other barley proteins has been followed through the various technological processes that convert barley to beer. Protein Z is in this context of particular interest because of its extreme heat stability—this protein survives all the processes of beer making relatively undamaged and was originally identified as a dominant beer antigen by crossed immunoelectrophoresis of a protein-rich beer fraction against beer antibodies (Hejgaard and Sørensen 1975, Hejgaard 1977). Subsequent studies showed that the amount of Z increases during malting. Several other unidentified components appear or disappear, whereas others are subjected to only minor changes (Hejgaard 1978). Protein Z was hypothesized to play a role in haze formation and/or foam stability (Hejgaard and Sørensen 1975), presumably complexing with proanthocyanidines to form haze particles (von Wettstein et al 1985).

The effect of malting, kilning, and mashing on the α-amylase inhibitor BASI was also investigated (Munck et al 1985). An ELISA technique used for this purpose showed that malting had only a very minor effect on the concentration of BASI, but during kilning about half of the immunological activity was destroyed. Accordingly, a decrease in content of α-amylase "III" during kilning was observed in an earlier study by other investigators (MacGregor and Daussant 1981). During mashing, the residual

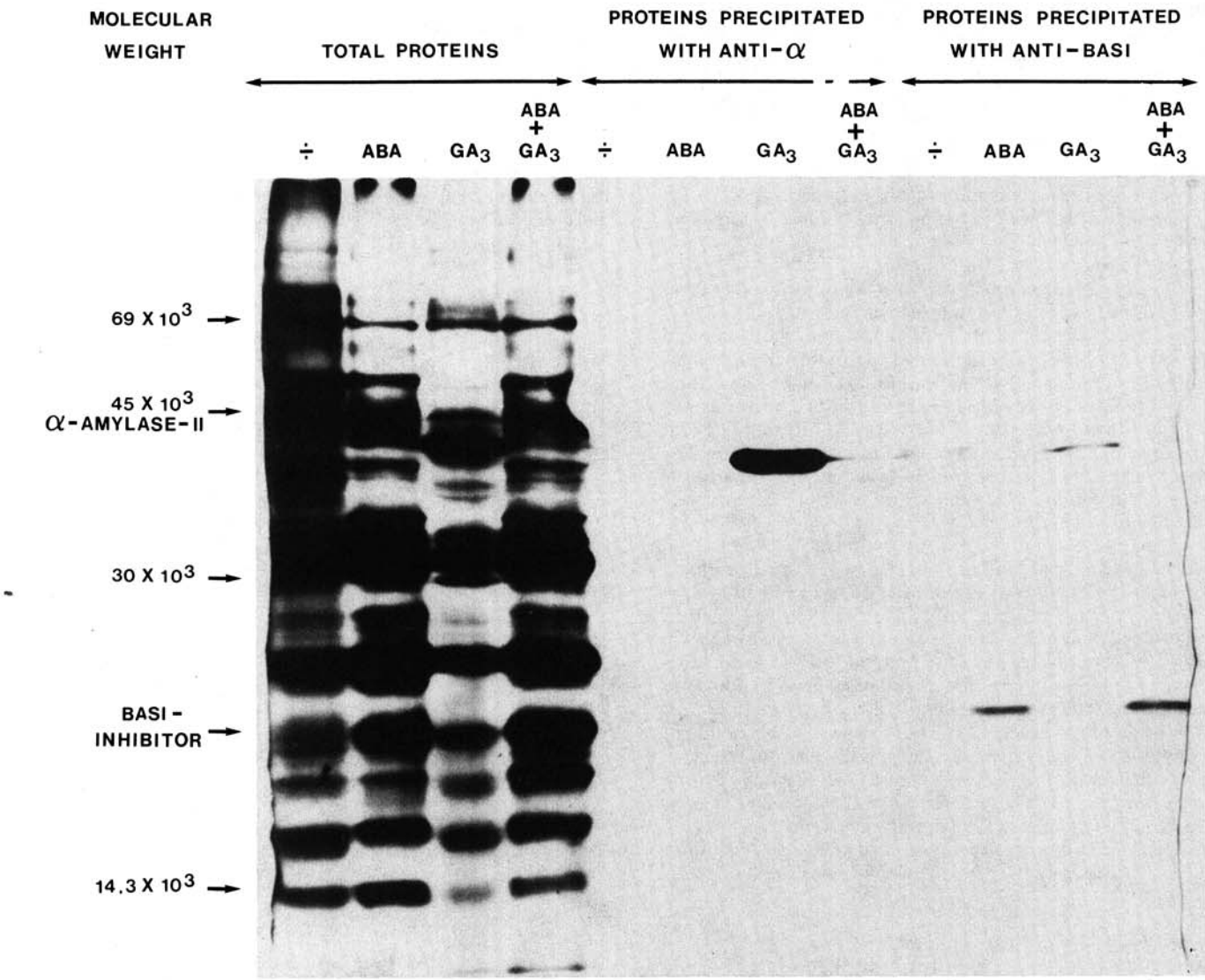

Fig. 9. Demonstration of hormonal regulation of α-amylase and barley amylase/subtilisin inhibitor (BASI). Aleurone layers of barley were organ-cultured either without hormones or in the presence of abscisic acid (**ABA**), gibberellic acid ($\mathbf{GA_3}$), or both hormones. ^{35}S-methionine was added and incorporated in the newly synthesized proteins. The total proteins (lanes 1–4) were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis followed by fluorography together with the proteins that were immunoprecipitated with antibodies against α-amylase II (lanes 5–8) or against BASI (lanes 9–12). Hormone treatment of the samples is indicated in the figure. The results are discussed in the text. (Reprinted with permission from Mundy 1984.)

amount of BASI was rapidly destroyed when the temperature in the mash tun exceeded about 55° C, and only traces were left when the temperature reached 70° C (Fig. 10). It is thus certified that common mashing procedures effectively destroy BASI and thus hinder any possible inhibition of α-amylase action on starch at this high temperature.

Other studies have focused on proteins responsible for the foaming capacity of beer. "Foaming proteins" from beer can be fractionated into three fractions with differing molecular weights. By various precipitation-in-gel techniques, these proteins were shown to react well with antibodies raised towards malt and only to a lesser extent with barley and yeast antibodies. A considerable decrease in the content of foaming proteins was observed during kettle boiling, especially in the presence of hops or humulones (Asano and Hashimoto 1980a).

Quality Control of Barley

Barley, like other cereals, is often attacked by fungi if stored under humid conditions, which may lead to production of toxic compounds. Mycotoxins are generally small molecules with molecular weights below 500, and they are thus not immunogenic per se. However, after conjugation of the toxins to a carrier protein, it is possible to raise antibodies specific against, for instance, aflatoxins (Bierman and Terplan 1980, Pestka et al 1980) and ochratoxin A (Pestka et al 1981), which can then be applied for analytical purposes. One study describes the establishment of an ELISA technique for detection of ochratoxin A in barley (Morgan et al 1983), a highly nephrotoxic secondary metabolite of fungi from the genera *Aspergillus* and *Penicillium*.

Concerning beer production, a special problem is associated with fungi of the genus *Fusarium*. Beer produced from *Fusarium*-infected barley has an excessive foam-forming capacity, which causes gushing (Haikara 1980). An ELISA method to detect *Fusarium* antigens in barley was therefore sought and established (S. B. Sørensen, Carlsberg Laboratory, Copenhagen, *personal communication*). However, the sensitivity of the assay was not sufficient to detect *Fusarium* in barley samples that had been naturally infected in the field, probably because the serum contained too low a titer of *Fusarium*-specific antibodies. If only specific antibodies to *Fusarium* antigens from gushing strains can be obtained, it should be possible to use ELISA or other immunochemical methods for this purpose.

A quite different problem encountered in evaluating barley for brewing purposes arises from the different malting behavior of typical winter and spring barley varieties. Generally, winter barley provides malts of poor quality (Narziss et al 1979), although altered malting conditions may give good malts from winter barley

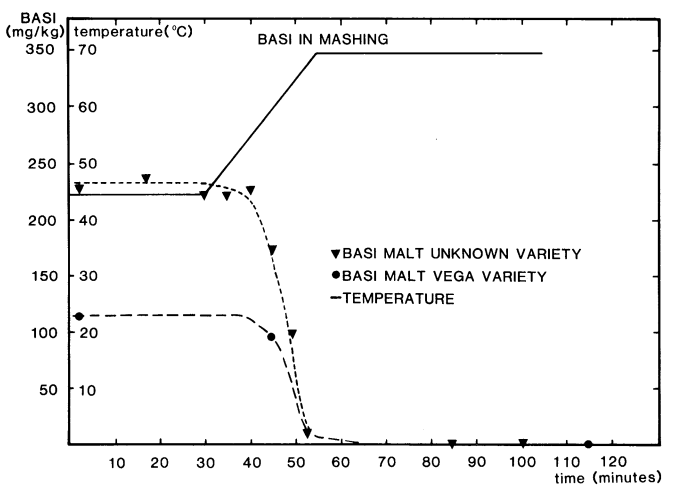

Fig. 10. Destruction of barley amylase/subtilisin inhibitor (BASI) during mashing. The destruction of BASI during pilot mashings of malts of the variety Vega and of an unknown variety was followed immunologically with an ELISA procedure. (Reprinted with permission from Munck et al 1985.)

(Hug and Pfenninger 1981). As the production of spring barley varieties is decreasing (Schildbach and Burbidge 1983, Hug and Pfenninger 1981), breweries may encounter problems in getting sufficient spring barley, or they may have to use spring barley mixed with unknown quantities of winter barley. In order to evaluate the content of winter barley in mixtures, an immunochemical test has been set up (Deichl and Donhauser 1985). An antigen that is specifically present in six winter barley varieties has been isolated, and with antibodies raised against this antigen, it has been possible to quantify the content of winter barley in mixtures by means of rocket immunoelectrophoresis. However, no description of the isolation procedure or nature of this antigen has been published. The antigen seems to be present in malted barley as well, but in a form with highly modified electrophoretic mobility. This makes evaluation by immunoelectrophoresis impossible, and an ELISA test is therefore claimed to be on its way.

Beer Proteins Originating from Yeast

Yeast proteins in beer have been studied relatively little. A minor component of the "foaming proteins" mentioned previously reacted with antibodies raised against yeast, but they were later found to be unimportant to the foaming properties of beer (Asano and Hashimoto 1980b). An antigen present in beer was demonstrated to be a yeast glycoprotein (Hejgaard and Sørensen 1975, Kaersgaard and Hejgaard 1979) but has not been further characterized.

Detection of Enzyme Additives or Cereal Adjunct in Beer

In the process of brewing, certain enzymes of nonbarley origin may be added, for instance in order to provide total conversion of starch and dextrines to glucose (amyloglucosidase from fungi) or in order to ensure the colloidal stability of the beer by degradation of high-molecular-weight proteins (plant proteases such as papain, ficin, and bromelin). Grits of maize, rice, wheat, or other cereals may also be substituted for part of the barley malt. Assays for such substances are of interest in quality control to ascertain that adequate doses have been added. Although use of these additives is common practice in most beer-producing countries, it is prohibited in a few such as Germany and Norway. Thus, there is also a legislative reason for establishing assays, in order to prove that additives have not been used.

Several radioactive immunoassays (RIA) and ELISA procedures for detection of added enzymes in beer have been described. RIAs are reported for ficin and bromelin (Donhauser 1979) and for papain (Donhauser and Biro 1984, Rauch et al 1984a,b). ELISAs have likewise been described for detection of papain (Donhauser et al 1985, Vaag 1985b, Zimova and Basarova 1985), ficin (Donhauser and Winnewisser 1985, Zimova and Basarova 1985), bromelin (Zimova and Basarova 1985) and amyloglucosidase (Vaag 1985a). The EBStrALISA variant, where biotin and streptavidin are incorporated in the original ELISA procedure, has so far only been applied for detection of papain (Vaag 1985b).

These techniques will surely supersede the various methods based on precipitation-in-gels, which have hitherto been used, because of their much higher sensitivity and speed—factors of equal importance in industrial quality control.

Some authors have suggested that the sensitivity of RIA should be superior to that of the ELISA (Ekins 1976, Watson 1976). Regarding the methods listed above for detection of enzyme additives in beer, the opposite appears to be true, although due care should always be exerted when comparing assays based on different antibody preparations and, for ELISAs, on different enzyme/substrate systems. However, in the RIAs described, the detection limits are generally on the order of 100–200 ng of protein per milliliter, whereas the ELISAs detect about 1–20 ng/ml. Moreover, one to five days are required for the RIA procedures, while the period needed for the ELISAs is typically 4–6 hr.

As regards ELISA and EBStrALISA, a direct comparison was made (Vaag 1985b), where the same preparations of antibodies and enzyme were used to develop assays of either type. Analyses of

comparable sensitivity could be performed at least three times faster with the EBStrALISA method than with the ELISA (Fig. 11).

Proteolytic enzymes are of legal importance even in trace amounts as previously described. It is, therefore, desirable that the immunological activity of these enzymes should not be destroyed by normal pasteurization procedures, whereas the actual enzymatic activity is of minor importance. Concerning papain, it has even been demonstrated that addition of enzymatically inactive papain ensures the colloidal stability of beer (Fukal and Kas 1984). In some reports, the effect of pasteurization on immunological activity of papain (Donhauser et al 1985, Vaag 1985b) and ficin (Donhauser and Winnewisser 1985) was investigated. In all cases, some destruction of the immunological activity was observed, but the destruction is far from complete at temperatures and times commonly used (Fig. 12A).

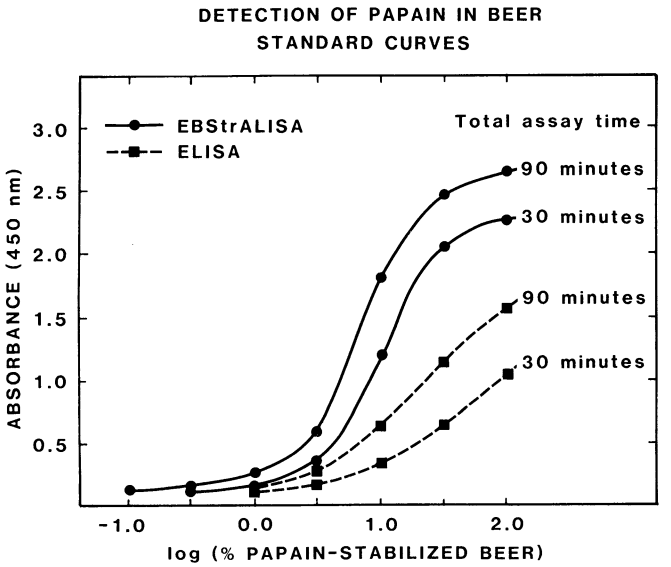

Fig. 11. Comparison of ELISA and EBStrALISA procedures for detection of papain in beer that were developed based on the same preparations of antibody and enzyme. Conditions were optimized for assays to be performed in 30 or 90 min, aiming at a blank value of 0.1 absorbance unit in all cases. (Reprinted with permission from Vaag 1985b.)

Concerning amyloglucosidase (AMG), the enzymatic activity of this enzyme is remarkably heat stable. Therefore, if beer containing even minor amounts of enzymatically active AMG is accidentally mixed with other beer types, the enzyme will degrade residual β-limit dextrins in the beer to free glucose, and an undesirable sweet taste will develop. The residual enzymatic activity after pasteurization is therefore in this case of great importance. In contrast, a precise quantification of AMG, independent of pasteurization, is relatively unimportant, as AMG is only used in production of certain low-calorie beers not normally exported. Fortunately, preliminary results (Vaag, *unpublished*) indicate that the immunologic activity of AMG is destroyed at a rate that almost equals the destruction of the enzymatic activity (Fig. 12B).

In most cases, cross-reactions between papain, ficin, and bromelin are reported, possibly reflecting the sequence homology between these plant proteases (Brocklehurst and Baines 1981). However, the cross-reactions of the reported assays are not pronounced and seem to be of no practical importance in beer control, if the immunogens for immunization are prepared with sufficient care.

Recently, an ELISA for detection of maize and rice in finished beer was published (Wagner et al 1986a). Addition of about 5% of either adjunct type to the barley malt could be detected if the adjunct cereals were not subjected to heating before mashing. In practice, however, adjuncts are often heat treated to facilitate the enzymatic degradation of starch. Further studies have shown that the detection of maize is not severely impaired by heating. Even

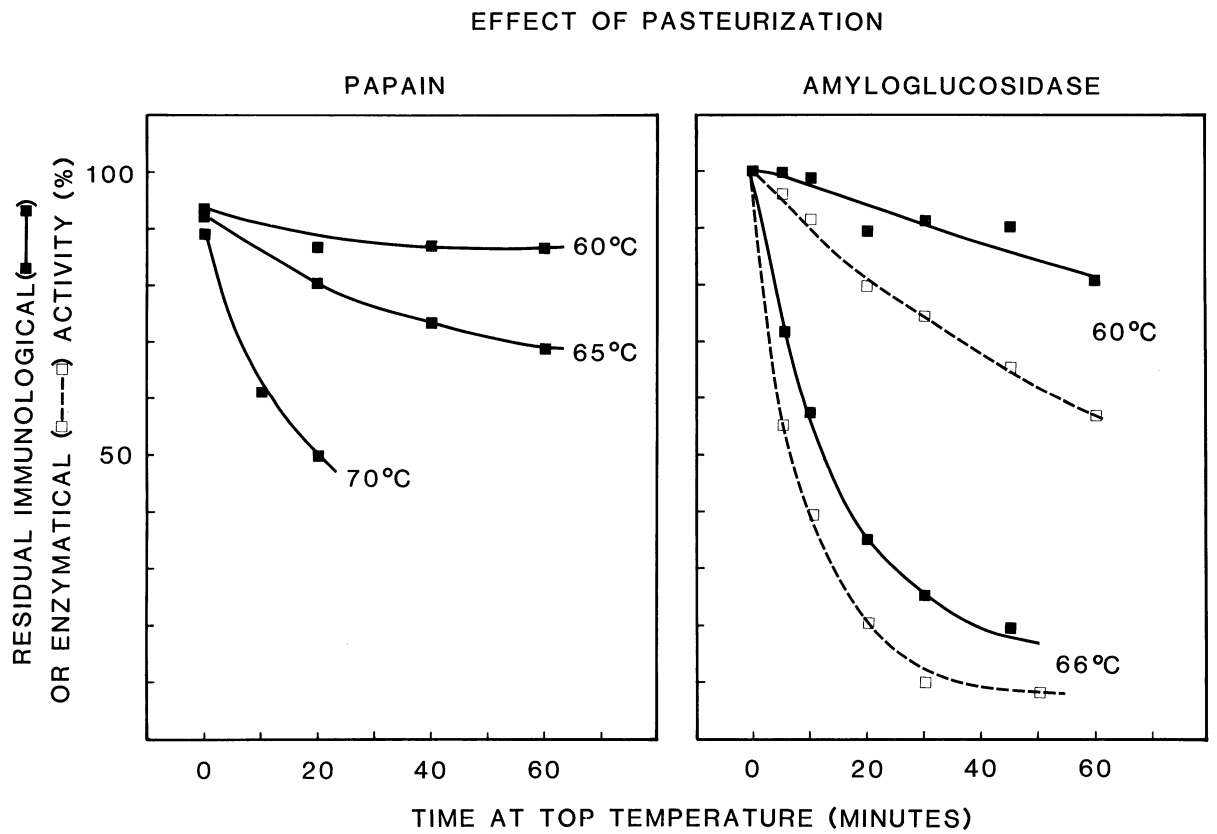

Fig. 12. Residual activity of papain and amyloglucosidase after pasteurization: ——, immunological activity; ---, enzymatic activity. Papain-stabilized beer was pasteurized in the bottles at 60, 65, or 70° C for up to 60 min, and residual immunological activity was determined with an ELISA-system. (From Vaag 1985b.) A small amount of low-calorie beer was mixed with ordinary beer and heated to 60, 63, or 66° C for up to 30 min. Determination of residual enzymatic activity was based on measurement of the release of free glucose. (Vaag *unpublished.*)

after high-pressure cooking at 120° C for 15 min, a 10% addition of maize is still detectable. The immunological detection of rice is affected somewhat more, although addition of 20% rice or more still gives a significant response in the ELISA (Wagner at al 1986b).

CONCLUSION

The examples given above from barley and barley-based technologies demonstrate the versatility of the various immunochemical procedures. They have been applied in basic research on the physiology of the barley grain as well as on the impact of technological processes such as malting and mashing. Furthermore, they have served as specific screening procedures for proteins of interest, and they have been applied in quality control of finished products. In some cases, immunochemical approaches have provided results that were unobtainable by conventional chemical procedures; in other cases they can support and be supported by other techniques. The immunochemical techniques described in this paper are also useful in basic and applied research on other cereals.

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