Immunochemical Characterization of Protein in Plant Studies

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The methodology of immunochemical methods in plant protein studies is reviewed. Applications of double diffusion, qualitative and quantitative immunoelectrophoresis, and absorption techniques are described, with special reference to the combination of these methods with those of enzymatic characterization, and with those of *in vivo* labeled amino acid incorporation into proteins. Applications of the microcomplement fixation technique are also reported. These applications are illustrated by examples concerning protein identification, isoenzymes, *in vivo* enzyme induction, and protein modification.

In the two past decades immunochemical methods have greatly accelerated the progress of investigations on animal and human proteins; they are now largely applied in medicine. For plant proteins, the specificity of immunological reactions has been recognized and used in taxonomic studies since the beginning of the century (Magnus, 1908; Wells and Osborne, 1913). More recently, after methods based on the antigen–antibody precipitation in gels were developed, the specificity and sensitivity of immunological reactions have been used in biochemical research on plant proteins. The decisive advantage of these methods [single diffusion (Oudin, 1946), double diffusion (Ouchterlony, 1949), immunoelectrophoretic analysis (I.E.A.) (Grabar and Williams, 1953), and related techniques] resides in their ability to visualize separately the reactions due to each different antigenic constituent present in a mixture.

This report will deal with studies concerning problems in plant physiology such as the identification of proteins, the nature of the molecular heterogeneity which often occurs within one enzymatic activity, the detection of *in vivo* protein synthesis, and modification of proteins. The capacity of these methods in quantitative evaluation of one constituent in a mixture will be illustrated. Since it has already been included in recent reviews (Fairbrothers, 1968; International Working Session of Phytoserologists, 1968; Moritz, 1964), the application of immunochemical methods in taxonomic investigations will not be reported here.

PRINCIPLE OF THE METHODS

The injection of one antigen (proteins are antigens) into a vertebrate induces in the serum of the animal the appearance of proteins called antibodies which will react specifically with the antigen which induced their formation. The specificity of these reactions between antigens and antibodies is related to the structures of the molecules. One antibody molecule has two identical antigen binding sites which react only with the same antigenic sites of two antigen molecules. One antigen may have several different antigenic sites. Consequently, after injection of one antigen, the antibodies occurring in the animal serum form a population of constituents which react specifically with different parts of the antigen. When a protein mixture has been injected, the immune serum of the animal contains several antibody populations specific for the different antigens injected. (Rabbits are most often used for immunization. Horses, goats, sheep, or guinea pigs also serve for production of immune serum.) The antigen–antibody reactions performed *in vitro* may occur in different forms: precipitation reaction of the antigen–antibody complexes; agglutination reaction between cells bearing the antigens and antibodies; complement fixation and hemolysis of the red cells; neutralization of virus and toxins; lysis of bacteria; and antigen opsonification, which favors phagocytosis. We shall deal here mainly with the first of these reactions, the specific antigen–antibody precipitation.

Specific Precipitation Reaction. When a protein solution and the corresponding immune serum are placed separately in a gel and allowed to diffuse, the occurrence of distinct precipitin bands in the gel reflects the presence of different antigens in the protein solution [double diffusion according to Ouchterlony (1949)]. When the proteins are first electrophoretically separated in the gel before reacting with the antibodies, the resolution of the different antigen–antibody precipitin bands is considerably increased. Moreover, proteins already characterized by their antigenic specificities can be defined by their electrophoretic mobilities [Immunoelectrophoretic analysis (I.E.A.) Grabar and Williams (1953)]. Characterization reactions of antigens in antigen–antibody precipitates may provide additional definition of the proteins from the same patterns. Characterization reactions of lipids, carbohydrates, nucleic acids, metalloproteins, and enzymes carried out on the specific precipitates in gels have been recently reviewed (Uriel, 1970).

Complementary techniques such as absorption techniques (Avrameas and Ternynck, 1967; Grabar and Burtin, 1964), serve to eliminate from an immune serum specific for one protein mixture that fraction of the antibodies which reacts with another protein preparation. Absorption consists of adding the protein preparation to the immune serum, letting it stand awhile to react, and then centrifuging. The procedure is repeated until no further reaction occurs between the treated immune serum and the protein preparation. Conversely, one can also eliminate one given protein from a protein mixture by adding the specific immune serum to the solution.

Complement Fixation. Complement is a series of serum proteins which fix onto antigen–antibody complexes. In the particular case where the antigens are red cells, complement fixation result in lysis of the cells.

The method of dosage (Levine, 1967; Wasserman and Levine, 1961) consists of two phases. First, the antigen and the corresponding decomplemented immune serum are incubated in the presence of a given amount of complement from guinea pig serum. During this period the complement fixes to the antigen–antibody complex. The amount of complement fixed is proportional to the size of the complexes. In the assay series, sizes of the complexes are maximum for

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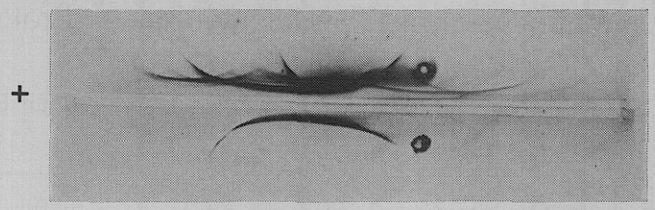

Figure 1. Immunoelectrophoretic analysis of total peanut cotyledonary extract (above) and of cryoprecipitated proteins from the extract (below). Immune serum, anticotyledonary extract

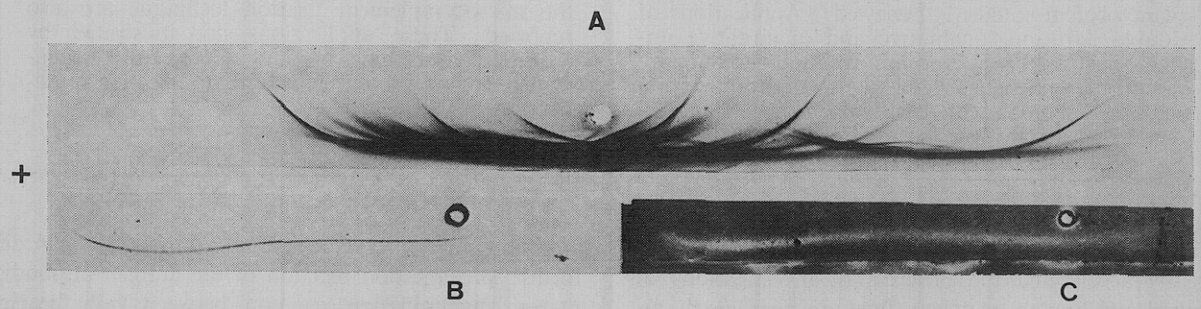

Figure 2. Immunoelectrophoretic analysis of wheat protein extracts. A: With antiwheat extract immune serum; B and C: With antiwheat β-amylase monospecific immune serum. Amidoblack staining after I.E.A.: A and B. Amylase characterization after I.E.A.: C. (The conditions for electrophoresis are identical for B and C but different for A)

a determined ratio between antigens and antibodies. This ratio is called the equivalent point. With an excess of antigen or an excess or antibodies, the size of the complex becomes smaller and less complement is fixed.

The second phase consists of evaluation of the nonfixed complement. Sheep red cells and corresponding antibodies are added to the samples, and the degree of the red cell lysis reflects the amount of free or nonfixed complement.

PROTEIN DETECTION, CHARACTERIZATION, AND IDENTIFICATION

Two types of immune serum can be used in methods of specific precipitation. The antisera can be specific for several proteins contained in extracts of the whole plants, plant organs, plant tissues, or liquids of plant origin. The immune sera are obtained from animals immunized with these complex extracts or liquids. On the other hand, antisera may be specific for one protein only. These monospecific immune sera are usually prepared by immunizing animals with a purified protein preparation. Figures 1, 2, and 3 provide examples of the use of both types of immune sera.

Use of the first reactant type is illustrated in Figure 1.

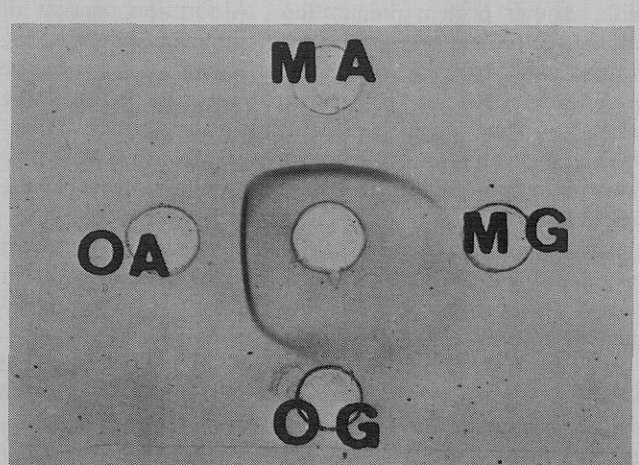

Figure 3. Double diffusion of albumin and globulin fractions of barley and barley malt extracts using antibarley β-amylase monospecific immune serum. OA: Barley albumins; OG: Barley globulins; MA: Barley malt albumins; MG: Barley malt globulins

This is the I.E.A. pattern of proteins in a peanut cotyledon extract using immune serum antitotal cotyledonary extract (Figure 1, top). These protein extracts exhibit the property of cryoprecipitation. In order to determine if this property was a feature of one or several proteins, a fraction prepared by cooling the solution (repeating the procedure twice) was submitted to the same I.E.A. (Figure 1, bottom). It was thus found that essentially only one protein displayed this property. This cryoprotein was further identified as α-arachin, a storage protein. The identification was obtained by a similar I.E.A. with a purified α-arachin. Applying this method to subcellular particle preparations from peanut cotyledons, the location of this protein in the aleurone grains was confirmed (Daussant *et al.*, 1969b; Neucere and Ory, 1970). It is noteworthy that the property of cryoprecipitation found for α-arachin was thereafter included in one procedure for purification of α-arachin (Neucere, 1969).

Figures 2 and 3 provide examples of the use of I.E.A. or double diffusion employing a monospecific immune serum. Figure 2A shows the I.E.A. of proteins in a wheat extract using an antiwheat extract immune serum. In contrast, Figure 2B represents another I.E.A. of wheat extract using monospecific antiwheat β-amylase immune serum. (Figure 2C represents an enzymatic characterization reaction for β-amylase, carried out on the pattern of Figure 2B. This verifies that the antigen detected by the monospecific immune serum bears β-amylase activity.) It is clear that use of monospecific serum makes easy the identification of β-amylase among all the other proteins present. Such monospecific serum was used, for example, on barley and wheat to detect the enzyme produced during germination of the seeds (Daussant, 1966; Daussant and Abbott, 1969) and to recognize it in different fractions obtained by exclusion chromatography from the whole extracts (Daussant *et al.*, 1966).

Figure 3 illustrates the use of a monospecific immune serum to barley β-amylase in identifying the enzyme in the albumin fraction of barley and malt extracts while demonstrating its absence from the globulin fraction of these extracts. The capacity for identification of a molecular species in different fractions among other proteins—even if the particular protein has no enzymatic activity—confers to the monospecific immune serum a particular technical interest. These methods, mainly I.E.A., are now being used on plant proteins in studies

of nomenclature, physicochemical investigations, localizations, and genetics research.

Nomenclature. Since some seed proteins, such as barley, soybean, and wheat, have been studied by great numbers of scientists, the need for a suitable reference system arose. A commission of the European Brewery Convention (1967) stated that a nomenclature for facilitating the study and comparison of the seed proteins must fulfill the following conditions. The proteins must be identified by the greatest number of criteria based on independent properties. The nomenclature must use only small quantities of test material for identification. The nomenclature must be sufficiently flexible to allow its extension and to permit the identification of one individual constituent, even while it is undergoing modifications in the course of natural phenomena such as germination, or in the course of industrial processes such as malting or brewing. I.E.A. was found best to fulfill these conditions, and a standard antibarley rabbit immune serum served in the development of this nomenclature. In the course of this development, an improvement was provided by substituting a horse antibarley and barley–malt immune serum for the standard rabbit immune serum when no more rabbit antiserum was available. This substitution presented several advantages. The horse serum detects more antigens. It is specific for both barley and barley–malt proteins. It is available in much larger amounts. In the course of development of this reference system, enzymatic activities have been detected on the precipitin bands, such as α-, β-, and other types of amylases, esterase, peroxidase, dehydrogenase, and peptidase activities.

A similar and in some ways a more complete reference system has been proposed by the Soybean Protein Nomenclature Committee sponsored by the Oilseed Division of the American Association of Cereal Chemists (Catsimpoolas, 1969). The particular feature of this proposal consists in presenting, in addition to an immune serum specific for water-soluble soybean proteins, another serum specific for globulins and two additional monospecific sera for glycinin and γ-conglycinin. The availability of these latter reactants would actually simplify the identification and the quantitation of the corresponding proteins in a protein mixture.

Physicochemical Studies. We have already shown as an example the identification of the cryoprotein in *Arachis hypogaea*. This property of cryoprecipitation is very widespread in the plant kingdom (Ghetie and Buzila, 1962). A function in regulation of germination has been suggested for these cryoproteins. Immunochemical methods served to identify the cryoprotein in pea as the main reserve protein vicillin. Physicochemical differences concerning molecular size and absorptive properties suggested that vicillin and the cryoprotein correspond to different forms of the same antigen (Buzila, 1966, 1967).

Investigations on the barley globulins defined by Quensel were carried out by combining exclusion chromatography on Sephadex gels with ultracentrifugation and I.E.A. These studies demonstrated that the α-, β-, γ-, and δ-globulins differing in their molecular sizes each contain several distinct structural constituents. Moreover, the presence in several fractions of the same antigen suggested that some of these constituents actually exist under different degrees of aggregation (Nummi, 1963a,b).

Immunochemical methods have been employed to elucidate problems related to structural features concerning gliadin and glutenin proteins. A major antigenic constituent of gliadin was identified in glutenin (Beckwith and Heiner, 1966; Benhamou-Glynn *et al.*, 1965; Escribano *et al.*, 1966; Ewart, 1966). When it appeared that a part of the identity reaction between gliadin and glutenin was due to entrapped gliadin in glutenin preparations the combination of immunochemical techniques with fractionation procedures nevertheless proved that a strong antigenic identity reaction does exist between gliadin and a typical glutenin fraction. This indicated that the main glutenin fraction has structural features common to some gliadin constituents (Ewart, 1966). The amino acid composition of antigenic constituents from gliadin has been reported (Escribano, 1967). An antigen often found in celiac disease has been identified in α- and β-gliadin fractions but not in γ-gliadin (Beckwith and Heiner, 1966). The action of reducing agents on the antigenicity of gliadin and glutenin has been reported (Beckwith and Heiner, 1966; Escribano and Grabar, 1966). Interesting conclusions of these studies suggested that the gliadin disulfide bridges were predominantly intramolecular (Beckwith and Heiner, 1966).

Localization. The use of immunochemical techniques in localization of plant proteins in specific organs or subcellular preparations has already been mentioned. Such methods served to show directly the localization of bound β-amylase in barley (Tronier and Ory, 1970). The enzyme exists in two forms in mature barley grains. One form, called free β-amylase, is extractable with water and saline solution. The other one, called bound β-amylase, is released from its bound form with reducing agents. Using a serum specific for β-amylase, it has been shown that the antigenic bound β-amylase was associated with preparations of protein bodies and was released from the protein bodies with reducing agents.

Genetic Investigations. The immunochemical characterization of proteins represents a valuable auxiliary tool in genetic studies. Utilization of serology in studies on virus diseases in plants has been recently reviewed (Vuittenez, 1966).

The identification of one antigen specific for the female leaf of *Mercurialis annua* (Durand-Rivières, 1969) permitted a study of the action of cytokinins on the synthesis of this specific protein (Durand and Durand-Rivières, 1969).

Among the many genetic problems, malignancy raises the question of the origin of the genome of tumor cells. Crown Gall is an example of specific plant tumor induced by a bacterium, *Agrobacterium tumefaciens*. For this induction, the bacterium, in conjunction with a wound on the plant, is necessary for a short time only. The detection of a bacterial antigen in the plant tumor cultures free of bacteria suggested that a part of the bacterial DNA is operative in Crown Gall tumors (Schilperoort *et al.*, 1969).

Some Practical Applications. In addition to their possible application in diagnosis of certain plant diseases, these methods may serve for testing the purity of products destined for food uses: 5% or more flour from barley origin can be detected in commercial samples of wheat flour (Liuzzi and Angeletti, 1969); 10% or more flour from *Vulgare* variety wheat can be detected in a durum wheat flour sample (Piazzi and Cantagalli, 1969). The source of protein in beer can be checked in the same way: for example, 5% or more of maize in the composition of the barley malt used for brewing could be detected in the finished beer (Schuster and Donhauser, 1967). These methods have also been employed in studies concerning heat stability of peanut proteins following different roasting processes (Neucere *et al.*, 1969).

IMMUNOCHEMICAL METHODS IN ENZYMATIC HETEROGENEITY STUDIES

The antigenic specificity of proteins confers to the immunochemical methods, particularly to I.E.A. and double diffusion,

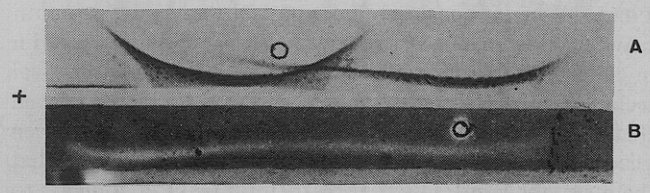

Figure 4. Immunoelectrophoretic zymograms. A: Peroxidases from plant tumor (Crown Gall of *Datura stramonium*), immune serum, antitumor proteins; B: β-amylase from wheat, immune serum, antiwheat β-amylase

the possibility of providing from a protein mixture comparison data on the structures of the proteins. These methods may, therefore, serve as one criterion for isoenzyme studies. The question arises when heterogeneity in physicochemical properties is detected in a class of enzymes catalyzing the same reaction and coming from the same origin. Does this heterogeneity reflect a variability in the same molecular species, or is it the result of expression of different structural genes? In the first case a structural relationship between the different constituents would be reflected by similar immunochemical reactions. In contrast, if this heterogeneity is due to the existence of proteins of different molecular species, they would present structural differences and consequently would possess distinct antigenic specificities.

Figure 4 shows two different examples which can be obtained.

Figure 4A reports the I.E.A. of plant tumor proteins using an antitumor immune serum. The peroxidase characterization carried out on the I.E.A. pattern detects the activity for two antigenic constituents. The corresponding precipitin bands cross each other, reflecting that the reactions are due to distinct antigen-antibody complexes. These enzymes correspond to different molecular species.

Figure 4B shows the I.E.A. of wheat extracts with an antiwheat β-amylase immune serum. The molecular heterogeneity of this enzyme is reflected by its wide range of electrophoretic mobility. The continuity of the precipitin band indicates that the enzyme molecules differing in their electrophoretic mobilities share a common antigenic structure. This suggests that the different constituents may be various forms of one molecular species (Daussant and Abbott, 1969). This result nevertheless constitutes only an indication, not a proof. Additional methods would have to be employed to verify this.

Another question related to this example is how can we know whether the enzymes precipitated by the antibodies of the serum constitute the total or only a part of the β-amylase present in the extract of mature wheat seeds. Other β-amylases, structurally different and for which no antibodies would have been produced, could also exist in this extract. This question can be answered by absorption of the enzyme in the extract with the immune serum employed in the I.E.A. To aliquot parts of the diluted extract are added increasing quantities of the diluted immune serum. After precipitation and removal of precipitates by centrifugation of the antigen–antibody complexes, the β-amylase activity is determined in the supernatants. The maximum percentage of decrease in activity indicates the percentage of enzyme precipitated by the antibodies. Experiments using serum nonspecific for the enzyme must also be carried out simultaneously. (Experiments on the wheat β-amylase presently underway indicate that the bulk of the enzymatic activity is present in the antigenic β-amylase identified in Figure 4B.)

The use of immunochemical protein characterization for localization of the barley bound β-amylase and for the antigenic identification of the bound enzyme to the free enzyme has already been mentioned (Tronier and Ory, 1970). In earlier studies, Nummi (1967) demonstrated the existence of a polymorphism related to molecular size in the free barley β-amylase. This heterogeneity in molecular size is found in several cereals (Nummi *et al.*, 1970) and includes, for barley, some constituents which could be transformed into each other by reduction and partly by oxidation (Nummi, 1967). A further structural identification between these constituents and with the bound β-amylase was provided by I.E.A. of all these fractions (Daussant *et al.*, 1966; Nummi, 1967). I.E.A. and the derived techniques have been used as auxiliary tools for peroxidase isoenzyme studies in maize (Alexandrescu and Calin, 1969) and in Crown Gall tumors of *Datura stramonium* where at least three distinct molecular species of peroxidase were identified (Daussant and Roussaux, 1969; Manigault *et al.*, 1970).

Molecular heterogeneity within one biological function may concern proteins other than enzymes; for example, the soybean trypsin inhibitor is polydisperse. Immunochemical studies of these inhibitors will probably indicate the nature of this heterogeneity (Catsimpoolas and Leuthner, 1969; Catsimpoolas *et al.*, 1969).

IMMUNOCHEMICAL METHODS IN STUDIES OF *IN VIVO* SYNTHESIS AND MODIFICATION OF ENZYMES AND OTHER PROTEINS

There are many examples of the occurrence of or the increase in enzymatic activity accompanying certain metabolic

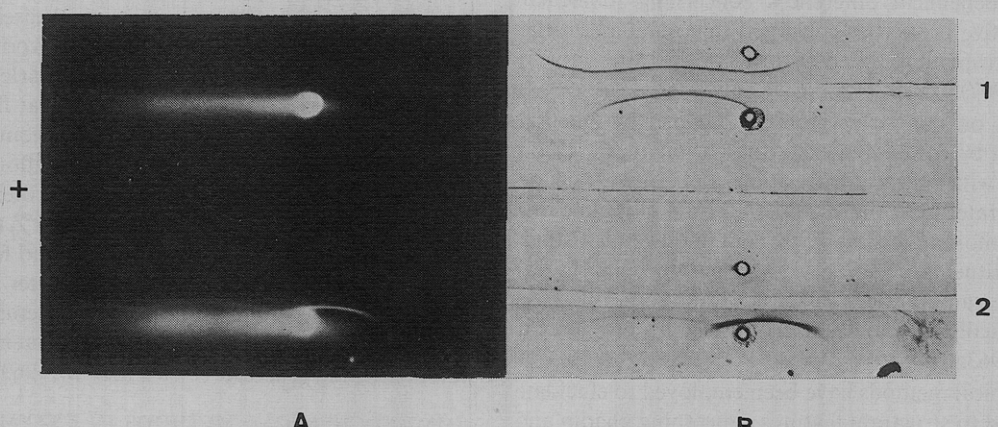

Figure 5. Immunoelectropherograms of proteins extracted from ungerminated wheat seeds (wells above the canals) and from germinated wheat seeds (wells below the canals). Immune serum used: (1) anti β-amylase immune serum; (2) anti α-amylase immune serum. A: Radioimmunoelectropherogram. B: Amido black staining of the immunoelectropherogram

changes in plant physiology and plant pathology. The identification of an increase in enzymatic activity as *de novo* synthesis of the enzyme, to the activation of a precursor or to a decrease in degradation of the enzyme, is a problem for which specific methods of investigation have been reported (Filner *et al.*, 1969). Combinations of different immunochemical techniques with *in vivo* labeled amino acids incorporated into proteins can also provide answers to these questions. Moreover, with immunochemical characterization methods, further identification of an enzyme or nonenzymic protein is possible even after this constituent has undergone changes in its physicochemical properties. Figure 5 illustrates study of the increase in α- and β-amylase activities during germination of wheat (Daussant and Corvazier, 1970). The seeds were germinated in water containing labeled amino acids. After 7 days the proteins extracted from the germinated seeds were submitted to I.E.A. and compared to proteins extracted from ungerminated seeds. The I.E.A. patterns using one serum specific for α-amylase and another specific for β-amylase are shown in Figure 5B. The corresponding radioimmunoelectrophoresis patterns are shown in Figure 5A. Radioactivity detected in the precipitin bands corresponding to α-amylase isoenzymes demonstrates that at least part of the molecules of both α-amylase isoenzymes have been newly synthesized. Moreover, the lack of reactivity between the immune serum and the proteins extracted from the ungerminated seeds indicates that there is no protein structurally analogous to the α-amylase present in the extract of mature seeds. An inactive precursor of the enzyme, because of its structural similarity to the enzyme, would have reacted with the antienzyme immune serum (Barett and Thompson, 1965; Lehrer and Van Vunakis, 1965). Therefore, it can be inferred that this increase in activity for most if not all of the α-amylase antigens is due to *de novo* synthesis. In contrast, the β-amylase precipitin band does not show any detectable radioactivity. Therefore, the increase in activity of this enzyme, generally attributed to the release during germination of the bound β-amylase, does not seem to be accompanied by further synthesis of the enzyme. The modification in electrophoretic mobility of the β-amylase suggests that the release of enzyme from its bound form is accompanied by modifications in the enzyme already present as the free form in ungerminated seeds.

Figure 6 illustrates another approach to this problem. Figure 6A represents an I.E.A. of plant tumor proteins extract with an antitumor immune serum followed by a characterization of peroxidase. The activity of these enzymes increased considerably during tumorization, wounding, or culture of the healthy plant tissue. A quantitative method has been reported (Laurell, 1966) which could determine whether the increase in enzymatic activity is accompanied by an increase in the amount of enzymic proteins (Figure 6B). The gel plate contains a small percentage of the antitumor immune serum, and increasing quantities of the tumor extract are deposited in the wells. After electrophoresis, peroxidase characterization is carried out on the precipitin bands. The areas of the peaks and their heights are proportional to the quantities of protein deposited. This relationship is quite apparent in Figure 6B. This example points out that the specificity of immunochemical characterization provides a possible quantitative estimation for a given protein in a mixture. This technique allows the independent determination of two easily identifiable proteins, one anodic and the other cathodic at the pH and with the gel employed. Another method described by Ressler (1960) allows the quantitation of several antigens simultaneously by combining a previous

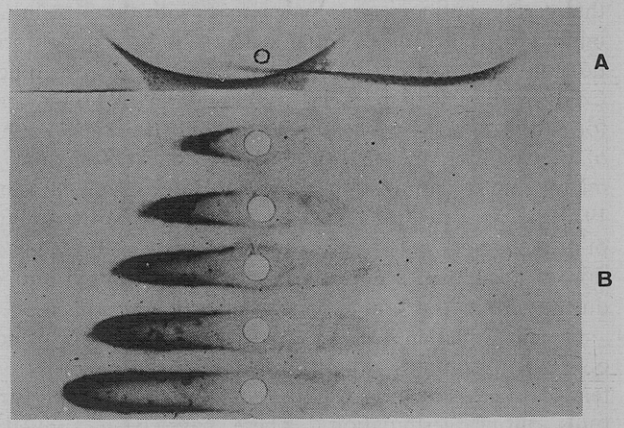

Figure 6. Qualitative (A) and quantitative (B) immunoelectrophoresis with staining of peroxidase. A: Ionagar gel, 1.2% in veronal, 0.025 *M*, pH 8.2, 4 v/cm, 2 hr. The serum is added in the canal after electrophoresis. B: Agarose, 1.2% in veronal, 0.025 *M*, pH 8.2, containing 4% immune serum. Electrophoresis, 6 v/cm. The wells were filled, respectively, from bottom to top with 25γ, 20γ, 15γ, 10γ, and 5γ of freeze-dried plant tumor protein preparation dissolved in veronal buffer

electrophoretic separation of the proteins with this procedure. Methods for single diffusion in gels (Mancini *et al.*, 1965) using monospecific immune serum can be also of practical use. The microcomplement fixation method (Levine, 1967) offers the advantage of requiring minute amounts of immune serum, but is rather laborious in use.

It is noteworthy that the microcomplement fixation method used for the quantitation of one antigen is particularly fitting for the detection of changes in protein conformation.

Figure 7 shows preliminary results on complement fixation of barley β-amylase and of the same enzyme after inhibition using an antibarley β-amylase immune serum. The reduction of complement fixation for the inhibited enzyme indicates

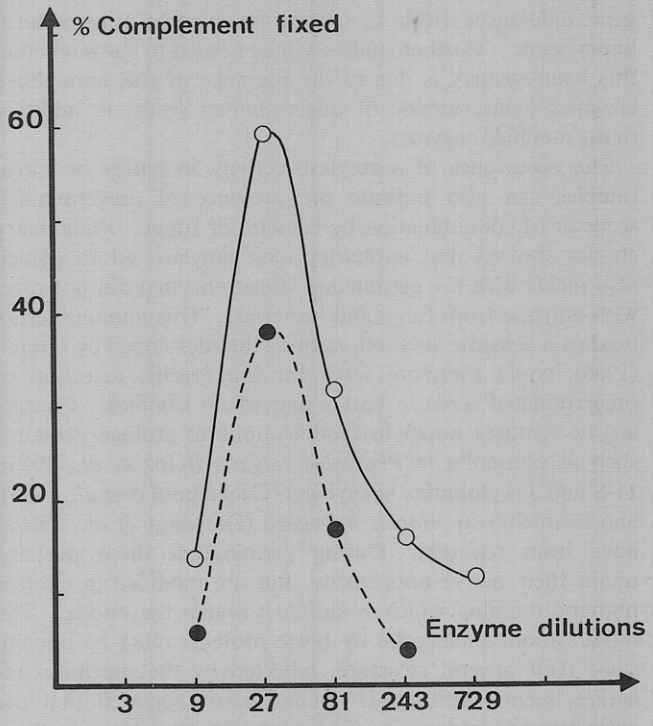

Figure 7. Microcomplement fixation as a function of antigen dilutions. Curves are: ——, β-amylase preparation; – – – –, inhibited β-amylase preparation. Anti-β-amylase immune serum was diluted 800-fold

that certain antibodies no longer react with the protein after inhibition. This suggests that a change in protein conformation has occurred or that antigenic sites have been hidden. Conformational changes in protein in phytochrome transformation, changes usually undetectable by the usual methods of precipitation in gels, have been readily detected by the microcomplement fixation technique (Hopkins and Butler, 1970). These methods, mainly techniques for precipitation of proteins in gels, have been applied to studies on the evolution of proteins (particularly enzymes and storage proteins) during germination and to their relation to morphogenesis.

Protein Evolution during Germination. Preliminary studies on protein contents of ungerminated seeds have been extended to investigations of the modifications in their structures during germination. These have been applied to proteins of wheat (Grabar *et al.*, 1962; Nimmo and O'Sullivan, 1967), of barley (Donhauser, 1967; Grabar and Daussant, 1964b; Grabar *et al.*, 1962; Hill and Djurtoft, 1964), of soybean (Catsimpoolas and Meyer, 1968), and of peanuts (Daussant *et al.*, 1969a).

Because of industrial implications, the modification of barley proteins during germination, malting, and brewing has been studied with particular emphasis on the identification of these proteins which cause difficulties in beer stability (Djurtoft and Hill, 1966; Donhauser, 1967; Grabar, 1960; Grabar and Daussant, 1966; Grabar *et al.*, 1968; Nummi *et al.*, 1969). Quantitative studies on these particular proteins have been initiated by Nummi (1970).

We have already given examples of α- and β-amylase induction and modification during wheat germination. Similarly, but without using labeled amino acids, I.E.A., double diffusion, and absorption techniques have shown the increase in α-amylase activity in germinating barley to be due to *de novo* synthesis of the enzyme (Grabar and Daussant, 1964a). The barley β-amylase was found to undergo physicochemical modifications during germination (Daussant *et al.*, 1966; Grabar and Daussant, 1964a; Nummi, 1967). A recent publication (Tronier and Ory, 1970) reported distinct antigenic differences within the β-amylase enzymes of germinated barley seeds. Further studies will be needed to show whether this heterogeneity is due to the presence of still unmodified enzymes (characteristic of ungerminated seeds) in addition to the modified enzymes.

The occurrence of α-amylase activity in barley or wheat samples can also indicate the presence of pregerminated seeds or of contamination by bacteria or fungi. Preliminary studies showed that antibarley malt amylase serum (which also reacts with the germinated wheat enzyme) did not react with amylase from fungi and bacterias. This immune serum used in a sensitive method, such as that developed by Laurell (1966), could therefore serve for the specific detection of pregerminated seeds in barley and wheat samples. Characteristic features noted in modifications of storage proteins, such as phaseolin in *Phaseolus vulgaris* (Kloz *et al.*, 1966), 11-S and 7-S globulins in soybean (Catsimpoolas *et al.*, 1968), and α-arachin in *Arachis hypogaea* (Daussant *et al.*, 1969a) have been reported. During germination these proteins retain their native antigenicity, but are modified in electrophoretic mobility which is shifted towards the anode. The modifications undergone by these proteins must be limited, since their general structure, reflected by the precipitin reaction, seems unchanged. It has been suggested that deamidation of asparagine and glutamine residues in these proteins, resulting in an increased negative charge by exposure of carboxyl groups, could be the cause of the observed shift in mobility.

Protein Evolution in Relation to Morphogenesis. Further investigations on antigens of *Phaseolus vulgaris* during maturation and germination (Kloz *et al.*, 1966), on *Vicia faba* (Ghetie and Buzila, 1963a,b), and on *Arachis hypogaea* during germination and growth (Daussant *et al.*, 1969a) were carried out using immune serum specific for proteins of plant organs at different ages. These studies provided evidence for the existence of both organ-specific proteins and for proteins common to different plant organs. They indicated that some proteins were characteristic of different stages in the development of the organ and showed that new proteins are synthesized during germination and growth in the storage organs of seeds. Immunochemical studies of cellular differentiation were conducted on tissues of tobacco stem cultures at different stages, including callus, organogenesis, and regenerated plant. The antigens studied were divided into several groups: those specific for one stage only, those present at several stages, and those detected in all stages under investigation (Boutenko and Volodarsky, 1968).

In plant pathology, the occurrence or increase of antigenic proteins has been reported. An antigenic peroxidase develops in potatoes infected with black rot, but this response does not seem to be specific for the infection since cutting of the potatoes produces the same antigen (Uritani and Stahmann, 1961a,b). Two antigenic peroxidases are synthesized in greater amounts in response to tumorization (Daussant and Roussaux, 1969). Here also the response does not seem to be specific for tumorization, since sterile wounds produce the same effect.

CONCLUSIONS

In addition to the methods described, it must be mentioned that the antigenic specificity of proteins can be used in cytology for direct localization of antigens and in preparative methods of protein purification.

This report illustrates the possibilities for immunochemical characterizations of plant proteins in both qualitative and quantitative analytical methods. Because of their high specificity and the various fields for their application, immunochemical techniques may provide a useful tool for studies on plant proteins.

ACKNOWLEDGMENT

The author thanks Robert L. Ory for reviewing the manuscript.

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Received for review October 26, 1970. Accepted January 11, 1971. Presented at the Division of Agricultural and Food Chemistry, 160th Meeting, ACS, Chicago, Ill., September 1970.