

BIOCHEMICAL STUDY ON THE PROTEINS OF CORN POLLEN

by

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INTRODUCTION

One of the greatest problems that confronts the world today is an inadequate supply of food. As a consequence of that, many countries are now suffering hunger and malnutrition which is evident from the increased frequency of sickness and the lowered level of physical fitness of their populations. The population explosion and economic crises are two of the main causative factors of those problems.

Agriculture, particularly the cultivation of cereals, has paralleled the civilization development from prehistory to the present. Cereal grains are the staple food of the majority of the people who live in underdeveloped countries. They provide 70-80% of the total calories in the diet and more than 67% of the proteins.

The improvement of cereals such as wheat, rice, maize, sorghum, barley and others constitutes a powerful tool in solving protein-calorie malnutrition and requires a multidisciplinary effort by numerous plant scientists throughout the world. International research centers are already working towards the selection of improved cereals cultivars. Cereal improvement, more specifically, has become a protein improvement problem since cereal proteins are deficient with respect to nutritional quality and quantity. Such deficiencies account for the basic protein malnutrition from unsupplemented cereal diets in many developing countries.

Interdisciplinary research teams have begun solving protein deficiency problems by screening genetic stocks of the major cereal grains. Genes for increased lysine, the limiting amino acid for animal

nutrition in most cereals, have been located in maize, barley and sorghum. Future food needs are expected to rapidly exceed even the great variability available in any one crop, thus requiring larger gene pools. Intergeneric hybridization, such as in triticale, can merge the wheat and rye gene pools. More extensive hybridization experiments focused on breaking barriers to crossability are necessary to meet future world protein demands. Fortunately, the apparent success of recent experiments on crosses between cereals of different genera suggests genetic recombination can be exploited more fully. The achievement of putative intergeneric barley x wheat, and barley x rye crosses encourages futuristic ideas of the possible incorporation of genetic material to obtain man made cereals with greatly increased nutritional quality and quantity.

Those crosses can not be obtained without disruption of the natural crossability barriers which have received a great amount of attention in the last years. Several recent reports studying the biochemical processes of plant fertilization support the hypothesis of a sort of immunochemical mechanism involved in at least part of the incompatibility phenomenon. The biochemistry of pollen grain and stigma interactions is gaining importance as a tool for understanding wide hybridization problems. Little information has been obtained on that topic. The objective of this research was to study the proteins present in maize pollen for clues to the involvement of such polymers in the incompatibility system.

REVIEW OF LITERATURE

General Pollen Chemistry

Introduction. The study of the chemical composition of pollen grains began in 1829 when the first detailed chemical analyses were published (1). Considerable progress has been made in succeeding years primarily due to the interest of bee nutritionists (2, 3) and of biochemists (4-9) for alleviating human allergenic reactions and more recently for understanding basic plant biosynthetic processes (10).

The data from proximate analyses shows that mineral and fat contents are the major components that vary the least (11-13). The water content of grass pollens at dehiscence is above 50% while other pollens, e.g., Typha or Pinus, contain about 20% or less water at time of shedding (14). Components such as carbohydrates and fibre vary more than other chemical constituents between and within species. The carbohydrate content of grass pollens may be more than twice that of angiosperm pollens. Fibre residue of most angiosperm pollens is 5-7% while in gymnosperms it averages 15-20% of the dry weight. The amount of protein varies widely between species and usually comprises 10-30% of the pollen dry weight. Higher protein content correlates to shorter lived and more rapidly growing pollens such as Zea mays and Typha. The average lipid content is from 1.5-4.0%. The ash content generally is about 2-4% but in some species it can reach 7%. In general the variation in chemical composition of pollen is accounted for by species differences and environmental differences during maturation.

Minerals. The first quantitative mineral analyses on pollen was reported for pine (15). The principal elements were potassium, phosphorus, calcium and iron. Studies on Zea mays (12) added chlorine and magnesium to the list of mineral pollen constituents. More recent analyses of inorganic ions and phosphate content in numerous pollens have been completed (16). The mineral content of gymnosperm and angiosperm pollen varies greatly. The significance of the variability is not well understood, since within species pollen mineral contents sometimes differ greatly. For example, corn pollen of the same variety grown in two successive years on the same field had a total ash of 4.9% in both years but phosphorus values ranged from 0.56-0.75% (17). There is also a considerable difference between mineral contents in pollens and in other plant tissue. In general, trace elements in pollen vary both due to genotype and environmental conditions during maturation. Iron and zinc are the most abundant minerals ranging from 100-400 and 30-300 $\mu\text{g}/\text{mg}$ dry weight, respectively, while other elements like aluminum, copper, manganese, nickel and titanium have also been detected (18). Even though the exact role of mineral nutrients in pollen has not been determined adequately, it has been already shown that calcium and boron are necessary in the germination medium. Calcium is associated with cell wall formation in the pollen tube (19) and boron is believed to be involved in nutrient transport and enzyme inhibition (20).

Carbohydrates. The concentrations of the major free sugars present in pollen are not species specific but generally are associated with a particular species. In pine pollen, 93% of the free sugar is sucrose

but in angiosperm pollen sucrose rarely represents more than 20-50% of the total amount of free sugars (21). The predominant angiosperm free sugars are glucose and fructose (22, 23). Arabinose, xylose, galactose and rhamnose (21) also occur free in grass pollens. Rhamnose occurs in many pollens particularly at maturity, but may, as in Rosa, disappear during storage (24).

Pollen starch content also varies widely between and within species. Date palm pollen lacks starch when shed in contrast to Typha pollen which contains sufficient starch that it sometimes is used as a flour substitute (21).

The cyclitols are an interesting group of compounds often related metabolically to sugars. Myo-Inositol frequently occurs as a free compound in pollen and it can also occur as phosphoinositol. Other cyclitols isolated from pollen include pinitol and sequoyitol, both occur only in trace amounts. Inositol can be incorporated into pectins during pollen germination (25, 26). Pentose sugars such as ribose and deoxyribose are isolated from pollen as free sugars and are probably hydrolysis products of nucleic acids.

During maturation pollen grains accumulate a high level of callose--a β -1,3 glucan polymer. This compound decreases and is re-synthesized during germination. Callose may occur in higher levels than cellulose and pectin (27). Normally cellulose does not exceed 2% by weight. Even though callose is a common and important carbohydrate in plants, its role is not understood. In pollen the functions of callose are as a storage carbohydrate and to seal off parts of the tube cytoplasm (21).

The pollen cell wall contains cellulose which can be extracted using 72% sulfuric acid and acetylation. When this extraction is completed a residue which varies from 2-5% in angiosperms to 20-25% in gymnosperms is obtained. This residue was first called pollenin in 1814 by John, later other workers named it modified cellulose (28). By 1920 this residue was thought to be a cuticle layer over a modified cellulose. Today we know this residue, called sporopollenin, is the principal component of the outer layer of the pollen grain wall (exine). The chemical composition of this substance is not well known. The exine is completely stable to organic solvents, hot aqueous alkalis and acids and will succumb only to strong oxidizing agents. This compound is believed now to be a unique and novel biopolymer. The first chemical studies of the polymer were done by Zetsche (29). He showed that sporopollenins from different sources had similar empirical formulas, that they were highly unsaturated, that they contained C-methyl and hydroxyl groups and that after oxidation they gave a mixture of dicarboxylic acids (C_2-C_6) as main products. More recent works with Lilium henryi (30) suggest that the monomeric substance from which the polymer is derived is of the carotenoid type; this research concludes that sporopollenins are copolymers of carotenoids and carotenoid esters with oxygen.

Various free organic acids, all the Krebs cycle acid intermediates (varying in concentration with the stage of development), and phenolic acids like p-hydroxy-benzoic, p-coumaric and gallic can be detected in pollen (21).

Fatty Acids. Fatty acids are very common in pollen grains. Large quantities of palmitic, linolenic and linoleic acids exist in several pollens (31). Pollen from gymnosperms like pine are very high in linolenic acid (32). Recent work by Lotti (33) results in fairly complete data. The major part of the fatty acids occur as esters combined with sugars, phosphates and other constituents (34).

Proteins and Amino Acids. All the essential amino acids are present in pollen. Bieberdorf (35) has recently analyzed pollen from 107 different plants. The maximum number of amino acids detected was nineteen, while half of the species tested had more than ten different amino acids. The level of glutamic acid and other free amino acids decrease markedly during long periods of storage (36); an observation also corroborated by Linskens (37) working with Zea mays.

Several efforts have been made to relate data concerning free amino acid content in pollen to an understanding of pollen fertility, taxonomic relations and pollen tube growth potential. In corn, sterile anthers have less proline and more alanine than viable, fertile anthers (38). Comparing fertile and sterile lines of Rosaceas (39) it has been found that aspartic acid and alanine accumulated in sterile pollen, while proline content was very low. In wheat the pollen of sterile irradiated plants contained more free amino acids, especially aspartic acid, asparagine, and glutamic acid and less proline than did pollen of fertile control or irradiated plants. Free lysine was detected only in sterile pollen (40). In sorghum pollen from fertile lines, the content of proline and tyrosine rose sharply as microsporogenesis proceeded while the content of asparagine decreased proportionally. In male sterile

sorghum lines, proline content remained the same and tyrosine and asparagine rose (41). Britikov and Musatova have studied more specifically the possible correlations between proline content and sexual fertilization in plants (42, 43, 44). They concluded that no correlation was found between free proline content and biological characteristics of plants such as monoecious, diclinous, entomophilus, anemophilus and self and cross pollinating nor was a correlation between the content of proline and fertilization rate found. Free proline may be required for synthesis of enzymes involved in pollen germination and development as well as for collagen synthesis.

Efforts to establish a taxonomic index based on free amino acid content in pollen have failed. Seven species of pine pollen studied in this aspect yielded no significant differences in free amino acid content (45). Less promising is the concept relating growth potential to the free amino acid content of pollen grains (46).

Germinating pollen grains can incorporate radioactive carbon dioxide from the germination solution into organic acids and amino acids (47, 48). The pathway for this fixation is primarily via phosphoenolpyruvate carboxylase. Addition of aspartic acid, glutamic acid and alanine stimulated germination of corn pollen, possibly due to the presence of their corresponding transaminases detected during the experiment (49). Transamination has also been suggested to have a very important role in fertilization (50). Free amino acid release from germinating pollen occurs after one minute of contact with the aqueous medium (51).

Most of what is known about the proteins in pollen grains is due to enzymatic research. The amount of protein in pollen ranges from

11-30% with the lower amount generally found in species with slower growing tubes and the higher amounts in rapid growing pollens. Proteins are among the constituents which rapidly diffuse from germinating pollen (52). Protein synthesis during pollen germination has been demonstrated and the role of functioning polysomes has been indicated (53). Evidence suggests that dormant mRNA and tRNA exist in mature microspores and are quickly activated upon germination (54, 55). Yamada, in 1973 (56), studied the developmental physiology in rice pollen. On the basis of his results, he assumes that there are three patterns of nitrogen metabolism in pollen development: 1) active protein synthesis at the nucleate stage, 2) active amino acid synthesis at the binucleate stage and 3) active amino acid and protein synthesis during the trinucleate stage.

The release of proteins and other chemical moieties to the solubilizing solution of the environment is at different rates depending primarily on the plant species and to a lesser degree upon the extracting solution (52). The growth of the pollen tube, the compatibility response of plants and the rate and type of allergenic reactions of people to pollen presumably will be influenced by such differences.

The fractionation of pollen proteins into albumins, globulins and glutelins was done in Poland (57). The protein and enzyme pattern of pollen, pollen tubes and styles was achieved by disc electrophoresis in self-incompatible cultivars of Lilium longiflorum. Twelve to twenty bands staining for protein were separated out of pollen tubes while 20-25 were observed from pollen grains. No apparent association was noted between the self incompatibility reaction and proteins which

migrate at pH 8.3 in 7.5% acrylamide gel (58). A disc electrophoretic study of pollen proteins from natural populations of Betulia was reported in 1973 (59) in which 14-16 protein bands were observed, depending on the population investigated.

A mucoprotein containing hexosamine has been found in corn pollen (60).

Enzymes. Contributions to pollen protein chemistry have been made through enzyme studies. Three major reviews of enzymes detected in pollen grains have been reported. The first one published in 1921 was by Paton (61) the second one by Makinen and Macdonald (62) in 1968 and the last one by Brewbaker (63) in 1971. Table I summarizes the enzymes reported to be active in pollen grains.

The separation of isoenzymes has been a favorite topic of study in recent years. Two glutamate dehydrogenases were resolved on gel electrophoresis from lily pollen (57) and eight different malate dehydrogenases have been separated from petunia pollen (65). In maize, polymorphism of peroxidase has been demonstrated (66). Zymogram patterns of transferases (67) and hydrolases (68) have also been achieved.

Comparatively few enzymes of plant tissue have been sought without success in pollen grains. Examples of enzymes which have not been detected are: maltase (69), lipase (70), and β -glucuronidase. The activity of ATPase and DNA synthetase in pollen grains is evidently very low or absent. Since germinating pollen can not synthesize DNA, histone proteins or most forms of RNA (71), the absence of appropriate enzymes in this tissue is obvious.

TABLE 1. ENZYMES REPORTED TO BE ACTIVE IN POLLEN GRAINS (66)

Enzyme class	Enzyme (trivial name)
Dehydrogenases	Glucose 6-phosphate dehydrogenase Glutamate dehydrogenases Isocitrate dehydrogenase Lactate dehydrogenase Lipoamine dehydrogenase Malate dehydrogenase 6-phosphogluconate dehydrogenase Succinate dehydrogenase Triosephosphate dehydrogenase UDPG dehydrogenase
Oxidases	Amino acid oxidase Catalase Cytochrome oxidase Peroxidase
Transferases	ADPG pyrophosphorylase Alanine aminotransferase Aspartate amino transferase (GOT) Hexokinase Nucleoside diphosphate kinase Phosphoglucomutase Phosphorylase Ribonuclease (RNase) UDPG pyrophosphorylase
Hydrolases	Acid phosphatase Alkaline phosphatase Aminoacylase Amylase Cellulase Esterases β -Fructo furanosidase (invertase) α -Glucosidase Leucine aminopeptidase Polygalacturonase (pectinase) Protase (trypsin+chymotrypsin) Trehalase
Lyases	Citrate synthetase Ketose 1-phosphate aldolase
Lygases	Carboxylases

NOTE: In 1972 Larson (64) reported the presence of glucosyl transferase in water extracts of corn pollen.

In general pollen enzymes are freely and rapidly diffused from the pollen grain. A possible role is suggested for these proteins in triggering their own germination processes, especially the trinucleate pollen types through action on the stigmatic fluid (72). Increased activity of amylase, transaminase and phosphorilase during germination has been reported but little evidence is available to explain the increase. The fact that many inhibitors of protein synthesis (methyl tryptophane, puromycin and chloramphenicol) fail to inhibit pollen germination (73) implies that germination can proceed without net enzyme synthesis at least in the binucleate pollen types. Pollen slowly loses certain types of enzymatic activity during storage (e.g., peroxidase, phosphatase, etc.) but that loss has not been correlated to the rapid loss of viability by corn pollen (74, 75).

Pigments. Even though the chemistry of pollen pigments has been relatively well investigated, little is known of the physiological role of these compounds. About 80% of several hundred randomly selected species of pollen were yellow (76); this yellow color has been characterized as due to carotenoids or flavonoids. The carotenoids in pollen are primarily α -carotene and some β -carotene, lycopene, xanthophyl and zeaxanthin. Chlorophyl has not been found. Free carotenes have never been isolated from pine pollen. While it is possible that carotenoids exist in pine pollen in some highly modified form, the visible pigments in the exine are primarily flavonoids. Quercetin, haempferol and isorhamnetin are the flavonoid pigments most commonly found in angiosperm pollens. Naringenin is another pigment only found in angiosperm pollens (77). Carotenes are removed from the pollen grains in the lipid

fraction. Some pigments, particularly flavonoids, are water soluble and readily diffuse from the pollen. Several functions have been assigned to the pigments present in pollen grains. In orchid pollen tubes, carotenoids appear to stimulate the sexual process (78). Flavonoids added in vitro also stimulated pollen tube growth (79). Another suggestion is that pigments protect the genetic content of the pollen; others postulate that the pigments assure transmission by the proper insect vector.

Vitamins. The vitamins present in pollen often behave chemically as enzyme cofactors. Some vitamins, like biotin, occur at very low levels in all species (17, 80). Others like nicotinic acid occur at much higher levels in pollen. Besides these last two vitamins, the amount of riboflavin, panthotenic acid, pyridoxine, folic acid and ascorbic acid have been also quantified. A decrease in pollen vitamin content can occur during storage with riboflavin and pantothenic acid being the most affected (81). The vitamin-like components ascorbic acid and inositol occur in the milligram per gram dry weight range. Inositol is particularly high in grass pollens, while ascorbic acid occurs at high levels in pine and palm pollen. When chromatographed, whole pollen extracts yield Rf values and indicator colours corresponding to indolacetic acid, auxin inhibitors and giberellins (82). In sorghum pollen, substances similar to indoleacetic acid have been isolated by paper chromatography from the grain pollen extract (83).

Steroids. The search for several steroid compounds in pollen grains has been successful. Cholesterol, sitosterol and several other related steroids are widely distributed in pollen. A mass spectrographic

study in pollen from fifteen species of plants of eleven families was published (84). The principal sterol of red clover, mustard, rye and sweet corn was 24-methylene cholesterol, while β -sitosterol was the principal sterol of juniper and scotch pine. Cholesterol was the main sterol of cottonwood.

Maize Pollen Chemistry

Each corn pollen grain is spheroidal in shape and about 90-100 microns in diameter. The pollen wall is composed of a thin outer layer known as exine and of a thicker inner layer known as intine. The intine is composed of cellulose and pectic materials, while the exine is composed of sporopollenin. The surface of the exine is continuous around the pollen grain except for the opening of the single pore through which the pollen tube grows during germination. Corn pollen is golden yellow in color due to the presence of a flavonoid pigment (85) known as glycoside. Both forms have been observed in corn pollen and the presence of an enzyme that adds glucose to quercetin to produce iso-quercetin was also demonstrated (87). The function of quercetin is still not known.

The amount of starch present in corn pollen grains is between 12-30%, depending upon the variety. The amylopectin percentage is very high (approximately 80%). The carbohydrate content, in percentage of dry weight, is given in Table II (11).

Free sugars in maize pollen have been reported to be predominantly fructose and glucose, with smaller amounts of sucrose and rhamnose (22, 88).

Sucrose has been found to be a good source of carbon for in vitro germination of corn pollen (89).

Major chemical and mineral components, in percentage dry weight, for corn pollen are given in Table III and IV, respectively.

The polymorphism of enzymes in corn pollen has been demonstrated by the observation of several isoenzymes (66-68). In addition to enzymes two mucoproteins have been identified (63).

Only limited lipid analyses have been done. Palmitic acid (54.1% of the total) and linolenic acid (34.4%) were the two principal fatty acids present in corn pollen (90).

The vitamin content in corn pollen, in micrograms per gram of dry weight, is shown in Table V (17, 80).

Sterols have been investigated only in sweet corn pollen (84). The principal sterol detected was 24-methylene cholesterol.

Fukui (91) isolated indoleacetic acid and two other growth promoting substances from the acid fraction of corn pollen in 1958. In the same year Tanaka reported the isolation of an auxin from corn pollen (92).

Incompatibility Reactions and Reactants

The importance of pollen and stigma interactions, particularly those of pollen wall proteins and the stigmatic exudate, has been recognized during the last decade as a consequence of great interest in incompatibility phenomenon, their reaction mechanisms, and application of this knowledge to breaking intergeneric crossing barriers. Resolution of such barriers could be a great aid for solving the food shortage prevalent in the world.

TABLE II. CARBOHYDRATE CONTENT^a OF CORN POLLEN GRAINS

Total sugars	36.6%
Reducing sugars	6.9%
Starch	22.4%
Non-reducing sugars	7.3%

^a Percent of dry weight.

TABLE III. MAIN CHEMICAL COMPONENTS OF CORN POLLEN

Component	Amount Present ^a	
	(11)	(12)
Ash	2.55	3.46
Carbohydrates	36.59	34.26
Proteins	20.32	28.30
Lipids	3.67	1.40

^a Percent of dry weight.

TABLE IV. MAJOR MINERAL CONTENT^{a,b} OF CORN POLLEN GRAINS

Potassium	0.67
Phosphorus	0.26
Calcium	0.10
Magnesium	0.21

^a Percent of dry weight.

^b Minor quantities of iron, aluminum, and sodium also have been reported.

TABLE V. VITAMIN CONTENT^a OF CORN POLLEN GRAINS

Riboflavin	5.7
Nicotinic acid	40.7
Panhotenic acid	14.2
Pyridoxine	5.9
Ascorbic acid	58.5
Biotin	0.52

^a Micrograms per gram of dry weight.

The first practical man made cereal obtained by an intergeneric cross between rye (Secale cereale) and wheat (Triticum aestivum), was named Triticale. This was the beginning of a completely new approach for plant improvement since it was the first demonstration of the potential available via wide hybridization. Scientists are now searching for ways to overcome incompatibility barriers to obtain increasingly wider crosses which would facilitate meeting increased human requirements for more and better quality protein. Application of animal-effective immunosuppressants suggests a new approach to breaking cross-incompatibility between wheat, barley and rye (93). Those results support the theory proposed by the same investigator with respect to the presence of an immunochemical system responsible, at least in part, for the incompatibility reaction in plants.

Research on incompatibility suggests chemical control of crossing barriers is possible. Evidence has been accumulating that the stigmatic exudate of plants is not a sugary solution, but a complex mixture of lipids (94) and phenolic compounds (95). Some of these compounds may account for the specificity of stigmas which permit certain pollens to germinate and inhibits germination of others. Knox, in 1972, studied the role of pollen wall proteins in poplar trees and concluded that these proteins also called "recognition substances," constituted a major part of the proteins located in the inner cellulose layer of the pollen grain wall and that these polymers affected the incompatibility systems on which pollen tube growth is dependent (96). The same investigators studied the pollen-stigma interaction in ragweed

and *Cosmos* and followed by scanning electron microscopy the release of wall-held material from the pollen into the stigma surface (97). They found that stigmatic papillae contained pollen wall antigens, proteins, lipids and carbohydrates with the incompatible pollen tubes being blocked, as expected with the polysaccharide callose. They considered that reaction very important for the incompatibility response. A rejection reaction has been induced in *Crucifera* (98) using agar or agarose gels into which pollen wall materials have been allowed to diffuse and also with isolated fragments of the tapetum from anthers of incompatible plants before transfer of its content to the pollen. Partial fractionation of the exine held materials using thin-layer chromatography and gel filtration suggests that the incompatibility reactions are mediated by one or more recognition proteins or glycoproteins with a molecular weight range of 10,000-25,000.

The protein recognition substances of Knox precisely fit the hypothesis of Bates (99) for stereospecific inhibition reactions (SIR).

This study was initiated to establish the functions of maize pollen proteins as a basis for further stereospecific inhibition reactant research.

MATERIALS AND METHODS

Sampling of Pollen Grains

Bulk pollen sampling was done during anthesis in corn plants grown at the North Agronomy Farm, located in Manhattan, Kansas. The material, collected in paper bags, was sieved through a series of metal screens with the final one being a 100-mesh (0.145 mm) sieve. Pollen was maintained in a freezer at -10°C in small glass vials until required for analysis.

Protein Extraction (also see Appendix)

The extraction of the water soluble proteins present in corn pollen was achieved by using 5.0 gms (fresh weight) of pollen with 60 mls of distilled water. The design used for the extraction procedure is unique, serving several functions, and is shown in Figure 1. The system permits continuous extraction of pollen grains, eliminates adsorption interference by continuously changing the extracting solution, and avoids microbial contamination in the extract during extraction.

To attain those desirable characteristics we used a specially designed system consisting of two Amicon (model #52) ultrafiltration cells. The first cell contained a reinforced Millipore filter (0.22 μ) and the second one contained a UM-2 ultrafiltration membrane with a nominal molecular weight cut-off of 1000. Pressure was supplied by nitrogen gas (20 psi). The whole system was constantly washed by fresh extracting solution from a stainless-steel reservoir connected simultaneously to the system and to the gas tank. A 4-way valve

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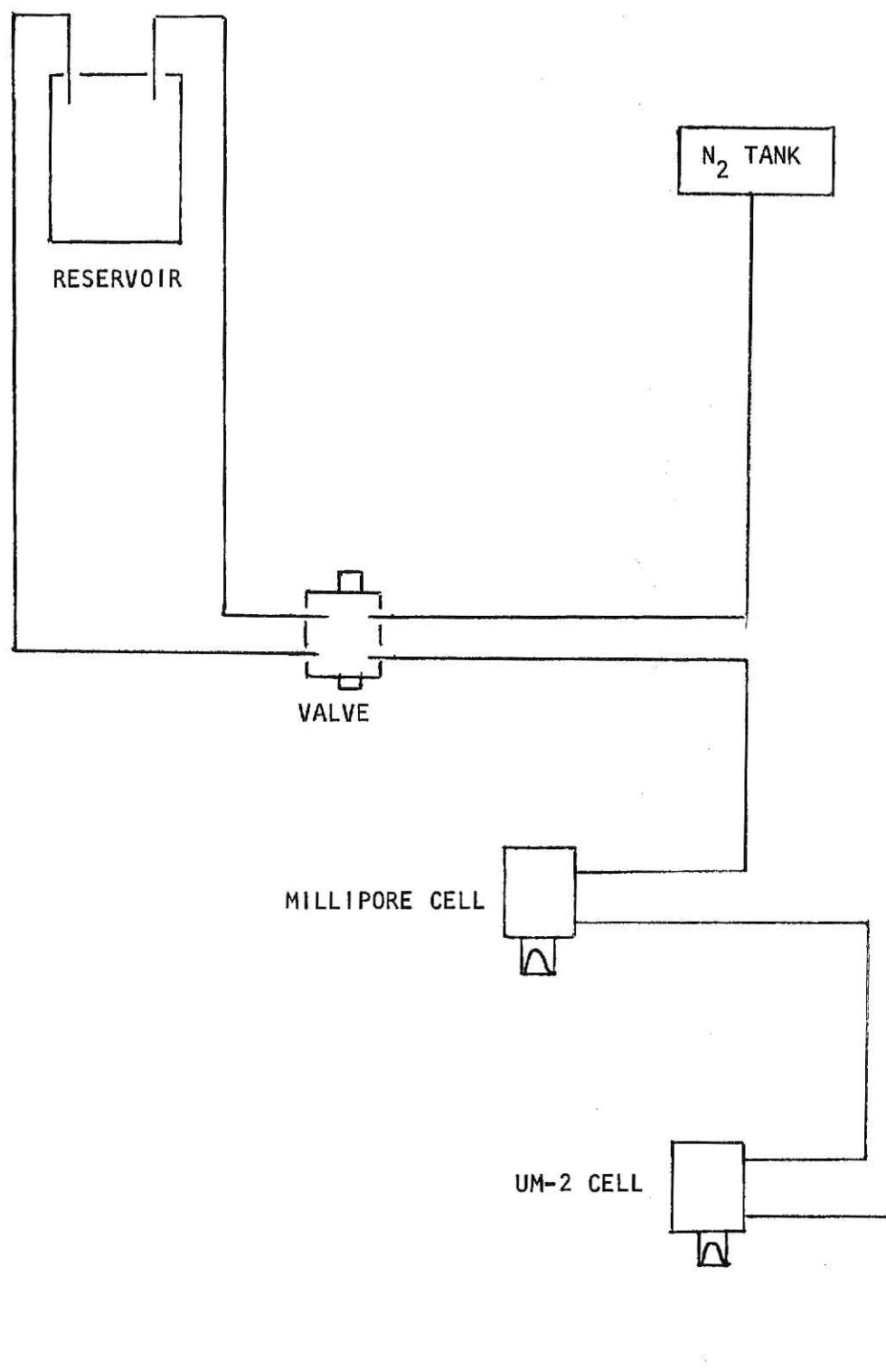


Fig. 1. Continuous system used to extract proteins from corn pollen grains.

permitted selection of continuous extraction (ultradialysis) or concentration of extracts. The valve was set initially to equally pressurize both reservoir and cells independently and then switched to include the reservoir in the flow-scheme for 24-hour extraction at 5°C. After extraction the reservoir was again valved out of the system and the extracts were concentrated to 40 mls.

All cells and connections were sterilized chemically with 70% ethanol and were assembled under sterile conditions in a laminar flow chamber. The membranes, stored in 10% ethanol, were washed with sterile, distilled water and the cells were filled with sterile water. Some contamination may develop in the extract after approximately 36 hours (time necessary for the whole process). This required a second sterile filtration through a 0.22 μ membrane to avoid interference and erroneous results.

Protein Fractionation

The whole extract was fractionated via continuous dilution through a cascade system composed of three tandem connected Amicon model #52 ultrafiltration cells. The cell membranes were selected to give fractions of greater than 100,000 molecular weight (Fraction I), less than 100,000 but greater than 10,000 molecular weight (Fraction II) and less than 10,000 but greater than 1000 molecular weight (Fraction III). The less than 1000 molecular weight fraction was discarded (Figure 2). Fractionation was carried out under similar conditions as extraction.

Separation of macromolecules by ultrafiltration is made possible by special microporous membranes which can discriminate between molecules

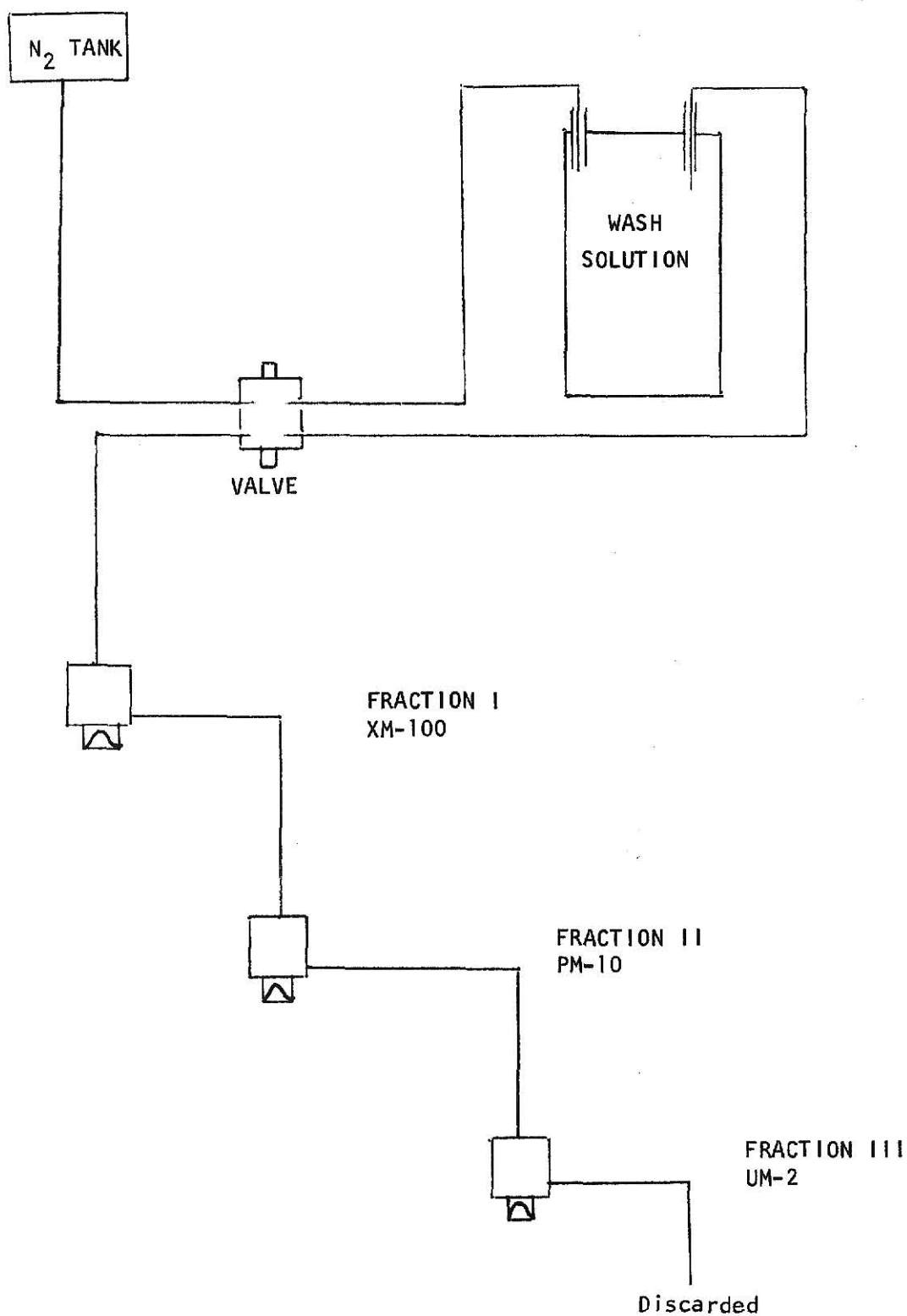


Fig. 2. Ultrafiltration cascade system utilized to fractionate protein extracts from corn pollen.

on the basis of their molecular weight. The membranes used were XM-100, PM-10 and UM-2, which "cut" at approximately 100,000, 10,000 and 1,000 molecular weight, respectively. The efficiency of the membranes was improved by continuous dilution. Molecular interactions, especially concentration polarization, was minimized by constant washing by fresh eluting solution. Contamination by smaller-than-pore-size molecules was limited to 1% or less by following Blatt's washout formula for microsolute (100). Fractionation time was also minimized although the whole process required approximately 78 hours.

Each fraction was concentrated by isolating the reservoir from the system and continuing ultrafiltration until the first cell reached a volume of 30 mls. That cell was separated from the system and the second and third fractions concentrated in the same manner. Each 30 ml fraction obtained was "cleaned up" by filtration through a 0.22 μ millipore membrane. The clear fractions were kept frozen until required for analysis. The fractions thus obtained were:

	<u>Filter</u>	<u>Molecular weight range</u>
Fraction I	XM-100	Proteins with molecular weight over 100,000
Fraction II	PM-10	Proteins with molecular weight between 10,000-100,000
Fraction III	UM-2	Proteins with molecular weight between 1,000-10,000

Isoelectrofocusing

Polyacrylamide gel electrofocusing (PGE) is a separation technique based on the ability of amphoteric substances to migrate in an electrical

field to their isoelectric point. Separation of proteins or other closely related macromolecular ampholytes requires a virtually continuous gradient of ampholytes.

The methodology employed follows Wrigley's description (101) with certain modifications which are described in the following procedure.

a) Gel formation by polymerization

Polymerization can be accomplished chemically or by light. Due to the undesirable effect of persulfate used in chemical polymerization, on certain proteins the following photopolymerization procedure was selected.

Solution "A" - Riboflavin, 0.014% in water

Solution "B" - Acrylamide, 30%
N,N methylene bisacrylamide, 0.8%

Solution "C" - Ampholytes, 40% (for the 5-7 and 7-9 pH range)

Solution "D" - Same as solution "A" plus 1% N,N,N'N'
tetramethylethylenediamine (TEMED)

Solutions "A", "B" and "C" were mixed in a ratio of 0.8:3.0:0.3 by volume to obtain a concentrated gel mix, which was protected against light before use. The protein solution to be separated was prepared in the concentration desired in glass tubes with a final volume not to exceed 1.2 mls. When those tubes were ready, an aliquot of 0.4 mls of the concentrated gel mix was added. The tubes were mixed by inverting the tube capped with a piece of "parafilm." Following mixing, an aliquot of 1.2 mls was transferred into a special electrophoretic tube which was capped with parafilm during photopolymerization. The time of polymerization depended on the components used to form the gel. When ampholytes in the pH 7-9 range were used, solution "D" was used rather

than solution "A" and time was increased to two hours for good polymerization. When using ampholytes in the 5-7 range the presence of TEMED was not required and a time of 75 minutes was sufficient.

b) Separation of components

After photopolymerization was completed, the tubes were placed in the electrophoretic chamber (Hoefer Instruments) and the chamber leveled. The catholyte used in the bottom tank was 0.02 M piperidine pH 10.8. Isoelectric focusing was initiated by adjusting the amperage to an initial output of two milliamperes per electrophoretic tube. The time required for separation varied depending on the concentration of the protein solution analyzed. That time ranged from 45 to 75 minutes at 10°C. Isoelectric focusing was completed when the milliampere value dropped to zero. The course of the separation, as well as the end point, was followed also by using an amphoteric marker dye, methyl red, included in one of the tubes before photopolymerization. The gels were carefully rimmed out of the tubes with the aid of an hypodermic needle.

c) Detection of the proteins separated

Protein bands were visualized by immersing the gels in 20% trichloroacetic acid (TCA) for 12-18 hours. Excess TCA was eliminated by rinsing the gels twice with distilled water. The gels were stained with 0.1% aqueous Fast Green FCF solution for one hour. Excess stain was removed from the gels by using a continuous destaining apparatus containing activated charcoal. Coomassie brilliant blue and Amido Black, commonly used electrophoresis dyes, form extremely insoluble

complexes with ampholytes in acidic gels and can not be used directly as can Fast Green FCF (102, 103).

d) pH gradient analysis

The pH gradient developed in any isoelectric focusing procedure can be measured by the following simple procedure. A gel which has just been removed from the electrophoretic tube was cut into symmetrical 0.5 cms sections with a razor blade; twelve sections were obtained from each 6.0-cms gel. Each 0.5-cm section was shaken in a tube containing 10 mls of distilled water. The mixtures were stoppered tightly and stored in a refrigerator overnight (12-18 hours) after which pH values were measured using an expanded scale pH meter.

Enzyme Detection

In order to detect separated enzymes, isoelectrofocussed gels were analyzed by histochemical techniques for the following enzyme activities: amylase, catalase, peroxidase, esterases and leucine aminopeptidases. Histochemical techniques used in this investigation were described in a review by Brewbaker (104).

General Methodology

The general methodology developed for study of corn pollen grains is diagrammed in Figure 3. Research was conducted in two main sections:

- 1) PGE separation and enzymatic studies of the whole extract.
 - a) Using ampholytes with a pH range of 5-7.
 - b) Using ampholytes with a pH range of 7-9.

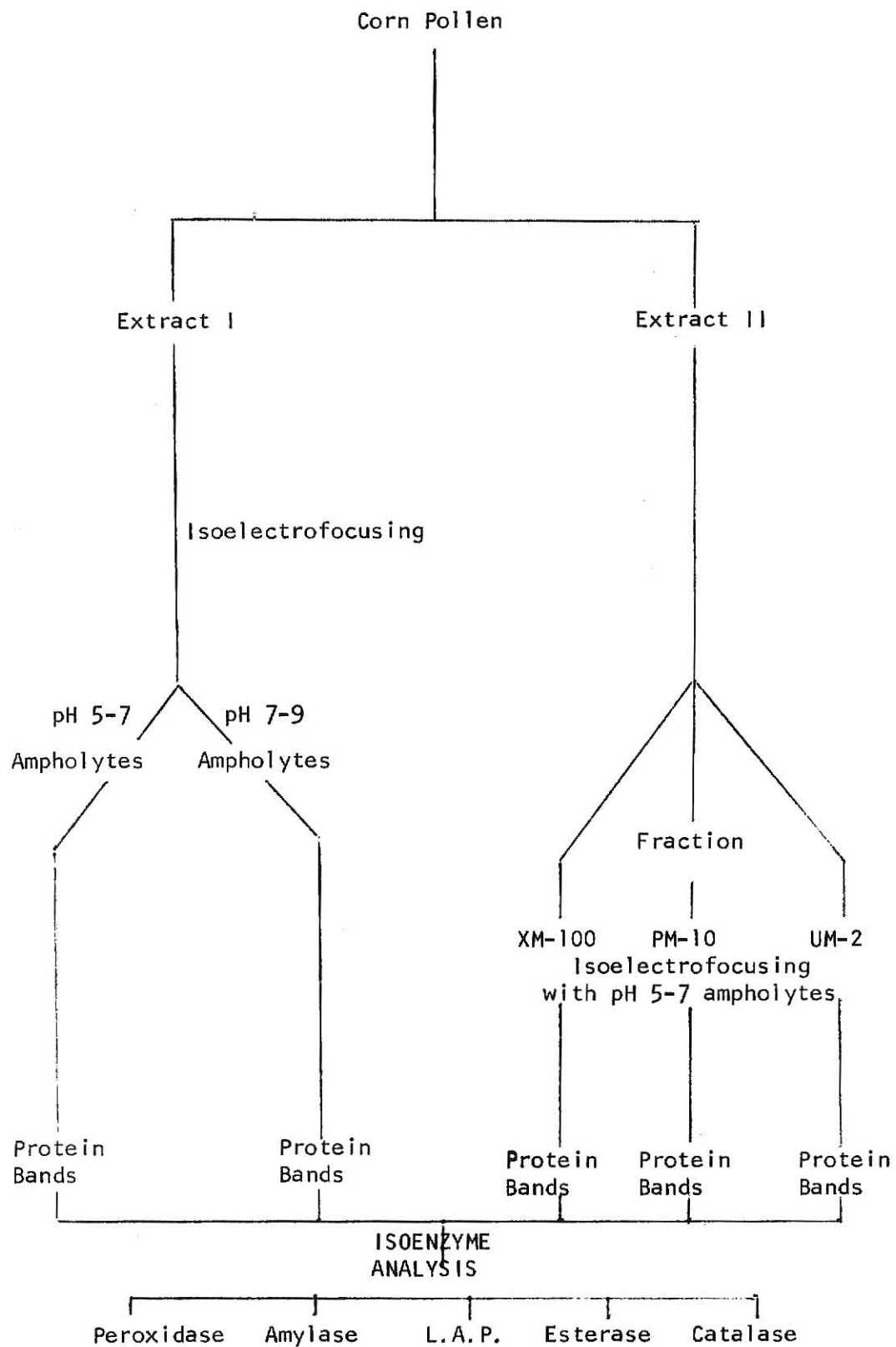


Fig. 3. Diagrammatic representation of experimental work on corn pollen grains.

2) PGE separation and enzymatic study of individual ultrafiltrate fractions obtained from the whole extract.

Proteins were first separated in a system with ampholytes in the pH 5-7 range. The banding pattern obtained was similar in repeated runs. After PGE, detection of the enzymes from separated bands was investigated by means of histochemical techniques. The same procedure was followed to investigate proteins separated in the pH 7-9 range. In all isoelectrofocusing experiments, one gel was used to obtain the corresponding pH gradient. Additionally, another isoelectrofocused gel was stained for protein. For each zymogram a "blank gel" (containing water instead of protein solution) was employed to assure proper control of PGE and enzyme detection.

Ultrafiltrate fractions were analyzed using ampholytes in the pH 5-7 range, in a manner analogous to the above scheme for whole extract, to obtain the corresponding protein patterns and enzyme locations. A pH gradient, as well as a protein pattern, was determined for each PGE run.

Table VI indicates the composition of a typical PGE experiment, containing a marker gel, in which catalases and peroxidases were investigated.

TABLE VI. COMPOSITION OF A TYPICAL PGE EXPERIMENT FOR THE INVESTIGATION OF CATALASES AND PEROXIDASES

Tube No.	Protein extract	Distilled water	Methyl red	4% Starch solution ^a	CGM ^b
<u>Whole extract analysis</u>					
1	1.2 ^c	-	-	-	0.4
2	-	1.2	-	-	0.4
3	1.2	-	-	-	0.4
4	-	1.0	0.2	-	0.4
5	1.2	-	-	-	0.4
6	-	1.2	-	-	0.4
7	1.2	-	-	-	0.4
8	-	1.2	-	-	0.4
9	1.0	-	-	0.2	0.4
10	-	1.0	-	0.2	0.4
<u>Protein fraction analysis</u>					
<u>Protein fraction</u>					
1	1.2 UM-2	-	-	-	0.4
2	1.2 PM-10	-	-	-	0.4
3	1.2 XM-100	-	-	-	0.4
4	1.0 PM-10	-	0.2	-	0.4
5	-	1.2	-	-	0.4
6	1.2 UM-2	-	-	-	0.4
7	1.2 PM-10	-	-	-	0.4
8	1.2 XM-100	-	-	-	0.4
9	-	1.0	-	0.2	0.4
10	1.0 UM-2	-	-	0.2	0.4
11	1.0 PM-10	-	-	0.2	0.4
12	1.0 XM-100	-	-	0.2	0.4

^a The 4% starch solution was required to form a polyacrylamide gel due to the conditions required for the detection of catalase.

^b CGM = Concentrated gel mix.

^c All values are milliliters.

RESULTS AND DISCUSSION

Rapid diffusion of proteins out of intact pollen grains has been well established (55). Extractions have been successfully completed with distilled water or dilute aqueous neutral salt solutions. A minimum extraction time of 19 hours has been recommended for most pollen (105). For convenience we used 24 hours of continuous distilled water ultra-filtration for extraction.

The viability of pollen grains is related to the presence of large quantities of enzymes and reserve substances in a balanced state (106). Germination of pollen grains is affected by the air temperature at pollen shedding time probably due to protein denaturation of the grains. The high amount of carbohydrates and the complete set of nutrients present in pollen grains and extracts are favorable for microbial contamination. Pollen and pollen extracts must be stored under appropriate conditions to avoid the undesirable effects described above. Fresh pollen samples as well as pollen extracts were kept frozen before being analyzed.

The storage stability of both pollen and pollen extracts at low temperatures was studied. Frozen samples kept for three weeks were analyzed periodically (each week) by PGE and their protein pattern compared. The number of proteins detected was the same each time although a decay in concentration, proportional to storage time, of the extract was noticed. Frozen pollen grains seemed to be much more stable than the extracts. The only problem encountered with the storage of pollen grains was the presence of microbial contamination when fresh pollen was stored frozen for five months.

Several protein fractionation techniques can be used in biochemical research. Fractionation by molecular weight differences can be successfully achieved using gel chromatography on columns of inert hydrated polysaccharide materials such as Sephadex. Fractionation of protein mixtures by membrane partition chromatography has been described recently (100). The separation is based upon the selectivity of various microporous membranes which function as partition media in ultrafiltration cells. To avoid membrane polarization (due to high concentrations of solute at the membrane surface consolidating into an undesirable secondary membrane) diafiltration techniques have been described where fresh solvent is constantly added to the system. Since the approach of our study was directed primarily to a search for "chemical clues" out of an enormous collection of compounds, it was appropriate to design a fractionation system using ultrafiltration which would provide a small number of fractions. Such broad molecular weight class fractions are more easily handled than a multitude of fractions from some more specific protein fractionations which result from using gel chromatography or Sephadex.

Polyacrylamide Gel Electrofocusing was selected for this research because of the inherent high resolution for separating proteins and other amphoteric macromolecules. Polyacrylamide gels have low electro-osmotic flow, high optical clarity and satisfactory mechanical properties. Gel rods were preferred to slabs due to their greater experimental flexibility and availability of equipment. Different PGE conditions were tested for the best possible protein separations.

Righetti and Drysdale (107) reported an improvement in the pH gradient produced when the concentration of ampholytes is increased above the normally used 2%. The maximum concentration of ampholytes permitted in our experiments was 2.56% which gave a pH gradient and separation similar to that obtained with 2% ampholytes. Since no differences or improvement in the pH gradient or in protein separation were observed, a concentration of 2% ampholytes in the final gel was used in all subsequent experiments.

Polyacrylamide gel electrofocusing with 0.01 M phosphoric acid and 0.02 M sodium hydroxide as anolyte and catholyte, respectively, was utilized by Righetti and Drysdale (107). We tested these electrolytes as a way of improving the linearity of our pH gradients. Two different PGE experiments were performed, one with 0.02 M sulfuric acid and 0.02 M piperidine solutions and the other with the new electrolyte system of Righetti and Drysdale (110). The results were not satisfactory since the same lag occurred in the acid side of the pH gradient of the gel when either of the two systems were used. The gradient obtained with the sulfuric acid-piperidine system gave slightly better results.

Long gels (12.0 cms) were used to separate the proteins from whole pollen extract. There was an improvement in the resolution of protein bands due to an increase of total gel volume as compared to shorter gels of 6.0 cms.

These longer gels had the disadvantage of being difficult to handle, especially in the zymogram techniques. Also undesirable was the need to double the amounts of extract, reagents and length of PGE time. Since

the better resolution possible on longer gels is only in the degree of separation and not in the number of protein bands, we decided to continue using short gels to save work, money and time.

Reports advising the use of recrystallized acrylamide and N,N'-methylene bisacrylamide (108) have been followed, after comparing the efficiency of pH gradients obtained with these purified reagents against ones obtained with commercial grade chemicals.

Localized heat produced during PGE is associated with protein denaturation and convective mixing in the gel and is a factor contributing to pH gradient instability (109). A circulating cold water (10°C) system was used in the electrophoretic chamber to avoid this problem.

There has not been any report of PGE on pollen grains. Electrophoretic analyses are limited. The study of pollen from poplar trees yielded 14-16 separated proteins by disc electrophoresis (62). Twenty to twenty-six different protein bands were detected by disc-electrophoresis of Lilium pollen extracts (61). A total of 21 protein bands, which compares favorably with electrophoretic results, was achieved from maize pollen extract separated by polyacrylamide gel electrophoresis. The combination of PGE and ultrafiltration provides biochemical information (approximate molecular weight and isoelectric points) which adds to zymogram data and permits a better characterization of the separated proteins.

Working with the whole protein extract in an ampholyte gradient from pH 5-7, we were able to separate and detect 15 different bands.

The intensity of the bands varied suggesting the presence of different concentrations of each protein detected. The isoenzymes identified from the separated protein bands were: one amylase in the anodic portion of the gel, two catalases, four peroxidases, six esterases and three leucine aminopeptidases (LAP). Zymogram band separation and identification were reproducible. Figure 4 presents a composite of information for a clearer understanding of data obtained in this portion of the study. In this graphic representation, pH values and distance migrated along the gel are correlated with zymograms to characterize protein bands.

The protein separation pattern and enzymatic characteristics of the whole protein extract using ampholytes in the 7-9 pH range was obtained in an analogous manner. A twelve-band pattern was obtained with a linear pH gradient from approximately 6.3-9.0. The distribution of these proteins and the detection of some of their enzymatic properties are shown in Figure 5.

Fractions obtained from the whole extract via ultrafiltration were studied by identical PGE and zymogram techniques, although only pH 5-7 gradients were used. Eighteen different proteins were located in fraction II while only five were detected in each of the other two fractions analyzed. The presence of similar proteins in some of these fractions (Figure 6) can be explained by the fact that ultrafiltration membranes are far from 100% efficient in their discrimination of polymer size. This could be due to protein-membrane interactions or more likely to different shapes of the protein molecules, since the discrimination characteristics of each membrane are based on calibration by spherical molecules.

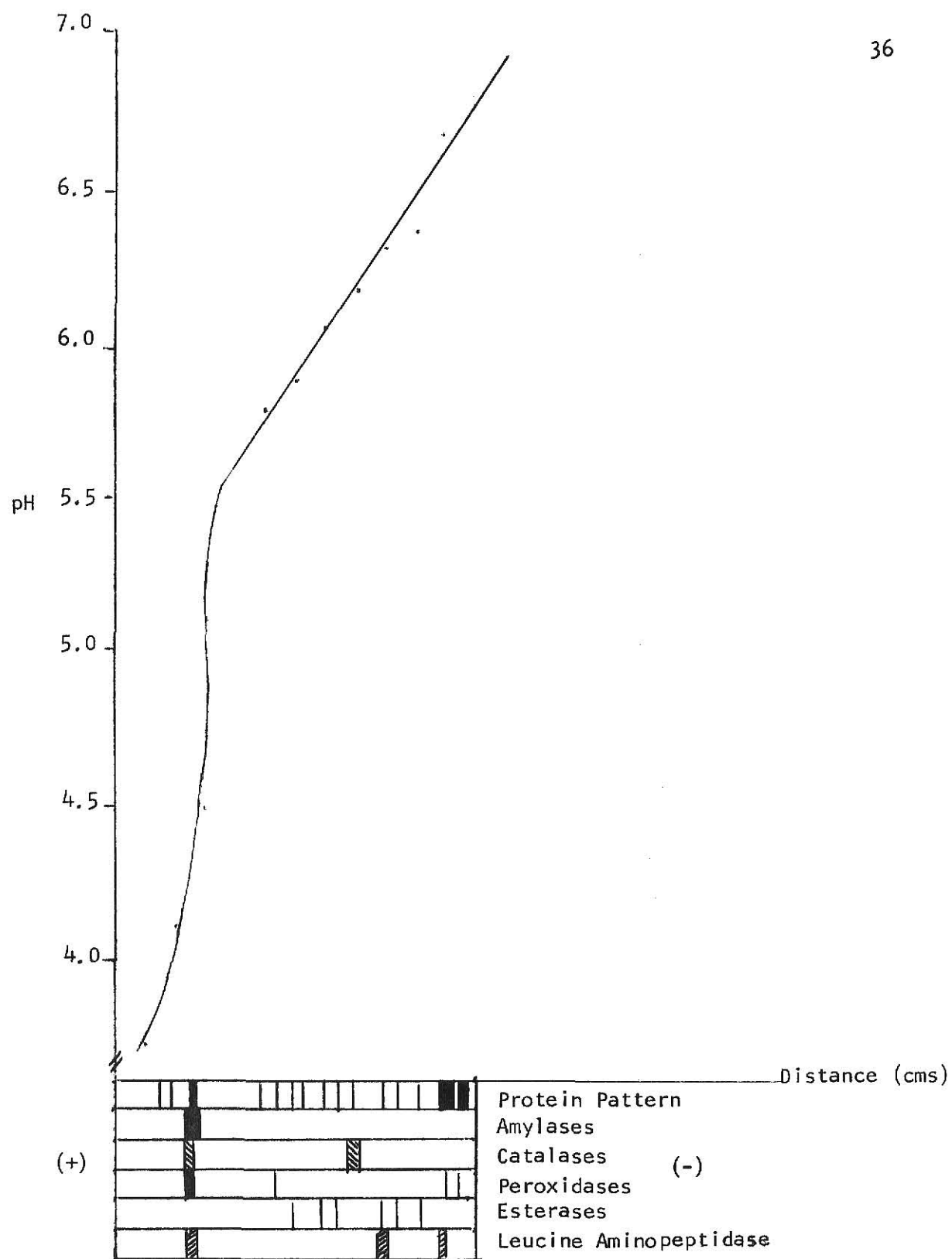


Fig. 4. Protein pattern obtained by isoelectrofocusing using ampholytes in the 5-7 range, and enzymes detected in the isoelectrofocused bands.

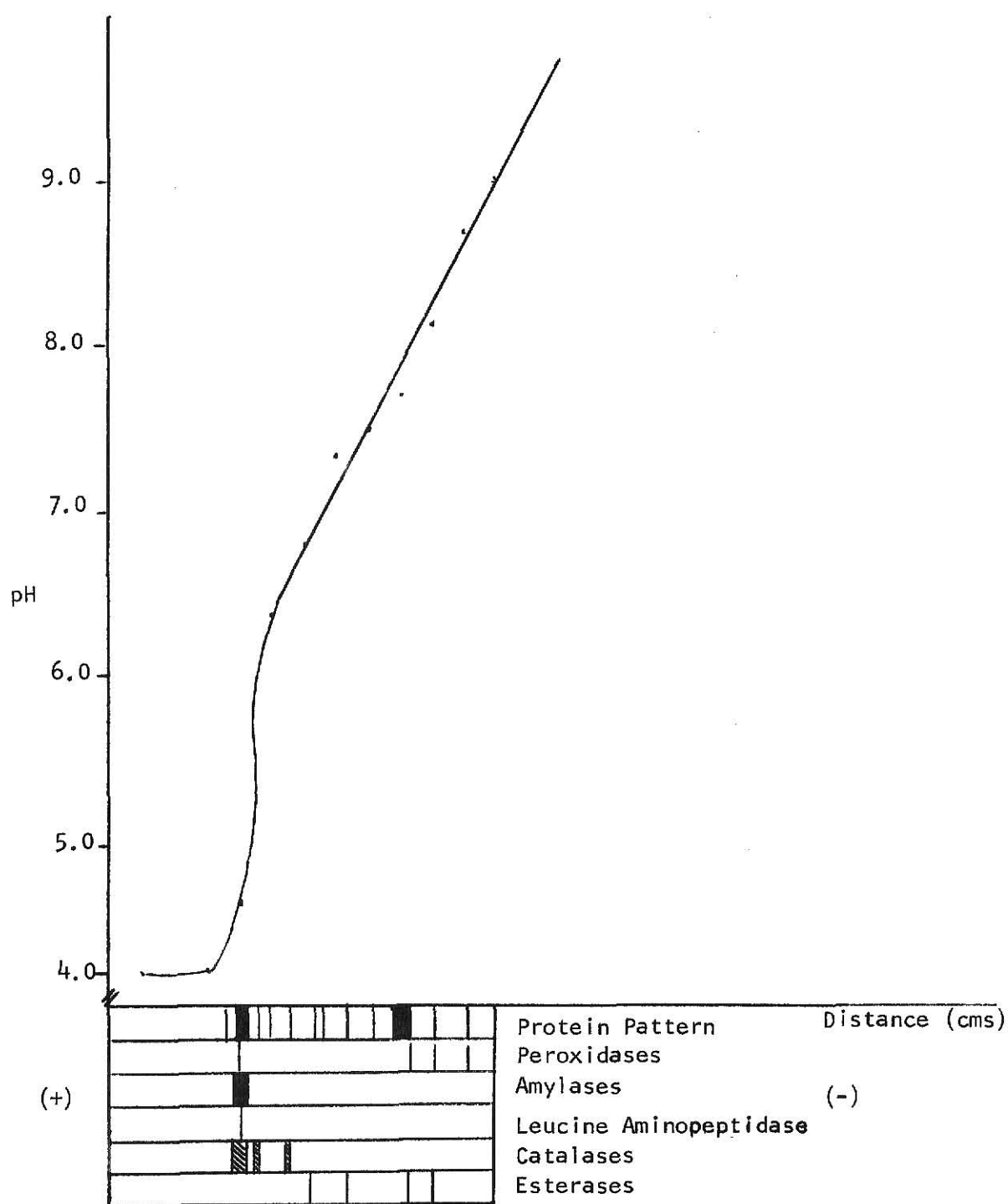


Fig. 5. Protein pattern obtained by isoelectrofocusing using ampholytes in the 7-9 range and enzymes detected in the isoelectrofocused bands.

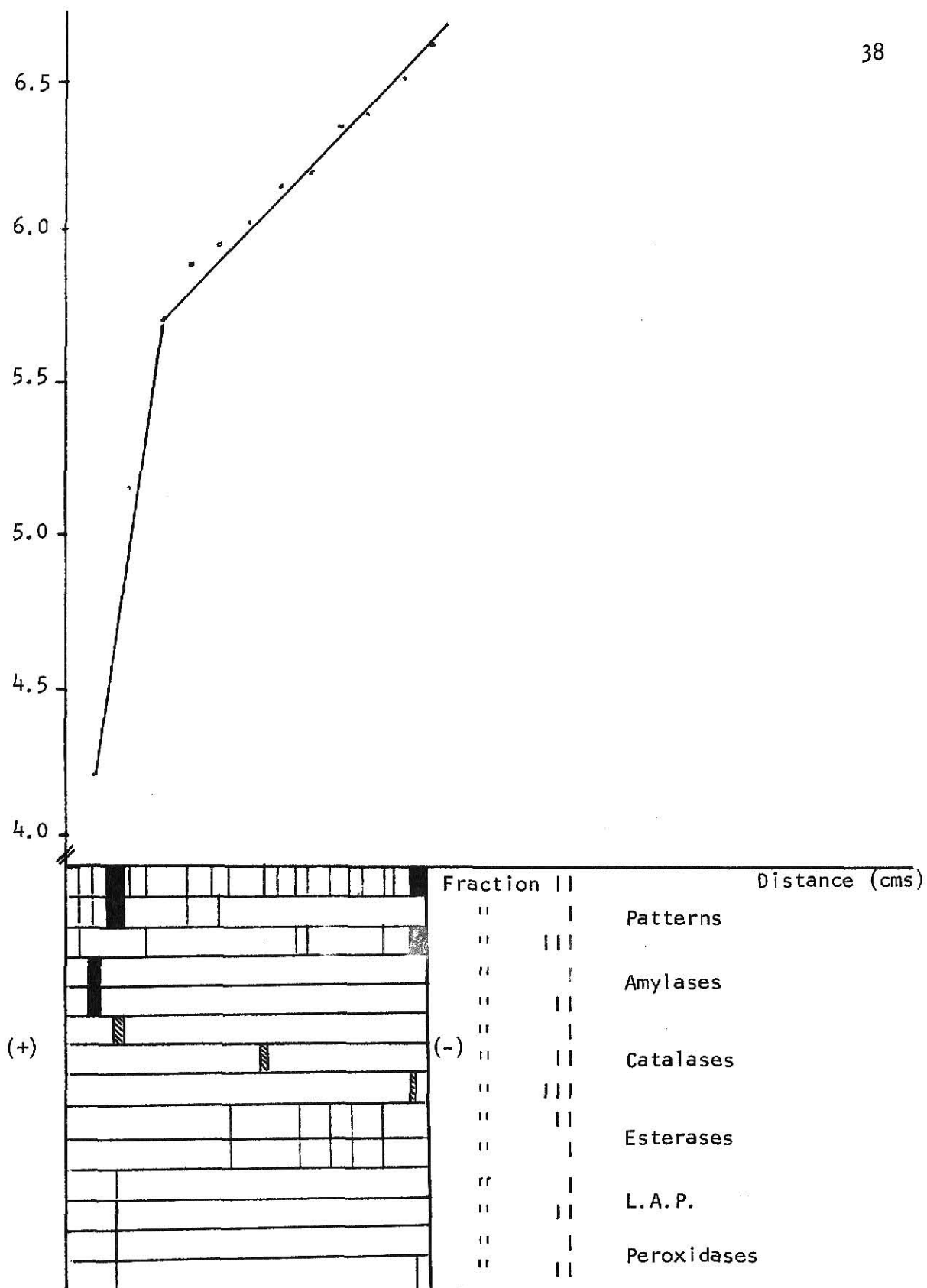


Fig. 6. Isoelectrofocusing protein pattern obtained by electrofocusing fractions obtained by ultrafiltration and enzymes detected in the isolated bands. Ampholytes in the pH 5-7 range were used.

Zymograms were developed from each of the fractions obtained by ultrafiltration (Figure 6). One amylase was detected in fraction I and fraction II. The concentration of this enzyme was higher in fraction I. Three catalases were detected, one in each of the three protein fractions. Five esterases were separated and detected in both fraction I and fraction II, the latter fraction apparently having a higher concentration of these enzymes. Finally, one leucine aminopeptidase (LAP) and two peroxidases were visualized. The LAP was located in both fractions I and II with higher concentration in fraction II. One of the peroxidases was exclusively found in fraction II while the second one was detected in both fractions I and II, being present in higher concentration in fraction I.

By compiling information collected from analyses of whole extract and ultrafiltration fractions analyses, we can produce a mapping pattern of the water-soluble proteins of corn pollen (Figure 7). Biochemical characteristics of these proteins are also indicated.

Most biochemical studies of corn pollen are related to the presence of isoenzymes and their possible genetic value (62, 66-68). These studies used starch gel electrophoresis as the separation procedure. In 1967 it was reported that corn pollen grains contained 3-6 esterase isoenzymes and 2-3 components with LAP activity. Both isoenzyme patterns were dependent of the inbred line of maize analyzed (62). Recently 17 bands with esterase activity were found in a maize pollen extract. The existence of tissue-specific polymorphism was demonstrated (68). Other reports include separation of five bands with peroxidase activity, the peroxidase isoenzyme pattern also being

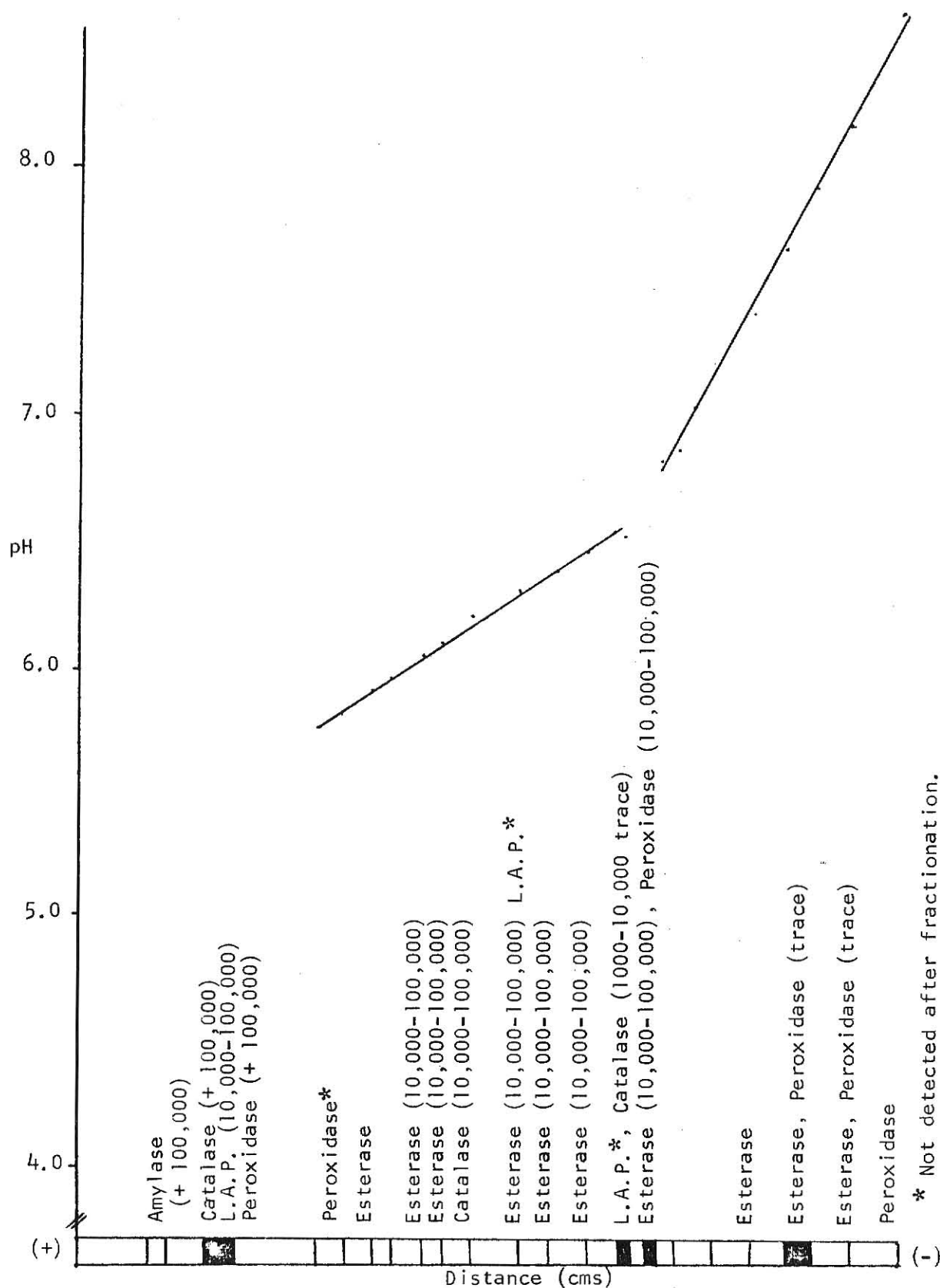


Fig. 7. Composite maize pollen protein map based on isoelectric point, enzymatic activity and approximate molecular weight.

tissue specific (66). Finally, one catalase and amylase band have been detected in maize pollen (73).

Using polyacrylamide gel electrofocusing we isolated and characterized, by isoelectric point, from maize pollen two bands (plus one very weak one) of catalase activity, ten different esterases, two LAP and five peroxidases. Our data and the above reported electrophoretic data agree with respect to the number of isoenzymes. This is significant since two different methodologies were employed. Nevertheless there are differences in the number of catalases and esterases. Isoelectrofocusing permitted separation of three distinct catalase isoenzymes. With regard to differences in the number of esterases detected, we must mention that, of the five histochemical techniques employed, only this enzyme assay gave detection problems due to partial interference by certain compounds in the pollen extracts. Great differences in zymogram patterns for esterase activity in maize hybrids have been demonstrated (62).

The molecular weight results are subject to the error expected from the nature of the ultrafiltration membranes (cut-off point, varies and is never 100%) and the unavoidable effect of the shape of the protein molecule upon the fractionation. The approximate molecular weight found by ultrafiltration fractionation of different enzymes was:

Amylase	> 100,000
Catalase	
(1)	> 100,000
(2)	10,000-100,000
(3)	1,000-10,000

Peroxidases		
	(1)	> 100,000
	(2)	10,000-100,000
Esterases		
	Five different esterases	
	all between	10,000-100,000
LAP	(1)	10,000-100,000

All other isoenzymes separated by PGE of the whole corn pollen extract which do not appear in the previous list were present in very low concentration after the fractionation process. As consequence of this, their detection was not possible on the zymograms of the fractions.

A result which attracted more of our attention was detecting catalase activity in a fraction where only very low molecular weight proteins were expected. This enzyme has a reported average molecular weight of 232,000 (110). Consequently it is hard to believe that a catalase of a molecular weight between 1,000 and 10,000 could really exist. One possible explanation could be that part of the catalase molecule, with enzymatic properties, was separated in the last fraction of the ultrafiltration system. This suggestion can be supported by reports (111) indicating that maize catalase may exist as a tetramer, although a 60,000 molecular weight monomer would still have difficulty passing the 10,000 molecular weight limit filter. Another catalase was detected in the PM-10 fraction. This enzyme could be one of the monomers of a 232,000 molecular weight enzyme or perhaps a new catalase with a molecular weight between 10,000-100,000. In this case, and assuming that this protein would also exist as a tetramer, the catalase present in the last fraction (1,000-10,000) could be explained as being

a monomer of this enzyme. The tetramer nature of maize endosperm catalase (111) supports the supposition that the catalase detected in the PM-10 fraction is a different isoenzyme with a molecular weight between 10,000-100,000.

Molecular weight reported (112) for the types of enzymes detected in our work include amylase (sweet potato) 152,000; leucine aminopeptidase (porcine kidney), 300,000; catalase (bovine liver), 244,000; peroxidase (horse radish), 40,000; peroxidase (myelocytes), 149,000; and arylesterases (sheep serum), 35,000-50,000. Our data differ only in the approximate molecular weight of the pollen leucine aminopeptidase and in the fact that two catalases with lower molecular weights were detected.

Several of the 21 protein bands detected by polyacrylamide gel electrofocusing were composed of at least two different types of enzymes with very similar isoelectric points. We suggest the use of electrophoresis and two dimensional techniques for separating and characterizing these proteins in a more complete manner.

SUMMARY

Analyses of corn pollen proteins were developed. Pollen proteins were studied by separation by polyacrylamide gel electrofocusing and by characterization of their enzymatic activity, isoelectric point and approximate molecular weight. Polyacrylamide gel electrofocusing was selected due to its high resolution and functionality. Ultrafiltration was the method of choice for separating the proteins into fractions based on molecular weight.

Twenty-one protein bands were detected in corn pollen extracts obtained by a continuous water extraction system. These proteins were separated by using ampholytes in the pH range 5-7 and 7-9. Zymograms for esterases, peroxidases, leucine aminopeptidases, catalases and amylases were prepared. Several protein bands from the isoelectrofocused gels contained at least two different enzymes with very similar isoelectric points. One large protein band in the anodic portion of the gel included three different enzymes. Those facts indicate that more than 21 proteins were present in corn pollen extracts.

All the enzymes analyzed, except amylase, presented polymorphism. Zymograms obtained were reproducible. The approximate molecular weight of several enzymes was estimated by their presence in ultrafiltration fractions obtained from a tandem system of three ultrafiltration cells containing different porous membranes.

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APPENDIX

The effect of a chemical preservative on extraction of protein from corn pollen was studied. The use of 2-phenoxyethanol as a protein preservative for animal tissue without greatly altering enzyme activity (113) suggested its possible use to preserve pollen grains stored in cold conditions. No protein bands were detected in extracts from 2-phenoxyethanol-preserved pollen, however. Pollen proteins must form insoluble complexes or be bound to cellular debris eliminating the possible use of 2-phenoxyethanol for pollen preservation.

The efficiency of extracting solutions containing dimethyl sulfoxide (DMSO), polyvinylpyrrolidone (PVP) and lithium diiodosalicylate (LIS) was investigated. The protein pattern obtained with DMSO was very similar to that obtained with water. Since oxidative properties of DMSO affect proteins, particularly in subsequent operations (114), we discarded the possible use of DMSO-water extracts. LIS was eliminated as a possible aid in pollen extraction due to the fact that gel formation did not occur even when the concentration of acrylamide was increased. Finally, a PVP-water extract was tested and compared to a water extract. The patterns obtained were very similar suggesting no improvement in the extraction procedure.

It was concluded that the use of preservatives or extraction adjuvants was ineffective and extraction was limited to distilled water via the continuous system described in the text.

BIOCHEMICAL STUDY ON THE PROTEINS OF CORN POLLEN

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Pollen biochemistry has become an area of great interest to scientists due to its participation in intergeneric crossability barriers. Pollen proteins were studied to enable future researchers to continue to search for biochemical "clues" responsible for incompatibility interactions with stigmatic tissue. Proteins were extracted with water in a continuous ultrafiltration scheme. Protein extracts were resolved by polyacrylamide gel electrofocusing using ampholytes in the ranges of pH 5-7 and 7-9. Fractionation of the whole pollen extract by membrane partition chromatography produced three different groups of proteins separated on the basis of their approximate molecular weight. These fractions were further resolved by polyacrylamide gel electrofocusing with ampholytes in the 5-7 pH range. Zymograms of both the whole extract and the fractions were determined for esterase, leucine aminopeptidase, catalase and peroxidase isoenzymes. One amylase was also detected from the pollen extract. A composite mapping pattern of the separated protein bands and some of their biochemical characteristics was developed.

Polyacrylamide gel electrofocusing in the 5-9 range yielded 21 protein bands, three of which include two or more enzymes which can not be resolved under the conditions used by this technique.