Korean Jour. Botany
Vol. 17, No. 4, 143—157(1974)

Studies on Protein Profiles and Isozymes in Germinating Seeds

Kwon, Oh Yong
(Andong Teacher's College, Andong, Korea)

種子發芽에 있어서 Protein Profile과 Isozyme에 關한 硏究

權 五 溶
(安東敎育大學)

ABSTRACT

The purpose of this experiment was to study one side of germination physiology based on that protein profiles and protease relating to protein metabolism, that peroxidase, catalase, α-amylase, β-amylase, and malate dehydrogenase involved in the carbohydrate metabolism of seed germination. All these experiments were divided into the two groups with and without acetone treatment, and were carried out.

The protein bands of each germinating stage between the groups treated with and without acetone showed certain basic pattern in polyacrylamide gel disc electrophoresis. However, there was a little difference in the number of protein band, optical density, and migration velocity between two groups.

The isozyme bands of peroxidase, and catalase between two groups in polyacrylamide gel disc electrophoresis did not show the numeral difference, but the optical density of certain germinating stage treated with acetone was higher than the group untreated with it and it showed their enzyme activity.

The α-amylase and β-amylase activities which involved in starch metabolism of seed germination were higher in the treated group than the other. On one hand, the protease activity of hydrolase occurred in the seeds for germination was also higher, more or less in the treated group than in the other.

The isozyme band pattern of malate dehydrogenase in TCA cycle of energy metabolism pathway was very different between two groups growing for 72 hours with and without acetone treatment in cellulose acetate electrophoresis. It indicated that two isozyme bands of malate dehydrogenase was high.

Consequently these experimental results mentioned above indicated that acetone treatment before sowing had an effect on dissolving certain complexed lipid substance involved in the seed coats, the activity of carbohydrate hydrolase increased with water absorption which was most comfortable in its germination, dissolved glycerin and fatty acid became certain energy source, and they stimulated the acceleration of respiration metabolism.

INTRODUCTION

The seeds of many plants will germinate as soon as ripe if environmental conditions are suitable. Pea seeds, for example, sometimes germinate within the pod, corn grains may sprout while still attached to the parent plant, the seed of some citrus species frequently germinate while still within the fresh fruits, and carrot seeds will gradually germinate while still imbibed adequate water. Seeds of many other species, however, will not sprout until after an interval of weeks, months, or years, even if environmental conditions are favorable for germination.

The intial step in germination is the imbibition of water by the various tissues within the seed. This generally results in an increase in its volume. The increase in the hydration of the seed coats usually causes a pronounced increase in their permeability to oxygen and carbon dioxide which is very low in the dry seed coats (Stiles, 1948). The swelling of the seed often ruptures the seed coat, but in some species this does not occur until the emergence of the primary root.

With an increase in the hydration of the cells, enzymes become activated. In seeds posessing an endosperm, enzymes apparently move into that tissue from the embryo. Stored foods, whether they occur in the endosperm or cotyledons, are digested and the soluble products of the digestion process are translocated toward the growing points of the embryo (Bibbey, 1948).

If chemical analysis are made of samples of seeds at successive stages during their germination, it is found that the quantity of starches, oils, or proteins in the seed decreases markedly. A large proportion of the fats present are usually converted, after digestion, into soluble carbohydrates. The soluble carbohydrates are not present during the process, indicating that a large proportion of these compounds is consumed in respiration or assimilated in the construction of the carbohydrates constituents of cell walls.

In oil seeds the soluble carbohydrates utilized in respiration result largely from chemical transformations of the products of the digestion of fats. Digested proteins are usually of amino acids, asparagine, etc. This indicates that proteins are not consumed in respiration but are utilized in the synthesis of the organic nitrogen compounds of the growing embryo (Jones, 1950).

Accordingly, Angei (1961) proposed on the inhibited matter in the germination of carrot seeds; he reported that the germination of carrot seed was delayed by certain yellow sterol. Therefore the author made an effort to investigate how many substrates accelerate the germination of carrot seed and how many substrate is well utilized in a practical part of agriculture. These experimental results indicated that the germination of carrot seeds was accelerated by many factors, such as acetone, gibberellin, penicillin, and mercron. In this study, the treatment with acetone or gibberellin was effective in the germination of carrot seeds.

For studying on the acceleration of germination, Kwon(1970, 1971, 1974) reported on the effect of X-ray and ultraviolet light in the germination of carrot seeds, effect of some inorganic compounds in the germination of carrot seeds, effect of some organic compounds in the germination of carrot seeds, On the other hand, Kwon(1974) demonstrated on the effect of acetone in the germination carrot seeds in order to study the respiratory mechanism of germination physiology. In these experiments, the oxygen consumption of carrot seeds treated with acetone before sowing was more increased than normally germinating carrot seeds.

It demonstrated that the imbibition occurred in the seed coats was well activated. Imbibition is usually considered to be basically a diffusion process, but capillary phenomena are probably also involved. Fundamentally, however, the cause of imbibition may be regarded as a difference in the difusion pressure between the liquid in the external medium and the liquid in the imbibant (Gornter, 1934).

It had been assumed to the author that the trea-

tment of acetone for 30 min before sowing had dissolved some kinds of lipid substances in the cell wall of carrot seeds and stimulated some kinds of inhibited matter in the endosperm.

In the present status of both physiology and genetics, it is especially desirable to be able to resolve the soluble proteins which are present in plants (Chang and Steward, 1962). Chromatography had led to great gains by the recognition and identification of the simpler nitrogen compounds that these techniques made possible. A similar degree of convenient resolution of the protein fractions may be fraught with similar gains.

Although there have been many studies(Evanri, 1949; Jones, 1950; Ohda, 1956; Susuki, 1959; Angei, 1961; Kwon, 1968), relatively little is known about studies on protein profiles and isozymes in germinating carrot seeds. Therefore, the mechanism of germination physiogy was analyzed for key isozymes of some pathway in carbohydrate metabolism, and nitrogen metabolism.

MATERIALS AND METHODS

Germination.

The seeds of *Daucus carota* Linne were used in these experiments. The methods used here for the germination of carrot seeds were similar to those described for *Nastutium* seeds by Fuji and Ishikawa (1962). Two hundreds seeds on the filter paper placed in each petri dish were used for the experimental group treated with and without acetone. Each dish for germination was wrapped with a thick black paper and that dish placed in an incubator was usually controlled at 24—25°C during the dark period.

Preparation of protein solution.

The seeds germinated in various stages (24, 48, 72, 96 hours) were prepared by rinsing it from 200 seeds on 40ml distilled water for 3 min, and were ground in a motar and pestled with 10ml phenol-acetic acid-water (2 : 1 : 1 W/V/V) at a temperature of 4°C. An equal volume of 10% acetic acid was added to the supernatant. The mixture was centurifuged at 12,000g for 30 min and the clear supernatant was used as crude proteins.

Disc polyacrylamide gel electrophoresis.

Disc electrophoresis on small column at polyacrylamide gels, a new method for the separation of serum proteins, has been developed by Ornsten and Davis (1964). The authors have modified their procedure to make possible separations of basic proteins and peptides. On the other hand, the modified method of Takayama(1964) was used for detecting the protein patterns of the seeds germinated in various stages.

A disc eletcrophoresis apparatus similar to that described by Davis (1964) was used. The glass tubes were 5mm i.d. and 70mm long. The height of the polyacrylamide gel colum was 45mm. The gel system consisting of 7.5% acrylamide, 35% acetic acid, and 5 M urea was prepared as follows.

Stock solution A contained; acrylamide, 6g; N,-N′ methylene bisacrylamide (BIS), 0.16g; urea, 12g; glacial acetic acid, 28ml; and water to a volume of 60ml.

Stock solution B(prepared fresh daily) contained: ammonium persulfate, 0.30g; urea, 12g; and water to a volume of 20ml.

The working solution was prepared by mixing Stock A, Stock B, and N, N, N′, N′-tetramethyl-ethylene diamine (TEMED)(3 : 1 : 0.02, V/V). The acrylamide solution was placed in a clean glass tube which was covered at one end with a triple layer of Parafilm: 75% acetic acid was carefully layered over the acrylamide solution and the tube was incubated at 50°C for 15 min to polymerize the acrylamide, the tubes were rinsed with 75% acetic acid and filled with the same solution. About 10—15μl of the protein sample (0.10—0.15 mg) was carefully layered between the acetic solution and the gel. Both upper and lower reservoirs of the electrophoretic apparatus(Davis, 1964) were filled with 10% acetic acid. The lower electrode served as the cathode. Electrophoresis was carried out at room temperature for one hour with a constant current of 5mA per

tube. After electrophoresis, the gel was carefully removed from the electrophoresis tube and placed in a test tube where it was stained for one hour in a solution, which was 7% in acetic acid and 1% in amido black. Excess stain was removed in 7% acetic acid for 24 hours. Each stained gels were photographed, and optical absorption curves of the protein were obtained directly with a recording densitometer.

Peroxidase and catalase zymograms.

The seeds germinated in various stages(24, 48, 72, 96 hours) were prepared by rinsing it from 200 seeds on 40ml distilled water for 3 min and were ground in a motar, and pestled 10ml cold 0.01 M tris-HCl buffer (pH8.5). The mixture was centrifuged at 12,000g for 30min, and the clear supernatant was used as crude enzyme (Loyster and Schramm, 1962; Tanaka and Akazawa, 1970). Glass tubes (7×0.5 cm internal diameter) are tightly closed at one end with flat-topped rubber stoppers. Standing upright on these stoppers, the tubes are each filled with 0.85 ml of small-pore solution at room temperature. This solution is carefully overlaid with 0.1 ml of distilled water and allowed to polymerize for 30 min. The water layer is removed, and the remainder of each tube rinsed once with large-pore solution, after which 0.15 ml of this solution is added and carefully overlaid with 0.1 ml of distilled water. A daylight flourescent lamp (15w) is placed behind the tubes about 3 inch away.

The large-pore solution becomes opalescent and polymerizes within 15 min. The water layer is removed and large-pore solution (0.15 ml) which is mixed with a sample of 50—200 μg of crude enzyme contained in 5-10 μl is added. Whenever this amount of sample is contained in a concentration sufficiently higher to off-set this dilution. The sample layer is photopolymerized for 15 min. The remaining space in each glass tube is filled with 0.2 M ethylenediamine, the rubber caps are removed, and the tubes, sample gel uppermost, are attached to the anode buffer compartment, which is then filled with tray buffer (0.2 M acetic acid containing 1 mM $CaCl_2$ and 0.2 M ethylenediamine). A hanging drop of buffer is attached to the bottom of each tube. The anode compartment, supported on a ring stand, is placed so that the tubes are immersed about 1/4 inch into the buffer contained in the cathode compartment. An alternative method of sample application is to layer the sample solution(up to 0.15 ml) on the spacer gel after the anode compartment is filled with tray buffer.

To prevent mixing the density of the sample solution is increased with sucrose. Electrophoresis carried out by applying a current of 6—8 mA per tube for 4 hours at 4°C.

When electrophoresis is completed, the gels are immediately romoved from the glass tubes by gently rimming them with a guage syringe neeedle through which a thin stream of water is allowed to pass.

Peroxidase activity was revealed by soaking the gels for one minute in a mixture consisting of equal amounts of 1% hydrogen perioxide solution and benzidine solution(1 g benzidine, 9 ml acetic acid, 33 ml water).

Visual observatin of catalase was made possible by first soaking the gel for one minute in 0.5% hydrogen perioxide, washing twice in water and then immersing it in a 1% solution of potassium iodide acidified with acetic acid (McCune, 1964; Scandalios, 1964).

Determinations of amylase and protease activity.

Preparation of enzyme solution. At each germination stage, 300 seed pieces were removed from the incubation dish and were ground at 0°C in a 0.05 M sodium citrate buffer(pH 5.7) with 0.2% $CaCl_2$(2 ml/g of grain) in a cold motar and pestle. The homogenate was filtered through cheesecloth and the filterate was centrifuged at 30,000 g at 0°C for 10 min. An aliquot of the supernatant solution was used for the assay of amylase activities by the method of Shuster and Gifford(Shuster and Gifford, 1962; Baun et al., 1970; Galsky and Lipincott, 1971).

α-Amylase assay. One ml enzyme solution was added to an equal volume of 0.5 M acetate buffer (pH 5.0) and incubated for 10 min at 37°C. After incubation, 2 ml of 1% soluble starch solution was added to it, and 5ml of 1 N acetic acid to the reaction mixture for ceasing enzyme activity. After it was diluted with 50 ml distilled water, 5ml of KI solution was added(Lee and Yoon, 1973a, b).

For the determination of α-amylase activity, Bernfeld's method (Shannon et al, 1973) was used. Absorbancy at 620 nm was measured by a DU-spectrophotometer.

β-amylase assay. The β-amylase activity was determined by the measurement of reducing sugar as glucose. For determination of reducing sugar, Nelson's method (1962) was used. 1ml enzyme solution diluted one hundredfold was added to an equal volume of 1% soluble starch solution buffered by acetate (pH 4.8). After incubation for 5 min at 40°C, 2ml of the low alkalinity copper reagent was added to the reaction mixture, and heated for 10 min in boiling water bath.

After cooling, 2ml of arsenomolybdate reagent was added. when all the cuperous oxide was dissolved after mixing, the solution was diluted to the 25 ml mark on the test tube and then allowed to stand for 15 min. Absorbancy at 540 nm was measured by a DU-spectrophotometer.

Protease activity. An aliqot of the supernatant solution gained in the preparation of crude proteins was used for the assay of protease activity (Nakamura, 1973). After the two test tubes were prepared, one was used for one reaction tube and the other tube was used for a blank tube. The two test tubes were filled with 2 ml buffer solution (7.2% Na_2HPO_4: 2.1% citric acid, 16.47: 3.53, pH 7.0) and then incubated for 10 min at 30°C. One of the test tube (blank) was added to 5 ml of 5% trichloroacetie acid and then shaked completely. Nextly the two test tubes were added to 1 ml crude enzyme solution and incubated for 10 min at 30°C. After promotion of enzyme activity, the reaction test tube was immediately added to 5 ml of 5% trichloroacetic acid, shaked completely, enzyme reaction was ceased, and each tube was let alone for 20 min so as to precipitate unisolated protein in the test tube.

Each test tube was added to 1 ml of filterated solution, 5 ml of 0.4 M Na_2CO_3, 1 ml Folin reagent, and it was colored for 20 min at 30°C. Absorbancy of 660 nm was measured by a DU-spectrophotometer.

Separation of malate dehydrogenase isozyme. The determination of malate dehydrogenase for cellulose acetate electrophoresis was performed according to the procedure of Park et al. (1971) as employed by Kohn (1968).

Crude extracts of each 300 seed pieces for use in preparing samples were ground at 0°C with 15 ml of deionized water in a cold motar and pestle. The homogenate was centrifuged at 12,000 g at 5°C for 20 min. The filterpaper (10×1.3mm) was laid on a clear glass plate, the other filter paper (2×2cm) was doubled on them, and 0.2 ml of homogenate was dropped. After the sample was absorbed under the filter paper (10×1.3mm), it was put on the cellulose acetate membrane (CAM) strip (5.6×6.6 cm, Beckman type) for 5 min and was excluded.

Electrophoresis was carried out in barbituric acid buffer (pH 8.6) at room temperature for two hours with a constant current of 0.8 mA per 1 cm width (Park and Cho, 1972). After electrophoresis, the cellulose acetate menbrane strip was removed from electrophoretic cell and the surface of CAM was doubled on the filter paper absorbed with dye solution. It was removed and was put on the filter paper absorbed water at 37° C for 60 min. The dyeing CAM strip was put into the fixation solution for 5 min, washed distilled water, and dried well. Afterward it was respectively photographed, and the bands of each dehydrogenase isozyme were obtained directly with schematic drawing and optical density curves of the MDH isozymes were obtained directly with a recording densitometer.

RESULTS

The determination of protein electrophoretic profiles.

The pattern for protein electrophoretic profiles, used as a standard, photographically enlarged to an arbitary length of 10cm. Since equal weights of the dry extract from differnt groups did not necessarily contain same amount of protein, optical densities were plotted as percentages of the total for each spectrum. The protein fractions which made up the spectrum between the seeds treated with and without acetone for 30min before sowing showed quantitative attributes and measurable dissimilarity(Figs. 1, 2).

As shown in Figs. 1—2, the group treated without acetone for 30 min before sowing had four fractions, but the group treated with acetone for 30 min before sowing had nine fractions only in the seed protein electrophoretic profiles. Therfore, the characteristic pattern for each Fig. 1—2 was obvious at once.

The protein pattern at the stage of the seed absorbed adequate water as soon as sowing were covered with the range of variability between the seed treated with and without acetone. Accordingly, it was assumed for the author that acetone treatment at the initial germinating stage had activated protein hydrolase in the nitrogen metabolism of seed germination.

In Fig.3—4, two groups had similarly five fractions with the exception of the migration situation of the fractions.

In Fig.5—6, two groups had also five fractions as refering to the curves of optical density and migration velocity. However, the migration situation of fractions was different.

In Fig.7—8, two groups had also six fractions with the conception of the optical density of the fractions.

Fig.9 gave a pattern with four bands in the fast series with centers at −5.8, −3.2, −2.3, and −0.3 cm.

Fig.10 gave a pattern with eleven bands in the fast series with centers at −8.8, −8.1, −7.1, −6.3, −5.8, −5.0, −4.2, −3.3, −2.4, −2.0, and −0.3 cm. In Fig.9—10, about 70 percent of the accessions showed 3—4 dense bands between −2.8 and −4.8 cm, whereas the rest of the accessions showed 0−0.7 dense bands, 2.0 −3.0 dense bands, 5.8—6.0 dense bands, and dense bands centered at −5.8 cm as common factor of two groups.

Fig.11 gave a pattern with six bands in the fast series with centers at −8.0, −6.2, −5.6, −5.3, −3.0, and −0.2 cm in the curves of optical density and migration velocity. However, Figs. 12 gave pattern with six bands similarly in the fast series with centers at −8.3, −6.7, −6.0, −3.8, −2.2 and −0.3 cm.

In Fig.11—12, about 80 percent of the accessions showed 1.4—4.9 dense bands between −1.3 and −5.3 cm whereas the rest of the accessions showed mainly 7.8 −8.7 dense bands, 5.4—6.2 dense bands and 0—0.4 dense bands.

Fig.13 gave a pattern with six bands in the fast series with centers at −7.9, −6.2, −5.7, −5.2, −3.0, and −0.2 cm. Accordingly, Fig. 14 gave a pattern with six bands in the fast series with centers at −7.5, −6.0, −5.3, −5.0, −2.5 and −0.1cm. In Fig. 13—14, about 70 percent of the accessions showed 1.2—4.3 dense bands between −1.1 and −4.5 cm, whereas the rest of the accessions showed 7.0—8.3 dense bands, 5.0—6.0 dense bands and a 0.1—0.4 dense band except for a 0.1—0.2 thin band.

Fig. 15 gave a pattern with six bands in the fast series with centers at −7.1, −6.1, −5.0, −4.7, −2.8, and −0.3 cm. In the other hand, Fig. 16 gave a pattern with seven bands in the fast series with centers at −7.0, −6.0, −5,7, −4.9, −4.6, −2.8 and −0.3 cm. In Fig, 15—16, about 40 percent of the accessions showed 2.0—4.0 dense bands between −2.0 and −0.4 cm, whereas the rest of the accessions showed mainly 6.8—7.8 dense bands, 4.5—5.3 dense bands and 0.1—0.5 dense bands as common factor of two groups.

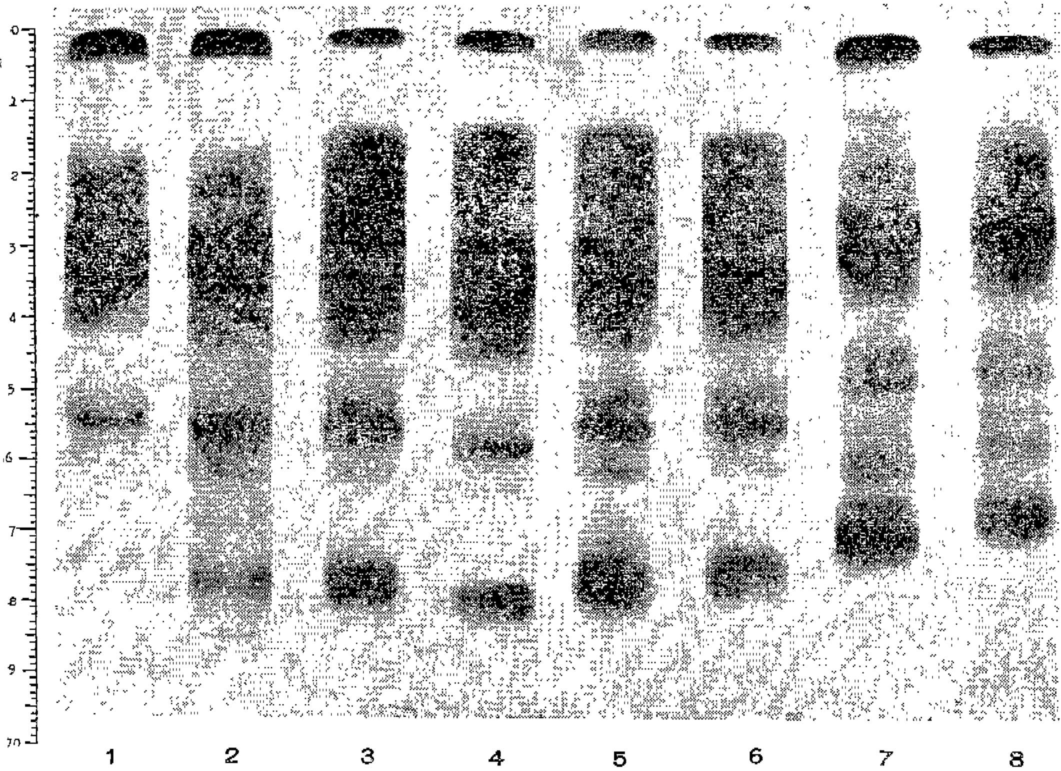

Fig.1~8. Seed protein electrophoretic profiles treated with and without acetone at various germinating stages.

Fig.1, seed treated without acetone for 30 min before sowing; Fig.2, seed treated with acetone for 30 min before sowing; Fig. 3, seed germinated for 24 hours without acetone treatment; Fig.4, seed germinated for 24 hours with acetone treatment; Fig.5, seed germinated for 48 hours without acetone treatment; Fig.6, seed germinated for 48 hours with acetone treatment; Fig.7, seed germinated for 72 hours without acetone treatment; Fig.8, seed germinated for 72 hours with acetone treatment.

The electrophoretic patterns of peroxidases and catalase isozymes.

The peroxidase zymograms revealed a main slow-migrating zone with little variability among the different germination stages. Accordingly, the seeds treated with and without acetone at each stage made no difference of electrophoretic pattern in a zymogram showing comparative rate of peroxidase isozymes.

Although the peroxidase isozyme band of seeds before sowing was not represented entirely, the seeds germinated for 24—48 hours had one band which stained with the same density. However, the seeds germinated for 72 hours had two distinct bands in this zone of which only two bands were also represented in certain distance. The peroxidases, like the catalase, seemed to be shown a small variability within the seeds germinated according to each different stage. There are several slow-migrating zones, but among them, few had a high enough activity to become visible.

Catalase isozyme bands between the seeds treated with and without acetone at each germinating stage were similar in number. Thus, the seed germinated for 24 hours without acetone treatment showed one very dense band which was also

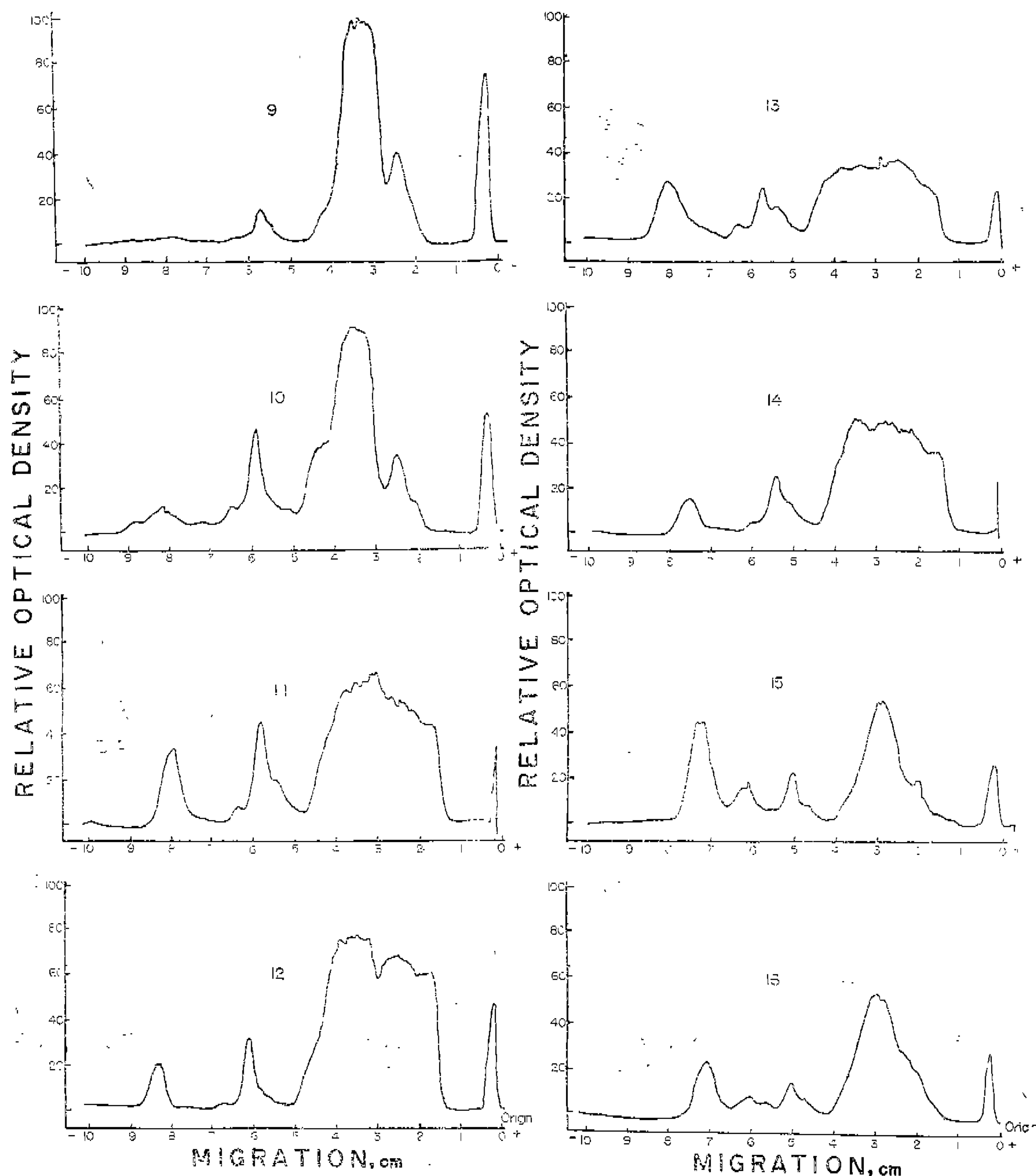

Fig. 9~16. Curves of optical density and migration velocity obtained from the electrophoretic gels. Fig.9, seed untreated with acetone before sowing. Fig.10, seed treated with acetone for 30 min before sowing. Fig.11, seed germinated for 24 hours without acetone treatment. Fig.12, seed germinated for 24 hours with acetone treatment for 30 min before sowing. Fig.13, seed germinated for 48 hours without acetone treatment. Fig.14, seed germinated 48 hours with acctone treatment for 30 min before sowing. Fig.15, seed germinated for 72 hours without acetone treatment. Fig.16, seed germinated for 72 hours with acetone treatment for 30 min before sowing.

presented in the seed germinated for 24 hours with it, but it seemed to be thin slightly. The seeds germinated for 48 hours with and without them had also one band which was represented in similar density as the groups germinated for 24 hours. The seeds germinated for 72 hours showed two bands of catalase isozyme regardless acetone treatment. The fast band between two bands was found in very thin form. Catalases here were the slowest anode enzymes.

Although the role of peroxidase is not clear, it has been speculated that they may play an inhibiting role in plant growth by limiting the amount of the auxin, IAA, through oxidation (Hunter et al., 1957).

Catalase activity was found to be completely lacking in the stem, and root homogenates, but presented in varied patterns and concentrations in the other five tissues. The endosperm showed one very dense band which was also presented in the leaf but in a dilute form (Scandalios, 1964).

The assay of α-amylase, β-amylase and protease activity.

Results presented in Fig. 17—18 showed the time sequence analysis of amylase activity at each germinating stage. In both the seeds treated with and without acetone. a marked increase in enzyme occurred from a lag period of about 12 hours. However, there was notable difference between two groups.

The breakdown of soluble starch by the crude extract from both the seeds treated with and without acetone at each stage as measured by the I_2—starch reaction (absorbancy at 620 nm) was hardly affected by heating at 70°C for 15 min.

The group treated with acetone for 30 min before sowing was more decolorized at each different stage than the other untreated group. Although physiological mechanism of the carrot seed germination with acetone treatment was not clear, it was indicated that the α-amylase activity of the seeds at each stage with acetone treatment was higher than those without acetone treatment.

An identical trend was observed by analyzing

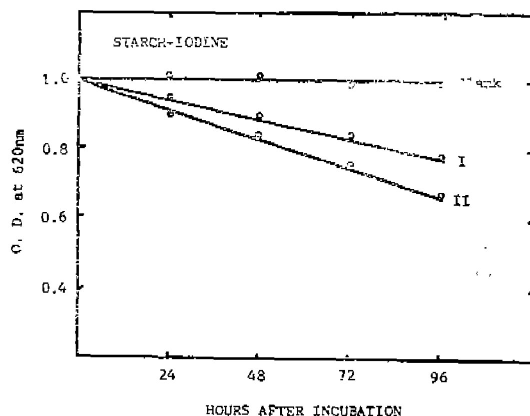

Fig. 17. α-Amylase activities of crude extract from the carrot seeds treated with and without actone.

Blank: no enzyme; Ⅰ. the germinating stage of seeds treated without acetone; Ⅱ: the germinating stage of seeds treated with acetone for 30 min before sowing.

the maltose formed (Bernfeld, 1955). It has thus implicated that β-amylase may play a minor role in hydrolyzing the reserve starch in germinating rice seed endosperm, unlike the case with barley seeds (Norris, 1968). In Fig. 18, the optical density of β-amylase activity in the group treated with acetone at each germinating stage was, more or less, also higher than the group treated without acetone. Therefore, the β-amylase activity of seeds treated with acetone was more increased than in the untreated group especially after the germination of 24 hours.

By comparing the results of detecting starch hydrolysis as presented in Fig. 18, it will be noted that the amount of low molecluar weight reducing sugar accounts for only a part of the total starch broken down in seeds.

Protease which hydrolyzed various proteins was detected in the seeds of various germinating stages, because protein metabolism was directly occurred in germinating seeds. Storage protein was hydrolyzed by protease and transformed into the essential amino acids needed for the developing of embryo. As shown in Fig. 19, the protease activity of the seeds treated with acetone for 30 min at each germinating stage was higher than the group

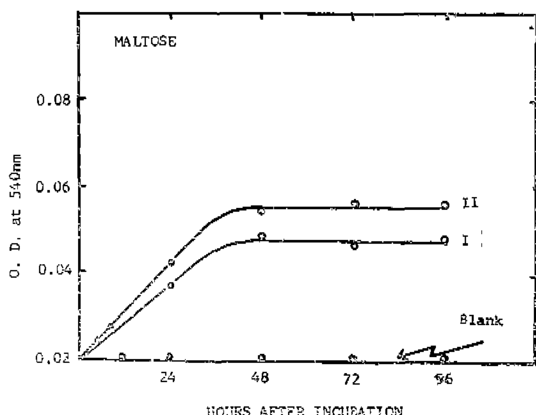

Fig. 18. β-Amylase activities of crude extract from the carrot seeds treated with and without acetone.

Blank: no enzyme; I :the germinating stage of seeds treated without acetone; II : the germinating stage of seeds treated with acetone for 30 min before sowing.

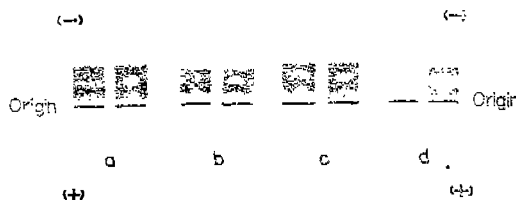

Fig. 20. Cellulose acetate electrophoretic profiles of malate dehydrogenase isozymes treated with and without acetone at various germinating stages.

a. Seed treated with (right) and without acetone (left) before sowing. b. Seed germinated for 24 hours with (right) and without acetone treatment for 30 min (left) before sowing. c. Seed germinated for 48 hours with (right) and without acetone treatment for 30 min (left) before sowing. d. Seed germinated for 72 hours with (right) and without acetone treatment for 30 min (left) before sowing.

treated without acetone.

The pattern of malate dehydrogenase isozyme. The pattern of malate dehydrogenase isozyme at various germinating stages of carrot seed was mostly separated from the two kinds of band. Park et al., (1973) reported that the activity of malate dehydorgenase isozyme was high in slow-migrating zones and was slow in fast-migrating zones in the muscle tissue of Vertebrata.

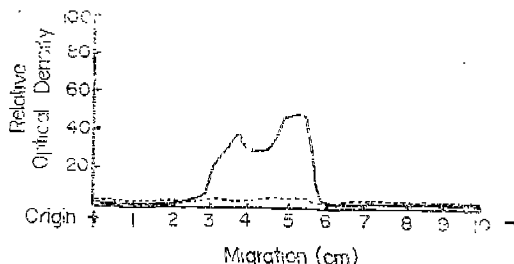

Fig. 21. Curves of optical density and migration velocity obtained from the membrane strip of cellulose acetate electrophoresis.

MDH isozyme of the seeds germinated for 72 hours with acetone treatment. MDH isozyme of the seeds germinated for 72 hours without it.

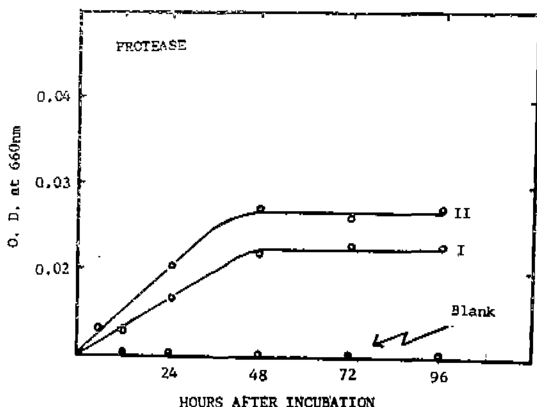

Fig. 19. Protease activities of crude extract from carrot seeds treated with and without acetone.

Blank: no enzyme; I, the germinating stage of seeds treated without acetone; II : the germinating stage of seeds treated with acetone for 30 min before sowing.

In Fig. 20, the group treated with acetone for 30 min before sowing (a) showed certain thick optical density in comparison with the untreated group. However, the groups germinated for 24—48 hours, regardless of acetone treatment, were scarcely similar though the untreated groups were thick a little. Thus, the group germinated for 72 hours with acetone treatment showed obviously two bands on the surface of CAM, but the group germinated for 72 hours without acetone treatment

did not appear. It indicated that the activity of malate dehydrogenase in TCA cycle was low in the group germinating for 72 hours without acetone treatment, but was high in the group germinating for 72 hours with acetone treatment.

Consequently, the activity of malate dehydrogenase in the seeds germinated with acetone treatment was activated, when they were initially sowed, and were germinated for 72 hours especially, as shown in Fig.20 and 21.

DISCUSSION

The present data indicated that some remarkable changes of protein metabolism were induced in the seeds treated with acetone such as in the vernalized winter wheat embryo (Pauli and Mitchell, 1960; Zech and Pauli, 1962). Such changes did not occur in the process of normal germination and were caused specifically by the vernalizing at a low temperature treatment. Lately, Steward (1966) reported that new proteins were formed in tulip bulbs treated with a low temperature and these proteins showed the highest mobility in disc electrophoresis.

The higher mobility implied that the induced protein by vernalization were of lower molecular weights as compared with the other soluble proteins. Wrigley et al. (1966) reported on the electrophoretic separation of soluble proteins prepared from wheat leaf. Although the age of their material (30 day-old leaves) was different from that of present ones (Teraoka, 1967), the number of protein bands they detected was approximately the same as obtained in the work.

In the present status of both physiology and genetics, it was especially desirable to be able to resolve the soluble proteins which were present in plants (Chang and Steward, 1962). A similar degree of convenient resolution of the protein fractions may be fraught with similar grains. Present-day ideas on gene-enzymic relationships and gene-protein relationships and on the mechanism of protein synthesis would endow a convenient and satisfactory procedure for separating and recognizing particular protein moieties with equal interest for the study of metabolism and genetics.

Polyacrylamide gel electrophoresis, which has been so successful in the fractionation of proteins and in which the gel pore size can be closely controlled, has been little used for RNA (Fredrick, 1964). Separation of small RNA molecules has been described by Richards, et al. (1965).

The potential of this approach to taxonomic problems is well illustrated by studies in successful work of Vaugham and his collegues on the seed proteins of various species of Brassica and Sinapis (Vaugham, et al. 1966; Noms, 1968). By a combination of serological and gel electrophoretic techniques these studies have thrown considerable light on some physiological status in germinating carrot seeds and indicated the effect on possible accelerated relationship.

It seemed reasonable to assume that the electrophoretic pattern of membrane proteins should reflect genetic identify or nonidentify of microorganisms. Our results appear to confirm this by showing the electrophoretic patterns of the various species to be specific and correlated with serological data (Rottem and Razin 1967). The electrophoretically abnormal stickle cell hemoglobin differs from normal by a single amino acid residue, since then numerous examples of the same type have appeared in the literature (Ingram, 1958).

Consequently, the accumulated gene mutation which distinguish species may be identified in part through their effects on the mobility of specific fractions of the protein spectrum (Johnson and Hall, 1965). Protein have lower constituent mobilities than most buffer ions, even in the substance of molecular sieving, making it necessary to minimize the lower stocking limit (Chrambach and Rodbard, 1971).

The electrophoretic patterns confirm cytogenetic evidence that Emmer and Timopheevi groups stem, respectively, from the Syric-Palestinian and Transcaucasian races of their wild progeniter, *T. dicoccorides* (Johnson, 1967). Bands in the electrophoretic patterns from different species are tested

by reference to for homology(that is equivalent migration velocity), the pattern obtained from a mixture of their proteins (Johnson et al., 1967).

It has an albumin pattern highly constituent among accessions with respect to a broad, dense band centered at −9.7cm that tends to fuse with a narrow one at −9.0cm(Johnson, 1972). The obvious dissimilarity of protein electrophortic profiles in germinating carrot seeds was showed in that Fig.1 (without acetone) had four fractions, Fig.2 (with acetone) had nine fractions, Fig.3—4 had similary five fractions, Fig.5—6 had also five fractions, Fig.7—8 had also six fractions, but fig.9 (without acetone) gave a pattern with four bands, Fig.10 (with acetone) gave a pattern with eleven bands, Fig.11 (the group germinated for 24 hr without acetone treatment) gave a pattern with six bands, Fig.12 (the group germinated for 24hr with acetone treatment) gave a pattern with six bands, Fig.13—14 gave also a pattern with six bands, Fig.15 (the group germinated for 72hr without acetone treatment) gave a pattern with six bands, and Fig.16 (the group germinated for 72hr with acetone treatment) gave a pattern with seven bands.

On one hand, the activities of α-amylase, β-amylase and protease in the groups treated with acetone were higher than in the untreated group, except for the activities of peroxidase and catalase regardless of acetone treatment.

On the other hand, the activity of malate dehydrogenase only when the seeds were initially sowed, and then were germinated for 72 hours with acetone treatment, was markedly activated as shown in Fig. 22.

It was suggested for the author that these results mentioned above had explained certain mechanism of activated isozymes in germinating carrot seed with acetone treatment.

In the other reports, according to Kay et al. (1967) the peroxidase isozymes of horseradish have molecular weight of approximately 45,000 but higher molecular weight forms have recently been detected in peas (Janssen, 1970), wheat embryos (Lanzant and Galante, 1964), and in tissue cultures which secreted complexes associated with hydroxyproline into the medium (Olson et al., 1969). There is no single explanation of the stable and multiple peaks of activity of peroxidase in extracts from Sorgham eluted from either Sephadex or Agarose columns(Stafford and Bravinder-Bree, 1972).

In the peroxidase zymogram, the seeds treated with and without acetone treatment at each stage made no difference of electrophoretic pattern in zymogram showing comparative rate of peroxidase isozymes. Especially, the isozyme band of the seeds before sowing was not represented entirely and it seemed to indicate that peroxidase activity was activated at the first stage in germinating seeds.

Although the rôle of peroxidase is not clear, it has been speculated that they may play an ihibiting role in plant growth by limiting the amount of the auxin, indoleacetic acid, through oxidation. (Hunter and Markert, 1957). Interestingly, the results of investigation show that peroxidase activity is greatest in the most actively growing tissues (leaves, stem, silks and husk) where IAA activity would be expected to be predominant (Scandalios, 1964).

Catalase, on the other hand, proved to be of little value since the catalase derived from a wide range of stage in germinating seeds had the same mobility in the polyacrylamide gel electrophoretic technique. Scandalios (1964) reported that the only case in this investigation where enzyme activity appeared to be completely absent from any tissue was that of catalase in stem, root and husk homogenates. It was recently found that there are two variants of catalase in maize endosperm under genetic control. Catalase activity was also found to be completely lacking in the seeds before sowing with and without acetone treatment. Accordingly, the difference of electrophoretic pattern in zymogram showing comparative rate of catalase isozy-

mes was revealed between the seeds treated with and without acetone at each stage.

Tanaka(1970) demonstrated that in both embryoless (+GA) and embryo-attached (−GA) half-seed endosperms, a marked increase in enzyme activity occurred after a lag period of about 1 day. However, there was a notable difference between the two systems.

Amylase activity in the medium of GA-treated, embryoless endosperm tissue showed an early linear increase between 3 and 8 days, while that from the extract increased slightly between 3 and 6 days and declined thereafter.

The solution of the group treated with acetone for 30 min was more decolorized at each different stage than in the untreated group. Although physiological mechanism in germinating seeds with acetone treatment was not clear, it was indicated that α-amylase activity of the seeds at each stage with acetone treatment was higher than in the untreated groups. Accordingly, the optical density of β-amylase activity in the treated groups at each stage was higher in comparison to the untreated groups.

Storage protein was hydrolyzed by protease and transformed into the essential amino acids needed for the developing of embryo. The protease activity of the seeds treated with acetone for 30 min at each germinating stage was greater than the other.

The alkaline proteinase from *Aspergillus oryzae* and a sojae have been purified by Subramanian and Kalnitsky (1964). Although the enzyme had many properties in common they differed in amino acid composition and molecular weight. These results are consistent with the observed differences in electrophoretgrams of the enzymes since electrophoretic mobility is considered to reflect the net charge, shape and dimension of an enzyme protein molecule (Subramanian and Kalnitsky, 1964).

ACKNOWLEDGEMENT

The author wishes to express his sincere appreciation and gratitude to Professors Min Jai LEE, Soon Woo HONG, Yung Ho CHUNG, and Sang Yun PARK for their invariable advice and guidence in this work.

The author would like to thank Mr. M.C. PARK and all faculties at Department of Biology, Atomic Energy Institute.

摘　　要

本研究는 당근 種子의 각 발아단계에 있어서 蛋白質의 消長과 거기에 관여하는 酵素學的 機作의 一端을 구명하여 發芽生理의 體系를 세우는데 그 目的을 두었다.

파종하기 전의 acetone 처리는 phytohormone, antibiotics, 農藥 등의 처리로서 보다 發芽促進의 効果를 나타내고 있는 것이기 때문에 이 모든 實驗에서 각 발아단계의 시료를 acetone처리구와 비 처리구로 區分하여 disc 電氣泳動裝置에 의한 polyacrylamide gel에 나타나는 pattern과 그 發芽過程에 있어서 효소의 活性을 측정하여 다음과 같은 結果를 얻었다.

1. Disc 電氣泳動 裝置에 의한 各 發芽段階의 protein pattern은 처리구와 비처리구 사이에 共通的인 basic pattern을 찾아 볼 수 있었으나, gel에 나타난 protein band의 數, optical density. migration velocity의 差異를 보였다.

2. Peroxidase와 catalase 活力의 消長에서 polyacrylamide gel에 의한 isozyme band는 처리구와 비처리구에 數的인 差異는 나타나지 않았으나 처리구의 特定한 發芽段階에서만 optical density가 높아 효소의 活性을 나타냈다.

3. 加水分解 효소 중에서, 澱粉加水分解 효소인 α-amylase, β-amylase의 活性은 처리구에서 더욱 높았고 또한 단백질 加水分解 粗酵素인 protease의 活性도 처리구에서 더 높았다.

4. Energy 代謝 經路의 TCA cycle에서 cellulose acetate 전기泳動裝置에 의한 malate dehydrogenase의 isozyme band pattern은 72時間의 처리구와 비처리구에서 뚜렷한 差異가 나타났다. 따라서 처리구의 isozyme band 數가 2개 뚜렷하게 나타난 것으로 보아 活性이 높음을 알 수 있었다.

以上의 實驗結果는 파종하기전의 acetone 처리가 種皮속의 lipid 複合物質의 分解에 作用되어 細胞內의 원활한 水分吸收로 加水分解 효소의 活性을 증진시켰

고, 다른 한편으로는 分解된 脂肪酸과 glycerin으로 energy源이 되어 呼吸代謝의 촉진을 誘發 시킨것으로 보아야 할 것이다.

REFERENCES

Angei, S. L. 1961. Repressible matters for the germination of carrot seeds. *Noko and Engei*. 36:150-153.

Baun, L. C. and E. P. Palmiano, C. M. Perez, and B. O. Juliano. 1970. Enzymes of starch matabolism in the developing rice grain. *Plant Physiol*. 46:429-434.

Bernfeld, P. 1955. Amylase, α and β. In:Method in Enzymology, Vol. I. eds. S. P. Colowick and N. O. Kaplan, Academic Press, New York. pp. 149-158.

Bibley,R.O. 1948. Physiological studies of weed seed germination, *Plant Physiol*. 23:467-484.

Chang, L.O.,and Steward. 1962. Electropheretic seperation of the soluble proteiins of Neurospora. *Nature* 193: 756-759.

Chrambach, A., and D. Rodbard. 1971. Polyacrylamide gel electrophoresis. *Science* 173: 440-450.

Davis, B. J. 1964. Method and application of human serum proteins. *Ann. N.Y. Acad. Sci*. 121:404-427.

Evenari, M. 1949. Germination inhibitors. *Bot. Rev.* 15: 153-194.

Fredrick, J.F. 1964. Conference on gel electrophoresis (ed.); *Ann. Sci*. 121: 251-257.

Galsky, A.G. and Lippincott. 1971. Induction of α—amylase in barely endosperm by substrate levels of glutamate. *Plant Physiol*. 47: 551—554.

Gornter, R.A. 1931. Water in its biochemical relationship. *Ann. Rev. Biochem*. 3:1—22.

Hunter, R.L., and C.L. Markert. 1957. Histochemical demonstration of enzymes seperated by zone electrophoresis in starch gels. *Science*. 125: 1294—1295.

Ingram, V.M. 1953. Abnoraml human haemoglobin I. The comparison of normal human and sickle—cell haemoglobin by "finger printing". *Biochem. Biophys. Acta*. 28: 539—545.

Janssen, M.G.H. 1970. Indolcacetic acid oxidase, peroxidase and polyphenol oxidase of pea roots. *Acta Bot. Neerl*. 19: 73—80.

Johnson, B.L., and O. Hall. 1965. Analysis of phylogenetic affinities in the Tritticinae by protein electrophoresis. *Amer. J. Bot*. 52: 506—513.

_______, 1967. Confirmation of the genome donors of Aegilops cylindrica. *Nature* 216: 859—862.

_______, 1972. Seed protein profiles and the origin of the hexaploid wheats. *Amer. J. Bot*. 59: 952—960.

Jones, H.A. 1950. Physiological study of maple seeds. *Bot. Gaz*. 69: 127—152.

Kay, E.,L.M., Shannon, and J. Y. Lew. 1967. Peroxidase isozymes from horseradish roots. 11. Catalytic properties. *J. Biol. Chem*. 242: 2470—2478.

Kohn, J. 1968. Cellulose acetate electrophoresis and immunodiffusion techniques,, In I. Smith (Ed.) chromatographic and electrophoretic techniques Vol 11;Zone electrophoresis, 2nd ed. pp. 84—146, John Wiley, New York, and London.

Kwon, O.Y. 1968. Effect of some reagents, antibiotics, phytohormone, and agricultural chemicals on the germination of carrot seed. The thesis of Andong Teachers Colllege. 1: 209—215.

_______, 1970. Effect of X—ray and ultraviolet light in the germination of carrot seed. *Kor. J. Bot*. 13:85—90.

_______, 1971. Effect of some incrganic compounds in the germ ination of carrot seed. *ibid*. 14: 1—6.

_______, 1974. Effect of some organic compounds inthe germin ation of carrot seed. The thesis of Andong Teachers College. under printing.

Lanzant, G.A., and E. Galantc. 1964. Peroxidases from wheat embryo ribosomes. *Arch. Biochem. Biophys*. 106: 20—24.

Lee, Y.N., and K.H. Yoon. 1973. Studics of the amylase of Rhisopus (11). *Kor. J. Microbiol*. 11:89—100.

_______, _______, 1973. Studies on the amylase of Rhizopus (111). *ibid*. 11.: 121—128.

Loyster, A., and M. Schramm. 1962. The glycogen amylase complex as a means of obtaining highly purified α—amylase. *Biochem. Biophys. Acta*. 65:200—206.

McCune, D.C.1964. Multiple peroxidase in corn. *Ann. N.Y. Acad. Soc*. 94: 723—729.

Nakamura, S.1973. Measurement method of protease. *Ferment. Chem*. 66: 679—681.

Nelson, N. 1962. Methods in carbohydrate chemistry. *J. Biol. Chem*. 153: 357—376.

Norris, J.R. 1968. The application of gel electrophoresis to the classification of micro—organisms (ed.). Systematic Association Special Volume. 2: 49—56.

Ohda, S. M. 1956. The protective method for the disease of carrot seed. *Noko and Engei*. 31:1423—1428.

Olson, A.C., J. Ewans, D.P. Frederick, and E. F. Jansen. 1969. Plant suspension culture media macromoleculespectic subssances, proteins, and peroxidase. *Plant Physiol*. 44: 1594—1600.

Paleg, L.G.1960. Physiological effects of gibberellic acid: 1. On carbohydrate metabolism and amylase activity of barley endosperm. *ibid*. 35: 293—299.

Park, S.Y., S.O. Hong, and D.H.Cho. 1971. A variant of lactate dehydrogenase in brains of several species. The thesis collection of Sungungan Univ. 16: 1—16.

_______, and D.H.Cho. 1972. Seperation of dehydrogenase isozymes of Korean Squamata. *Kor. J. Zool*. 16:101—104.

_______, S.O. Kim, and D.H. Cho 1973. Lactate and malate dehydrogenase isozymes of Korean Squamata. *ibid*. 16: 55—65.

Pauli, A.W. and H.L. Mitchell. 1960. Changes in certain nitrogenous constituents of winter wheat as rel ated to cold hardness. *Plant Physiol*. 35: 539—542.

_______, B.J. Kolp, and F.C. Stickler 1961. Relationship of cold hardness to soluble protein nitrogen content and epicotyl growth rates in winter wheat. *Crop. Sci*. 1: S137—138.

Reisfeld, R. A., U. J. Lewis, and D.E. Williams.