Proteolytic Enzyme Activity and Storage Protein Degradation in Cotyledons of Germinating Peanut (*Arachis hypogaea* L.) Seeds

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Most of the storage proteins in peanut cotyledons from *Arachis hypogaea* L., cv. "Florunner", disappeared quantitatively by day 10 (91 to 5 mg/cotyledon). Gel electrophoresis showed a decrease of major storage globulins by day 10 in extracts and simultaneously a number of new polypeptides appeared which also became difficult to resolve by day 20. Rapid protein depletion between days 4 and 10 was associated with increasing levels of acid (pH 5.0)—and neutral pH—sulfhydryl proteolytic activities (major activity was at acid pH); incubation mixtures containing 10 mM 2-mercaptoethanol showed optimum activity. The proteolytic enzymes hydrolyzed substrates of total, arachin, and nonarachin proteins from ungerminated peanut seeds; the latter was most readily hydrolyzed. This proteolytic activity was heat labile at reaction temperatures above 60 °C and became inactive within 4 weeks when stored at 4 °C; it was very stable when extracts were frozen stored at −18 °C. Addition of ungerminated seed extract to 10-day enzyme preparation inhibited proteolytic activity. Changes in leucine aminopeptidase activity detected by gel electrophoresis coincided with increasing proteolytic activity during seed development.

In peanut seeds, arachin comprises most of the storage protein and is stored in subcellular bodies or aleurone grains, whereas, conarachin has been suggested to be mainly in the cell cytoplasm, but can be particle bound (Altschul et al., 1964). During germination, storage proteins such as these are degraded to polypeptides and free amino acids by proteolytic enzymes and translocated to the developing embryo axis (Bagley et al., 1963; Beevers and Splittstoesser, 1968; Daussant et al., 1969). Degradation of storage proteins during the early stages of seed germination was shown by changes in their gel electrophoretic mobility due to removal of amino groups by a deamidase enzyme (Basha and Beevers, 1975). Ghetie (1966) proposed that during this early stage of seedling development, peptide bonds were hydrolyzed after initial "double activation" of both substrate and a specific proteolytic enzyme. This activity depolymerizes proteins from their native conjugates and dissociates them into component polypeptides for proteolytic hydrolysis to free amino acids.

Proteolytic activity measured as the capacity to hydrolyze proteins of animal origin such as casein and (or) hemoglobin (Beevers, 1968) and globulins of plants (Basha and Beevers, 1975) was shown to occur in germinating seeds. An active proteolytic enzyme was isolated from germinating peanuts and shown to be capable of hydrolyzing *N*-benzoyl-D,L-arginine-*p*-nitroanilide (BAPA) (Irving and Fontaine, 1945; Mainguy et al., 1972). Apart from this, little is known about proteolytic enzymes of germinating peanuts.

In the present study, fractions containing proteolytic enzymes were extracted from germinating peanuts and partially characterized relative to their ability to hydrolyze seed storage proteins. Attempts were also made to determine the possible presence of inhibitors of proteolytic enzymes in peanuts. Identifying and characterizing proteolytic enzymes in peanuts can possibly lead to use of these native activities to hydrolyze storage proteins in seed isolates to new functional forms.

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MATERIALS AND METHODS

Plant Material. Peanuts (*Arachis hypogaea* L., cv. "Florunner") were germinated at 30 °C in the dark in sterile vermiculite. Cotyledons were obtained from seedlings at 2-day intervals during a 3-week test period.

Protein Extraction. Testa-free cotyledons from seeds germinated for various time intervals (0 to 20 days) were ground for 2 min at high speed in a polytron homogenizer with 1 M NaCl–20 mM sodium phosphate buffer, pH 7.0. The homogenates were stirred for 30 min and clarified by centrifugation at 20 000*g* for 20 min. The pellets were reextracted twice and the resulting supernatants pooled and dialyzed against deionized water. Protein content of these extracts were determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Polyacrylamide Gel Electrophoresis. The dialyzed protein extracts from cotyledons of germinating seeds were examined by disc electrophoresis on 10% polyacrylamide gels according to the methods of Canalco (1973), Cherry et al. (1973), and Basha and Cherry (1976).

Preparation of Enzyme Extracts. After removal of testa and embryo axis, cotyledons were chilled, washed in cold deionized water, and ground for 2 min at medium speed in a polytron homogenizer with cold 0.02 M sodium phosphate buffer, pH 7.2 (tissue to buffer ratio was 1:5, w/v). These preparations were made with or without 10 mM 2-mercaptoethanol. The homogenates were squeezed through cheese cloth and miracloth (Calbiochem) and then centrifuged at 40 000*g* for 10 min. The resulting supernatants were used to determine the protease activity. In further studies, the centrifugation speed was varied from 900 to 40 000*g* to determine the distribution of proteolytic activity in various extracts. Thermal stability of extracts was determined by exposing them to temperatures from 40 to 100 °C for various time intervals prior to enzyme assay. Enzymatic activity in extracts was followed during storage at 4 and 18 °C for various time intervals to 4 weeks.

Preparation of Substrates. Testa-free cotyledons from ungerminated seeds were ground in a Wiley mill (60 mesh), and the meal was defatted with hexane, air-dried, and stored at −18 °C. The method of protein extraction was developed from Basha and Cherry (1976). In general, defatted meal was homogenized in a Sorvall Omni-Mixer with 1 M NaCl–20 mM sodium phosphate buffer (pH 7.0)

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and stirred for 30 min. The homogenate was centrifuged at 20 000*g* for 20 min. The pellet was reextracted twice, and the resulting supernatants were pooled (total protein) and dialyzed against deionized water for 2 days. The dialysate was centrifuged at 20 000*g* for 15 min at 4 °C and the supernatant designated as nonarachin protein. The pellet was suspended in 0.02 M sodium phosphate buffer (pH 7.0) and designated as arachin. These three protein substrates were each adjusted to levels of 10 mg/mL.

Standard Protease Assay. Enzyme extract (1 mL) from germinated cotyledons was incubated with 1 mL of either total protein, nonarachin, or arachin fractions (10 mg/mL) and 1 mL of 0.2 M sodium citrate buffer, pH 5.0, in a shaker bath at 40 °C for 3 h. The reaction was terminated by the addition of 1 mL of 20% trichloroacetic acid. A reaction mixture in which 20% trichloroacetic acid was added immediately after the addition of enzyme extract served as a zero time control. These samples were allowed to precipitate overnight at 4 °C and centrifuged. Aliquots from the trichloroacetic acid soluble supernatants were analyzed for increases in ninhydrin positive material (proteolytic activity) by the method of Moore and Stein (1954). Appropriate modifications of this assay were made to establish optimum incubation pH, temperature, time, and sulflhydryl requirements. In studies on pH effects, 0.2 M sodium phosphate buffer ranging in pH from 6.5 to 9.0 and 0.2 M sodium citrate buffer, pH 2.0 to 6.0, were used. The sulfhydryl content of the reaction mixture was modified by adding various amounts (0 to 1 M) of 2-mercaptoethanol.

Leucine Aminopeptidases. Enzyme extracts were electrophoresed in 7.5% polyacrylamide gels according to the procedure of Cherry et al. (1973). After electrophoresis the gels were incubated in a mixture containing 50 mL of 0.2 M Tris–malate buffer, pH 2.0, 10 mL of 0.15 M NaOH, 40 mL of deionized water, 50 mg of Black K salt, and 40 mg of 1-leucyl-β-naphthylanilide–HCl (Scandalios, 1969). All gels contained approximately 100 μg of protein and were incubated for 50 min. The reaction was stopped by placing the gels in 7% acetic acid.

RESULTS AND DISCUSSION

Proteins of Germinated Cotyledons. Evaluation of protein content (mg/cotyledon) in extracts of germinating cotyledons showed a decline in these constituents from 91 to 69 mg during the first 4 days after imbibition, then a drop from 69 to 5 mg between days 4 and 10. After day 10, the rate at which extractable protein decreased was small (5 to 2 mg). Thus, these data showed that approximately 95% of the protein had disappeared from cotyledons by day 12 after germination. Similar changes in protein content of germinating peanut cotyledons have been reported (Dechary et al., 1961; Bagley et al., 1963; Daussant et al., 1969).

The breakdown of these cotyledonary proteins was further investigated qualitatively by polyacrylamide gel electrophoresis (Figure 1). Protein extracts from ungerminated seeds (0 day) consisted mainly of the monomeric (1.5 cm) and dimeric (1.0 cm) components of arachin. Except for intensification of two bands in region 0–0.5 cm, no major changes in seed proteins were observed during the first 2 days of germination. Major changes in protein composition became evident at day 4. The arachin components and the bands in region 2.0–3.5 cm decreased quantitatively in the gel patterns and simultaneously there appeared numerous proteins in region 2.5–7.0 cm. The appearance of new components in the gel patterns indicate the hydrolysis of the major storage proteins of peanuts by proteolytic enzymes to polypeptides of various sizes and

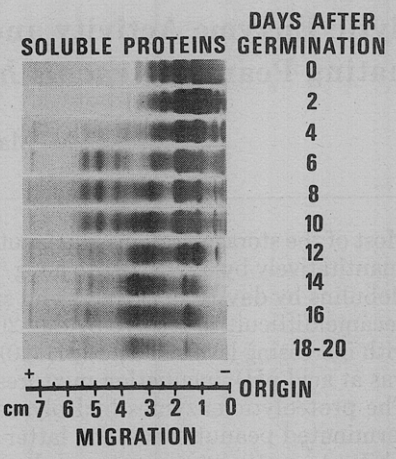

Figure 1. Gel electrophoretic patterns of total protein content of extracts from cotyledons during germination of peanuts.

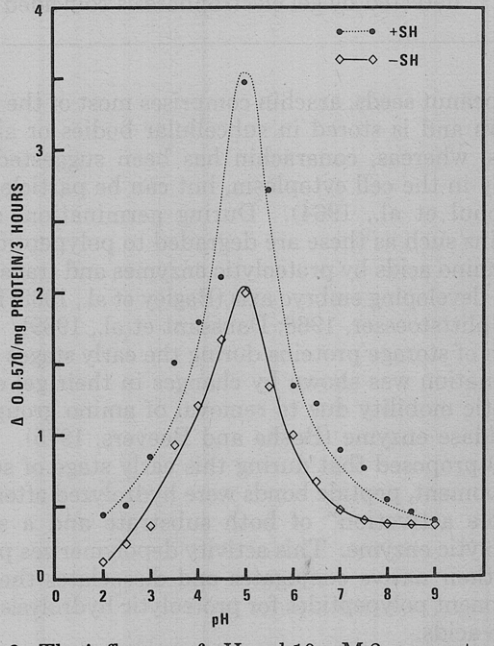

Figure 2. The influence of pH and 10 mM 2-mercaptoethanol on the capacity of extracts prepared from cotyledons of 10-day germinated peanuts to hydrolyzed total proteins of ungerminated seeds.

free amino acids prior to their transportation to the embryo axis. After day 14, no further major changes were noted in the few remaining proteins detectable by gel electrophoresis. This coincided with the observation that a consistently low quantity (2 to 5 mg/cotyledons) of protein remained in the cotyledons between days 12 and 20.

Characterization of Proteolytic Enzymes. Preliminary studies suggested that cotyledons from seedlings germinated for 10 days contained high amounts of proteolytic activity using storage proteins from dormant seeds as substrate. This proteolytic activity reached optimum values when the extracts contained 10 mM 2-mercaptoethanol (Figure 2). Proteolytic activity increased as the sulfhydryl content was raised from 0 to 10 mM (ΔOD from approximately 1.6 to 3.5 units), then decreased rapidly (to 0.7 unit) between 0.01, 0.1, and 1.0 M reagent (Figure 3). Maximal activity was observed at the incubation pH 5.0 (Figure 2; cf. ΔOD 570/ (mg protein)$^{-1}$ (3 h)$^{-1}$ of approximately 3.5 to 2.0 units with and without the thio-reducing reagent, respectively). The enhanced hydrolytic activity suggested that low levels of thio-reducing reagent may protect sulfhydryl groups in proteolytic enzymes

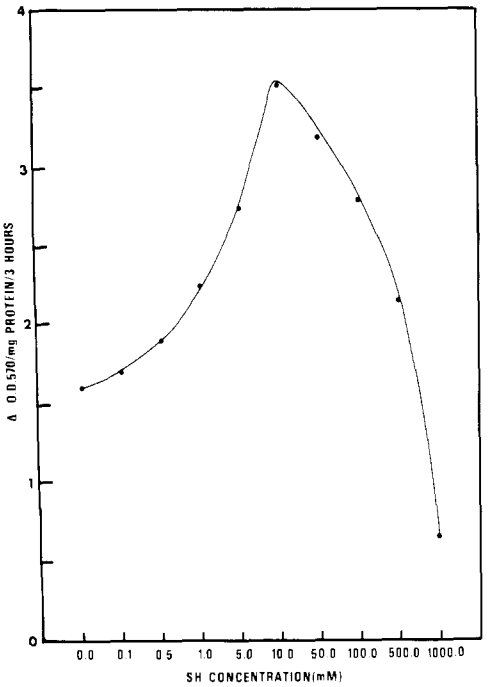

Figure 3. The effect of various concentrations of 2-mercaptoethanol (SH) in the incubation mixture on the proteolytic activity of 10-day cotyledonary extracts.

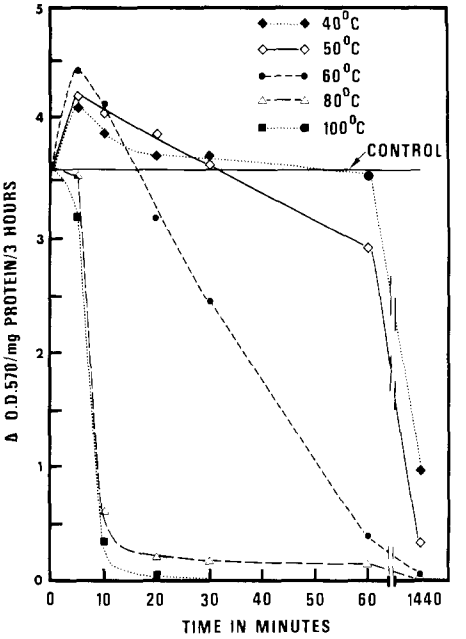

Figure 4. Thermal inactivation of proteolytic activity in 10-day cotyledonary extracts to hydrolyze total proteins of ungerminated seeds. Thermal stability of proteolytic enzymes were determined by preincubating extracts at temperatures 40 and 100 °C for time intervals to 24 h prior to assay.

during isolation from peanut seeds as was shown with peas by Basha and Beevers (1975). However, at high concentrations it prevents the oxidation–reduction reaction associated with sulfhydryl group(s) in the enzymes and/or substrates (Cherry and Ory, 1973a; Basha and Beevers, 1975).

Varying the incubation temperature (-20 to 100 °C) showed that 10-day extracts contained maximal proteolytic activity, in a typical bell-shaped curve, at 40 °C. In addition, thermal stability of proteolytic enzymes was determined by preincubating cotyledonary extracts at various temperatures for different time intervals prior to assay (Figure 4). Preincubating enzyme extracts at 40–60 °C for 10 min increased proteolytic activity. Although preincubation at 60 °C initially stimulated activity, subsequent loss of activity was more rapid than at 40 and 50 °C. Inactivation increased rapidly as the temperature was raised above 60 °C, becoming virtually instantaneous by 100 °C.

Proteolytic activity of the enzyme extract from 10-day germinated cotyledons stored at 4 °C declined gradually from 10 to 70 h and then rapidly afterwards to 4 weeks (Figure 5). Within 5 days, most of the enzyme activity was lost from this extract. In contrast, the preparation placed at -18 °C remained essentially unchanged during the 4-week storage interval.

When varying amounts of 10-day extract were used to hydrolyze total protein, a linear relationship was observed between enzyme concentrations of 0.2 to 1.0 mL (Figure 6). The initial velocity lag was possibly due to the presence of inhibitors in the incubation mixture. Evidently, enzyme levels above 0.2 mL in the incubation mixture were great enough to react with the substrate. Ungerminated seeds from which substrate protein was extracted have been shown to contain inhibitors of proteolytic enzymes (Astrup et al., 1962; Cepelak et al., 1963; Dechary, 1970; Cherry et al., 1973). This will be discussed in more detail in a later section of this paper.

A typical substrate concentration curve was observed when the enzyme extract from 10-day cotyledons of germinated seeds was incubated with various amounts of substrate (total protein). Maximal activity was attained at a substrate level of 0.4 mL.

Proteolytic Activity in Cotyledons of Germinating Peanut Seeds. Enzyme extracts prepared from cotyledons of germinating seeds and tested for proteolytic activity at pH 5.0 and 7.0 showed varying capacities to hydrolyze total protein, arachin, and nonarachin components prepared from dormant peanuts (Figure 7). Since the cotyledons varied in weight and protein content at each time interval during germination, the proteolytic activity was expressed both on protein and cotyledonary bases. The enzyme extracts from seeds germinated up to 4 days showed low amounts of proteolytic activity regardless of substrate used. On the other hand, enzyme activity (expressed on milligram of protein basis) increased rapidly from day 4 onward, reaching a maximum in seeds germinated for 10 days. After day 10, the proteolytic activity decreased rapidly. During the test period of 0 to 20 days, similar activity profiles were observed with total protein, nonarachin, and arachin as substrates. However, proteolytic activity was lower with arachin than with total protein and nonarachin fractions as substrates.

Interestingly, when proteolytic activity at pH 5.0 was expressed on a cotyledonary basis, maximal enzyme activity was observed on day 8 rather than on day 10 of germination. This is in contrast to the activity profiles obtained by expressing the activity on a milligram of protein basis. This variation is expected since cotyledons contain higher amounts of enzyme proteins at day 8 than at day 10 after germination.

Further studies show that proteolytic enzymes with optimal activity at pH 7.0 may also be present in extracts from peanut cotyledons at other stages of germination. Figure 7 shows that enzyme preparations from cotyledons 10 days after germination did contain high proteolytic activity at pH 7. However, this activity was consistently lower than that at pH 5.0 throughout the test period (0 to 20 days), suggesting that the activity at neutral pH may be residual activity of the enzyme with optimal activity

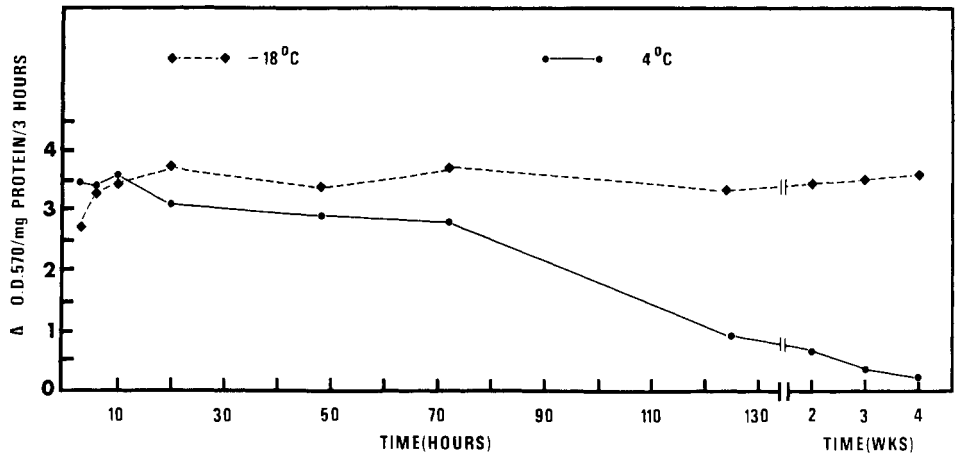

Figure 5. Effect of storage temperature and time on proteolytic activity in 10-day cotyledonary extracts.

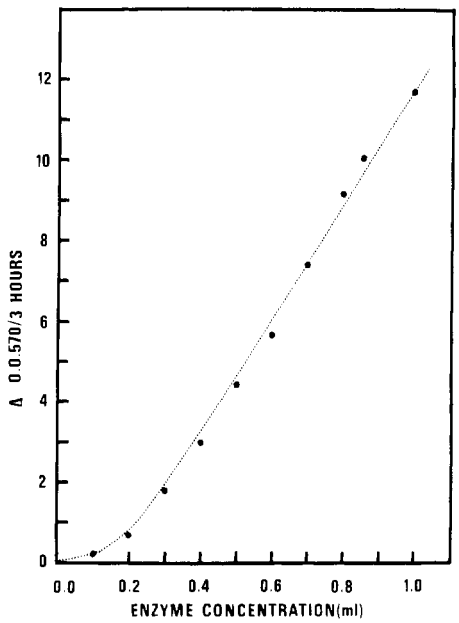

Figure 6. Effect of enzyme concentration in extracts of 10-day cotyledons on the rate of hydrolysis of total proteins in peanuts.

at pH 5.0. Interestingly, when proteolytic activity examined at pH 7.0 was expressed on a cotyledonary basis, enzyme extracts from 6 and 10 days germination showed peak activity. These data suggest that enzymes with optimal activity near or at pH 7 may be present in cotyledonary extracts from seeds germinated for 6 days. It is possible that proteolytic activity in cotyledonary extracts of germinated peanuts may contain two or more sulfhydryl proteases having optimal activities at pH 5.0 or 7.0; the pH 5.0 enzyme(s) may efficiently hydrolyze proteins of dormant seeds while those active at pH 7.0 would be active with partially hydrolyzed polypeptides. These observations are consistent with findings for peas by other investigators (Beevers, 1968; Basha and Beevers, 1975).

Proteolytic Inhibitors in Cotyledons of Germinating Peanuts. Several studies have shown the presence of proteolytic inhibitors in legume seeds (Vogel et al., 1966). This was also indicated by the lag in proteolytic activity discussed earlier in this paper (Figure 6) when low concentrations of 10-day enzyme extracts were incubated with the total protein fraction of ungerminated peanut seeds. To examine this possibility further, 10-day cotyledonary total protein extracts were mixed with equal amounts of each similar preparation from dormant (0 day) and germinated seeds (2 to 20 days) and incubated at pH 5.0

Table I. Effect of Mixing Enzyme Extracts from Cotyledons of Seeds Germinated for Various Time Intervals (0 to 20 days) with that of the 10-Day Preparation[a]

Mixtures 1:1 (v/v)	Total ΔOD 570/mL of enzyme extract	Percent increase over control
10 (control)	10.80	100
10 + 0	9.76	−9.3
10 + 2	11.60	+7.4
10 + 4	11.76	+8.8
10 + 6	20.80	+92.59
10 + 8	19.52	+80.73
10 + 10	19.64	+81.85
10 + 12	15.60	+44.44
10 + 14	13.12	+21.48
10 + 16	12.80	+18.51
10 + 18	12.64	+17.08
10 + 20	9.04	−16.30
10 + testa	3.60	−66.67

[a] Inhibitor activity in an extract of testa from ungerminated seeds was also evaluated. Total protein from ungerminated seeds was used as substrate.

(Table I). These data showed that proteolytic activity of 10 day germinated seeds could be inhibited by preparations from dormant seeds. Mixed extracts from 6- to 10-day germinated cotyledons contained higher amounts of activity than other combinations. Percentage proteolytic activity progressively decreased when 10-day extracts were mixed with preparations from cotyledons of seeds germinated for 12 to 18 days; proteolytic activity was inhibited in the mixtures containing 20-day cotyledon and testa extracts. Extracts of testa have been shown to contain protease inhibitors (Cepelak et al., 1963). Further studies are needed to determine if increased proteolytic activity of germinated peanut seeds was due to (a) breakdown of stored inhibitors of proteolytic enzymes following germination (Pusztai, 1972), (b) de novo synthesis of enzymes (Varner, 1965), or (c) to activation of enzyme precursors (Shain and Mayer, 1968).

Leucine Aminopeptidase Activity in Cotyledons of Germinating Peanuts. Changes in leucine aminopeptidase activity in cotyledonary extracts of seeds germinated for 0 to 20 days were examined on polyacrylamide electrophoretic gels (Figure 8). The peptidase activity shown in region 3.5–4.5 cm from these preparations remained consistently high during early stages of germination to day 6. After day 6, this peptidase activity decreased and was replaced by peptidase activity appearing in gel region 5.0–5.5 cm. This change coincides with high proteolytic activity noted during the time interval of 6 to 10 days after seed germination (cf. Figures 7 and 8). These

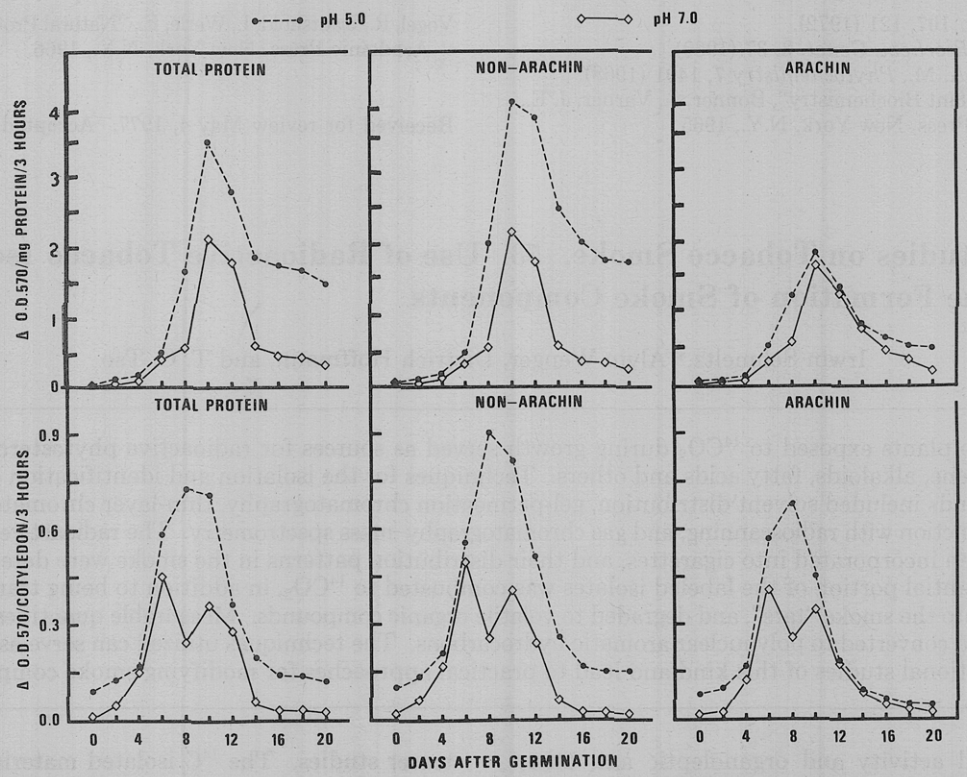

Figure 7. Changes during seed germination in the capacity of cotyledonary extracts to hydrolyzed total proteins, nonarachin proteins, and arachin from ungerminated or dormant peanut seeds. Proteolytic activity is presented both on a protein and cotyledonary basis.

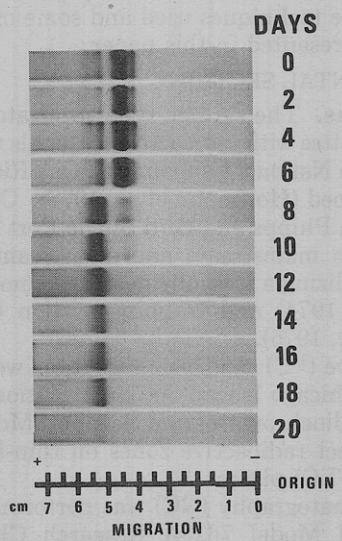

Figure 8. Changes in leucine aminopeptidase activity on polyacrylamide electrophoretic gels of cotyledonary extracts from seeds germinated for various time intervals to 20 days.

data suggest that two types of peptidase activity may be responsible for the reserve protein breakdown depending on the type of proteolytic activity occurring in the germinating seed. These peptidases correspond with the two peaks of proteolytic activity at days 6 and 10 examined at pH 7.0 and expressed on a cotyledonary basis (Figure 7). High peptidase activity has also been reported in maturing and dormant peanuts (Cherry and Ory, 1973b), while increases in amounts of these enzymes have been reported in germinating squash cotyledons (Penner and Ashton, 1967).

Information from research to isolate and characterize peanut proteolytic activity could result in their utilization to partially hydrolyze proteins to forms with more desirable solubility properties (or other functional characteristics) than the normally extracted protein forms. Such processes of protein modification are presently completed by methods of acid hydrolysis or using microbial proteolytic enzymes. Use of endogenous proteolytic systems of peanut flour derivatives, or partially purified or concentrated fractions of these constituents, would seem more appropriate for altering protein properties for their expanded food use than extraneous enzyme sources.

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Chemical Studies on Tobacco Smoke. 53. Use of Radioactive Tobacco Isolates for Studying the Formation of Smoke Components

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Tobacco plants exposed to $^{14}CO_2$ during growth served as sources for radioactive phytosterols, neophytadiene, alkaloids, fatty acids and others. Techniques for the isolation and identification of these compounds included solvent distribution, gel-permeation chromatography, thin-layer chromatography in conjunction with radioscanning, and gas chromatography–mass spectrometry. The radioactive isolates were then incorporated into cigarettes, and their distribution patterns in the smoke were determined. A substantial portion of the labeled isolates was combusted to $^{14}CO_2$, in addition to being transferred intact into the smoke "tars", and degraded to volatile organic compounds. Measurable quantities (<1%) were also converted to polynuclear aromatic hydrocarbons. The techniques utilized can serve as models for additional studies of this kind and lead to practical approaches for modifying smoke composition.

The biological activity and organoleptic and other properties of cigarette smoke are a function of the chemical compounds that comprise the smoke. These compounds originate from the leaf, by direct transfer (e.g., distillation, sublimation), by thermally degradative processes, and/or by pyrosynthesis. By identifying precursors in the leaf that give rise to undesirable smoke constituents, one could ultimately modify the levels of these precursors in the leaf and consequently control the composition of the smoke.

Previously, precursor–product relationships were established by pyrolysis studies. In these, selected leaf components or extracts were subjected to appropriate high temperatures, and the products thus generated were identified (Higman et al., 1970; Schmeltz et al., 1972; Wakeham, 1972; Schlotzhauer et al., 1976). However, such studies were models of only one process in an ignited cigarette, i.e., pyrolysis, and did not reflect the complex phenomena occurring there.

In order to understand better the pathway of individual tobacco constituents from the leaf into the smoke, a number of workers have used cigarettes containing isotopically labeled compounds (Jenkins et al., 1976; Wakeham, 1972; Houseman, 1973). By such techniques, these workers have studied the distribution of certain leaf constituents and their thermally altered products in the particulate and vapor phase of smoke. However, studies such as these have limitations because many tobacco constituents (e.g., terpenoids) containing label are hardly accessible. Moreover, those that are available are usually not of sufficient activity for tracer studies or are labeled in only one position.

To overcome these difficulties, we isolated various ^{14}C-tobacco constituents from tobacco plants grown in a $^{14}CO_2$ atmosphere. These constituents naturally formed in the plant should theoretically contain random labeling in all C atoms and be especially suitable for extensive tracer studies. The ^{14}C isolated materials were used in smoke formation studies, not only to determine how they distribute in the smoke, but also to identify, with some quantitation, the compounds they give rise to in the process. The techniques used and some of the data obtained are presented in this paper.

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EXPERIMENTAL SECTION

Apparatus. The syringe-type applicator for impregnating cigarettes with radioactive materials was developed at Oak Ridge National Laboratories, Oak Ridge, Tenn. Its use is described (Hoffmann et al., 1977). Cigarettes were smoked on a Phipps and Bird Single Port Smoking Machine. Both mainstream and sidestream smoke were collected utilizing a specially designed smoking chamber (Liu et al., 1974) and trapping system (Marmor and Minnemeyer, 1975).

Radioactive (^{14}C) samples, in solution, were counted in a Nuclear-Chicago Isocap 300 Scintillation Counter. A Packard Radiochromatogram Scanner, Model 1201, was used to detect radioactive zones on thin-layer chromatographic (TLC) plates.

Gas chromatography (GC) was performed on a Hewlett-Packard Model 7620A Research Chromatograph equipped with flame-ionization detectors. Gas chromatography–mass spectrometry (GC–MS) was accomplished on a Hewlett-Packard system (5980A mass spectrometer interfaced with a 5710 gas chromatograph and a 5933A data system).

Reagents. All organic solvents were spectrograde, and the other chemicals were reagent grade. Counting solutions were Toluscint I (ICN Pharmaceuticals, Inc.) for materials soluble in organic solvents and Aquascint I (ICN Pharmaceuticals, Inc.) for water-soluble materials. Plant fibers were solubilized with NCS Solubilizer (Amersham Searle) prior to counting. Sephadex LH-20 was obtained from Pharmacia Fine Chemicals, Inc. GC columns and TLC plates were obtained from various commercial sources.

Gas Chromatography. The GC column conditions used for the various tobacco isolates were as follows: sterols, 3% OV-17, 10 ft × 0.13 in. o.d., 230 °C; fatty acids, 3% OV-17, 10 ft × 0.13 in. o.d., 160 °C; fatty acids, 10% DEGS-PS, 8 ft × 0.13 in. o.d., 165 °C; neophytadiene, 3%

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