Table V. Ranges of Water Activity at Which Intersection of Li-2 and Li-3 Occurred

Range of A_w	% total[a] A	B
0.40	5.17	5.0
0.40-0.50	17.24	15.0
0.50-0.60	40.23	51.66
0.60-0.70	29.31	20.0
0.70-0.80	5.74	8.33
0.80-0.90	1.72	

[a] A, percent values over 174 isotherms comprising various temperatures for several of the products tested; B, percent over 71 isotherms corresponding to the same number of products.

forces is given by the Kelvin equation:

$$\ln A_w = -(2\gamma/r) \cos \theta A \quad (2)$$

where A_w = water activity, γ = surface tension of water, θ = contact angle, A = constant, and r = radius of capillary. Considering eq 2 and the capillary radius which are more probably common in foods, Karel (1973) and Loncin (1973) suggest that definite capillary effects could only be expected above 0.9 water activity.

The depression of water activity in the region defined by Li-3 could also be due to dissolved solutes. The minimum activities which can be expected from solutes which are commonly found in foods (sugars and sodium chloride) have been reported by a number of authors (Bone, 1969; Kaplow, 1970; Cakebread, 1973; Karel, 1973; Loncin, 1973). Considering those reported values, it is doubtful that dissolved solutes in foods would be able to depress water activity to values well below 0.60, as it should be if vapor pressure is lowered by this kind of effect.

In view of the above considerations, it might be concluded that it is difficult to accept that Li-3 defines a region in which capillary forces and/or solute depression are responsible for the depression of water activity.

CONCLUSIONS

Rockland's (1969) suggestion that localized isotherms represent the three accepted modes of water binding in foods looked attractive because of the recognized relationship between modes of water binding and stability of dehydrated foods (Labuza et al., 1970; Karel, 1973). However, the results found here indicate a lack of agreement between the interception of Li-1 with Li-2 and B.E.T. monolayer values. Besides, in many cases the region defined by Li-3 begins at a water activity too low to consider that moisture is present as unbound, free water as suggested by Rockland (1969). We may conclude that, although in a broad sense the local isotherms proposed by Rockland (1969) may be related to the different modes of water binding, they cannot be used to give a precise and unequivocal definition of the physical state of water in foods.

Supplementary Material Available: Table I (Henderson's parameters n and k for Local Isotherms in Foods and Food Components) and Table II (Intersections of Local Isotherms in Foods and Food Components); 44 pages. Ordering information is given on any current masthead page.

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Protein and Amino Acid Changes in Peanut (*Arachis hypogaea* L.) Seeds Infected with *Aspergillus oryzae*

John P. Cherry,* Larry R. Beuchat, and Clyde T. Young

Proteins and free and total amino acids in peanuts (*Arachis hypogaea* L., C.V. Florunner) inoculated with *Aspergillus oryzae* (Ahlburg) Cohn were examined at various time intervals over an 18-day test period. Quantities of proteins from infected peanuts which were soluble in aqueous buffer declined markedly shortly after inoculation and then increased rapidly during the later stages of the test period. Gel electrophoresis showed that proteins were converted to many low molecular weight components in infected seeds. Most free amino acids from infected seeds increased to levels greater than those of uninoculated peanuts. Amino acid profiles distinguished inoculated from uninoculated whole seeds as well as from their soluble and insoluble fractions at various intervals of the test period.

It is well known that saprophytic fungi degrade dormant, dead, and decaying plant tissues to yield products which are metabolically acceptable for the maintenance of life. In storage organs such as seeds and tubers, for example, fungal invasion results in deterioration of cellular components and ultimate loss in viability (Christensen and Kaufmann, 1965; Harman and Granett, 1972; Harman and Pfleger, 1974). Several saprophytic storage fungi, including some aspergilli, produce mycotoxins which, when ingested,

Department of Food Science, University of Georgia Agricultural Experiment Station, Experiment, Georgia 30212.

may be detrimental to human health (Goldblatt, 1969). Research with these saprophytes has mainly centered on determining environmental conditions suitable for growth, developing methods to isolate and characterize toxic metabolites, and determining effects of toxins on biochemical mechanisms in living animal and plant tissues (Wogan, 1968; Asahi et al., 1969).

On the other hand, many saprophytic fungi are useful in fermentation processes to upgrade the nutritional quality and flavor of certain foods (Hesseltine, 1965). These fungi have been shown to contain a complex array of enzymes which hydrolyze substrate components such as proteins, lipids, and carbohydrates during the fermentation process (Beuchat and Worthington, 1974; Beuchat et al., 1975; Quinn et al., 1975; Worthington and Beuchat, 1974). The sequence of biochemical changes attributed to the activities of "food fungi" during fermentation of various substrates is complex and not fully understood.

Cherry and coworkers (1972, 1974, and 1975) have studied interrelationships between the storage fungus *Aspergillus parasiticus* and peanuts during various infection periods. Data showed that biochemical mechanisms of this saprophytic (or weakly pathogenic) organism function efficiently and systematically for the benefit of the fungus at the expense of the seed. Systems studied included proteolytic degradation of storage proteins to small polypeptides and free amino acids, enzymatic hydrolysis of ester-like linkages, and decomposition, hormonal interaction, and/or oxidation of organic substrates with fungal hydrogen peroxides. Many of the new isozymes associated with these biochemical processes in infected peanuts were also observed in zymograms from extracts of fungal tissue. Proteolytic degradation of the large storage globulins of germinating peanuts required 2 weeks to reach the same stage of proteolysis as observed 2–5 days after infection with *A. parasiticus* (Bagley et al., 1963; Cherry and coworkers, 1972, 1974, 1975).

The present work introduces studies into complex biochemical interrelationships between *Aspergillus oryzae* and peanuts which it infects. *A. oryzae* was chosen for study because it exhibits biochemical and morphological characteristics similar to yet distinct from *A. parasiticus*. Both species are members of the *A. flavus-oryzae* group of the aspergilli and both are associated with spoilage of stored peanut seeds. It was desirable to have definitive information on similarities which might exist with respect to proteolytic activities of the two species as they grow on viable seeds. In addition, comparisons could be made between the rate and extent of protein hydrolysis by *A. oryzae* as it grows on living peanut seeds and on peanut meal. We have previously reported on the proteolytic activity of *A. oryzae* on peanut meal (Beuchat et al., 1975). With these objectives in mind, experiments were carried out to determine changes in composition of proteins and free and total amino acids during the growth and development of *A. oryzae* on viable peanut seeds.

EXPERIMENTAL PROCEDURES

Aspergillus oryzae (Ahlburg) Cohn NRRL 1988 was cultured on potato dextrose agar slants for 9 days at 24°C and conidia were harvested by gently washing the culture surface with 0.005% Span 20 in sterile deionized water. Testa-free Florunner peanuts were soaked in an aqueous dilution of the conidia suspension for 1 min. Seeds were deposited in petri dishes which were placed in a container lined with water-saturated absorbent cotton to maintain high humidity and incubated at 29°C for time intervals ranging to 18 days. Uninoculated control peanuts were exposed to identical incubation conditions. Two independent replications were performed (experiments 1 and 2).

Methods for preparing soluble and insoluble protein fractions and free and total amino acid samples were previously described (Cherry et al., 1974, 1975). To summarize, duplicate samples of three control and three uniformly infected peanuts were collected after inoculation periods of 0, 2, 4, 7, 9, 11, and 18 days and individually ground in 7 ml of sodium phosphate buffer (pH 7.9; I = 0.01) using a mortar and pestle. Fungal mycelia and conidia, if present, were carefully removed from the surface of infected peanuts prior to grinding. Ground peanut and mycelium samples were centrifuged for 30 min at 43500g and the soluble (supernatant) and insoluble (pellet and fat pad) fractions analyzed for protein, and free and total amino acid content.

The nitrogen content of lyophilized whole peanuts (with fungal growth removed from the surface) and soluble and insoluble fractions of seeds and mycelia/conidia were evaluated using the macro-Kjeldahl technique. Crude protein content was calculated using a conversion factor of 5.46. In addition, the method of Lowry et al. (1951) was used to determine the content of soluble protein in extracts of seeds and fungal mycelia. Gel electrophoresis of proteins in the soluble fractions was performed on 10% polyacrylamide disc gels according to the procedures outlined by the Canalco Catalog (1973) and Cherry et al. (1970).

Free and total amino acids were determined in fat-free peanuts and/or soluble and insoluble seed fractions by ion-exchange chromatography using a Durrum Model D-500 Amino Acid Analyzer as previously described (Cherry et al., 1975; Young et al., 1974; Spackman et al., 1958). Total amino acid profiles were determined for soluble and insoluble fractions of mycelia/conidia. All experiments were conducted in duplicate.

RESULTS AND DISCUSSION

Soluble and Insoluble Proteins. Protein content (as measured by the method of Lowry et al., 1951) in soluble fractions of uninoculated peanuts varied between 50.0 and 65.0 mg/ml of extract during the test period of 18 days (Figure 1). The protein content in the soluble fraction of *A. oryzae* infected peanuts declined from 51.0 to approximately 19.5 and 18.0 mg/ml between 2 and 4 days after inoculation. At days 7, 9, and 11, protein levels increased to average values of 32.0, 47.0, and 56.5 mg/ml, respectively. The protein content then declined to 37.0 mg/ml of extract at day 18. An analysis of variance showed that in these experiments the treatment (infection with *A. oryzae*) variable was highly significant ($P \leq 0.01$). During the infection period, soluble protein in fungal mycelia/conidia collected from the surface of peanuts showed only minor quantitative changes, averaging between 2.5 and 5.5 mg/ml of extract.

Changes in percent of crude protein in lyophilized whole seeds and soluble and insoluble fractions during the test period are presented in Figure 2. In this study, the analysis of variance showed that the various fractions analyzed were significantly different ($P \leq 0.01$). The data showed that the percentage of proteins in the soluble fraction rapidly declined from 61.0% at day 0 to an average of 42.5% after 2 days of infection. Percent protein in lyophilized soluble extracts then increased to 60.5% at day 7 and values of approximately 67.0% between days 11 and 18; the latter percentages were greater than those of uninoculated seeds. These observations support those made using the technique of Lowry et al. (1951). Simultaneously, protein levels in insoluble fractions increased

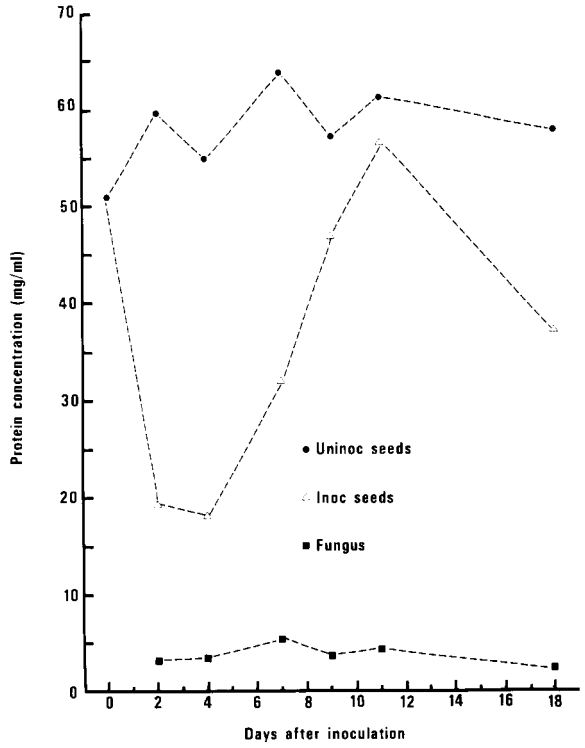

Figure 1. Quantitative changes in soluble protein concentrations (Lowry et al., 1951) in extracts of uninoculated (uninoc.) and inoculated (inoc.) peanuts of combined duplicate data of two experiments from test periods within 18 days. Included are the values for soluble protein from *A. oryzae* mycelia/conidia collected from the seed surface during the infection period.

from 33.5% at the time of inoculation to 49.5% at day 2 and then declined thereafter to 29.0% by day 11. At day 18, percent protein in the lyophilized insoluble fraction increased slightly to an average of 33.0%. Protein levels in insoluble fractions from peanuts infected for 9 to 18 days were similar to those of uninoculated seeds. Percentages of proteins in soluble and insoluble fractions of uninoculated peanuts varied between 54.5 to 63.0% and 31.5 to 36.0%, respectively, throughout the test period. Total protein content from inoculated whole seeds remained slightly higher (47.0 to 49.0%) than those of uninoculated preparations (42.0 to 45.0%) during the test period.

Soluble and insoluble fractions of *A. oryzae* mycelia/conidia contained varied percentages of protein during the infection period. In soluble preparations, protein levels increased from approximately 11.0% on day 0 to 62.0% at day 11, while levels of protein in insoluble preparations were between 16.0 and 32.0% during the infection period of 18 days.

Similar studies with *A. parasiticus* showed that initial (2 to 4 days after inoculation) levels of proteins in soluble fractions also declined rapidly (Cherry et al., 1975). Simultaneously, the percentage of protein in insoluble peanut fractions increased. Changes in soluble and insoluble fractions of *A. parasiticus* infected peanuts continued through the later stages of the test period but at a much slower rate than that noted initially. In contrast, the data in Figures 1 and 2 of this study show that percentages of protein in the soluble fraction of peanuts inoculated with *A. oryzae* initially decreased and then increased during the 18-day infection period. This trend was reversed for insoluble protein.

These observations imply different rates and/or mechanisms of peanut protein hydrolysis for *A. oryzae* and *A. parasiticus*. Both organisms are known to exhibit strong proteolytic activity (Hesseltine, 1965; Klapper et al., 1973; Nakadai et al., 1971). The sustained increase in

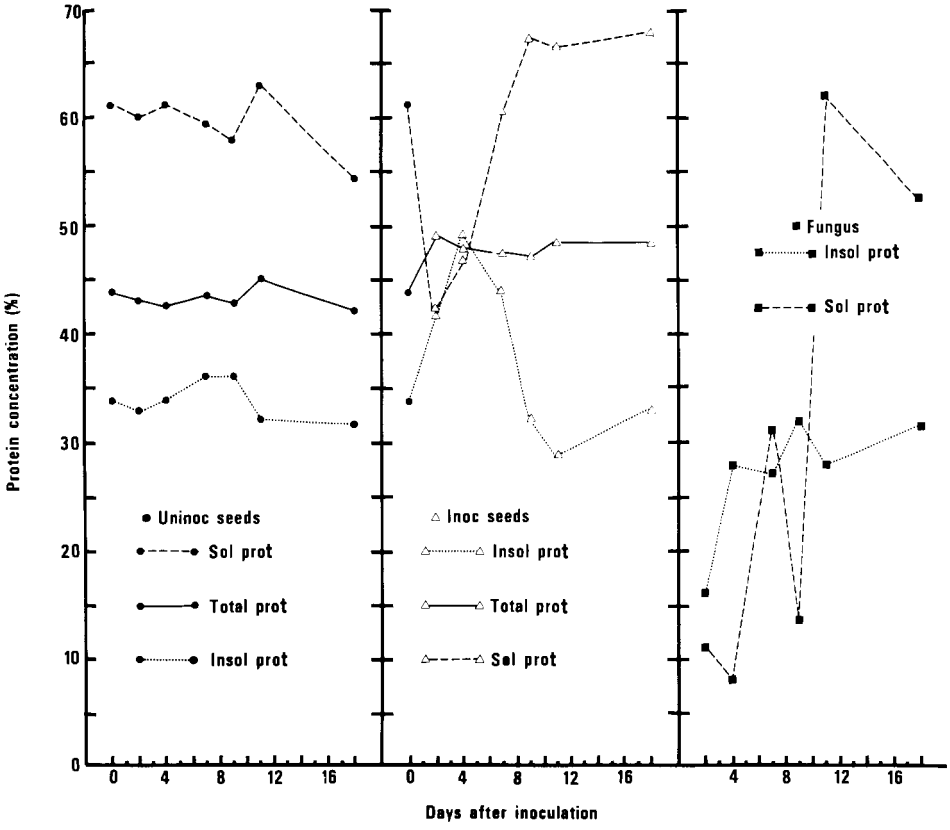

Figure 2. Percent changes in protein (prot) levels (macro-Kjeldahl) in whole peanuts (total) and soluble (sol) and insoluble (insol) fractions of uninoculated (uninoc.) and inoculated (inoc.) seeds and the *A. oryzae* mycelia/conidia from the surface of seeds from test periods within 18 days.

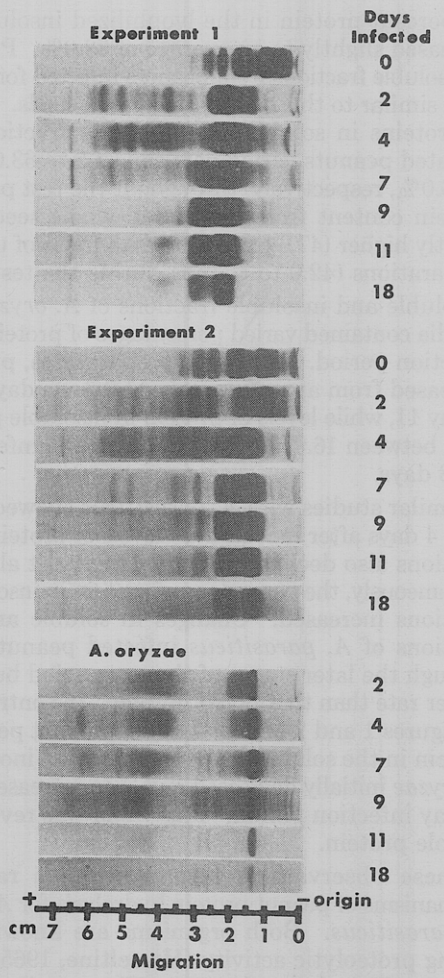

Figure 3. Polyacrylamide disc gel electrophoretic patterns of soluble proteins of inoculated peanuts and *A. oryzae* mycelia/conidia from the surface of seeds from experiments 1 and 2 after incubation for 0 to 18 days.

percentages of protein in soluble fractions during late infection stages suggests the accumulation of low molecular weight proteins, peptides, and amino acids. This may be due in part to increased levels of mycelial enzymatic protein and free amino acid pools which are undoubtedly present in peanut fractions late in the test period. A significant contribution to the apparent increase in soluble protein in *A. oryzae* infected seeds, however, is more likely due to the accumulation of peanut protein hydrolysates which are not efficiently utilized by *A. oryzae* for growth and metabolism.

Gel Electrophoresis of Soluble Proteins. Coinciding with quantitative changes of proteins in soluble fractions of *A. oryzae* infected peanuts, gel electrophoresis showed that arachin was degraded and two new major components appeared in region 1.0–3.0 cm (Figure 3). Proteins in gel patterns of uninoculated seeds did not change significantly during the 0 to 18 day test period. A number of small proteins (or more negatively charged components) appeared in region 3.0 to 6.5 cm in gels of extracts from peanuts infected for 2 to 7 days which then gradually disappeared from the patterns during the later stages of the test period. The two major components in extracts from experiment 1 showed a gradual increase in mobility on electrophoretic gels from region 1.0–2.5 to 2.0–3.0 cm during the test period. The increase in mobility of these components was not as great in experiment 2.

The gel patterns of extracts from *A. oryzae* mycelia/conidia contained a major dark-staining component in region 1.0–1.5 cm and a number of minor bands in regions 0–1.0, 1.5–3.0, and 3.5–6.5 cm. The major component gradually decreased in quantity between days 7 and 18; simultaneously, bands in region 0–1.0 and 1.5–3.0 cm increased. Proteins in region 3.5–6.5 cm varied both quantitatively and qualitatively during the entire test period. A number of these bands had mobilities similar to those noted from proteins of infected peanuts, indicating that proteins from hyphae which penetrated peanut tissue during *A. oryzae* growth may have been present at low levels in soluble peanut protein extracts.

Gel electrophoresis showed that changes in protein composition of soluble extracts of *A. oryzae* infected peanuts differed from those inoculated with *A. parasiticus* (Cherry et al., 1975). Qualitatively, buffer-soluble proteins of inoculated peanuts were continually degraded by *A. parasiticus* to smaller polypeptides and components not distinguishable by gel electrophoretic techniques. *A. oryzae* also degraded peanut proteins, producing many small components, but at the same time caused the accumulation of two major polypeptides which increased quantitatively throughout the test period. These polypeptide components probably account in part for the increase in protein content of the soluble fraction (Figures 1 and 2).

Apparent differences in activities of proteinases produced by *Aspergillus* spp. support earlier reports. Although certain proteolytic enzymes are common to both *A. oryzae* and *A. parasiticus* (Dworschack et al., 1952; Kulik and Brooks, 1970), each species and strain within the *A. flavus–oryzae* group has the capacity to produce an array of proteinases different from other members of the group. Thus, differences in electrophoretic patterns of soluble proteins from peanuts inoculated with *A. oryzae* and *A. parasiticus* are a result of types and amounts of proteinases produced by the fungi during the 18-day test period.

Free Amino Acids. Analysis of variance of quantitative changes in free amino acids in uninoculated and inoculated whole peanuts showed that the variable for experiment was significant for only Ala, Val, Tyr, and Phe. The summary of mean square values and level of significance obtained from free and total amino acid analyses of various preparations from peanut seeds uninoculated and inoculated with *A. oryzae* will appear in the microfilm edition of this volume of the journal. The first-order interaction of treatment times experiment was significant for only Ser and Glu. Since only the whole peanut preparation was examined for free amino acids, the experimental design was not complex; therefore, although some of the components showed experimental variation, we felt justified in combining experiments 1 and 2 to simplify their presentation. The combined statistical analyses showed that treatment, time, and the interaction of these variables had the greatest effects on free amino acids in peanuts during the test period.

Quantities of Thr, Gly, Ala, Ile, Leu, Tyr, and others remained higher than those of uninoculated seeds throughout the test period, while Ser, Val, Glu, and Met increased to levels greater than those of uninoculated seeds after 9 days of infection (Figure 4); only the changes in Val and Met were not significant. Free Tyr, Lys, and unidentified (others, Figure 4) amino acids increased to levels above that of uninoculated seeds shortly after inoculation. During the test period, quantities of Phe and Asp of infected seeds remained consistently less than those of uninoculated peanuts. Levels of Pro, His, and Arg (the latter two being not significant) in uninoculated seeds were

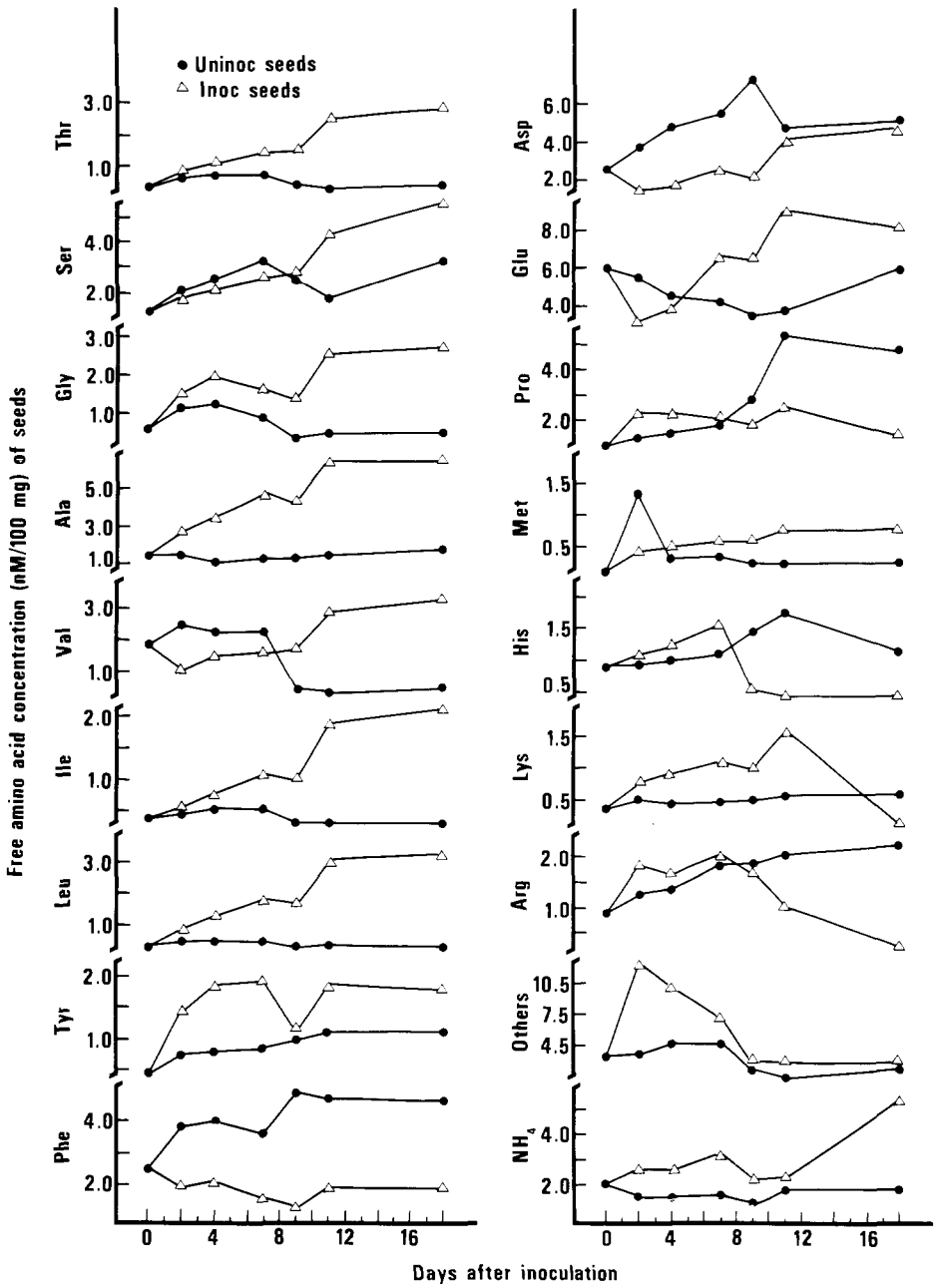

Figure 4. Quantitative changes in free amino acid content of uninoculated (uninoc.) and inoculated (inoc.) whole peanuts. Averages were taken from experiments 1 and 2 during the test period of 0 to 18 days.

greater than those of inoculated peanuts during the later stages of infection.

Increased levels of free amino acids noted in inoculated peanuts as fungal growth progressed coincided with the formation of the two major peanut proteins in region 1.0–3.0 cm of electrophoretic gels (Figure 3). After prolonged infection, a number of small polypeptides gradually disappeared from the extracts of inoculated peanuts. Increases in certain free amino acids undoubtedly result from complete and/or partial hydrolysis of certain peanut proteins.

Changes in levels of free amino acids in *A. oryzae* infected peanuts differed from those of seeds inoculated with *A. parasiticus* (Cherry et al., 1975). Quantities of most free amino acids in peanuts infected with *A. parasiticus* increased to levels above that in uninoculated seeds shortly after inoculation (days 2 to 4). Thus, the rate of protein catabolism in peanuts differs considerably for these two fungi.

Specific enzymes responsible for breakdown of soybean proteins by *A. oryzae* have been studied. Nakadai et al. (1972b) reported that neutral proteinase degrades soybean proteins to oligopeptides and liberates only small quantities of free amino acids. The same researchers (Nakadai et al., 1972a) state that carboxypeptidases play an important role in the digestion of peptides to yield free amino acids. Although we did not characterize specific enzymes in the present study, data from electrophoretic analyses as well as from soluble protein and free amino acid studies infer a sequential degradation of peanut proteins by *A. oryzae*.

Total Amino Acids. A preliminary analysis of variance of the total amino acid data showed many first- and second-order interactions within experiments 1 and 2. Because of these significant interactions, a statistical combination of experiments 1 and 2 was not appropriate (statistical data on microfilm). During both experiments, significant treatment effects (*A. oryzae* infection) were

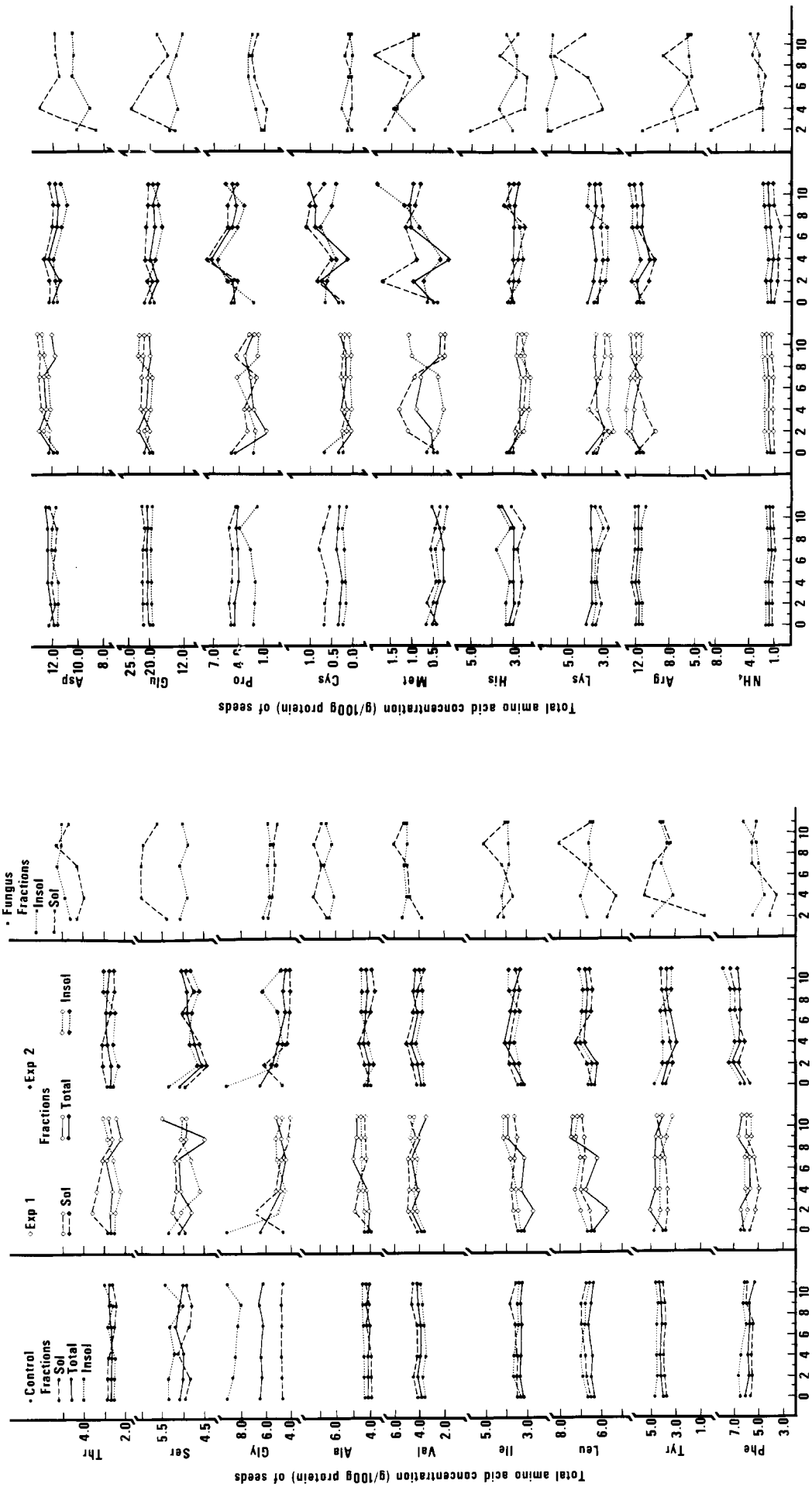

Figure 5. Quantitative changes in total amino acid content (parts A and B) of whole seeds and soluble and insoluble fractions of uninoculated (control) and inoculated (experiments 1 and 2) peanuts and *A. oryzae* mycelia/conidia during the test period of 0 to 11 days.

noted on Ser, Gly, Met, Leu, and Tyr. Several other amino acids were affected in either experiment 1 (Asp, Val, Ile) or experiment 2 (Phe, His, Lys, Arg). Only a few total amino acids were affected significantly by the time interval of the infection (experiment 1: Gly, Leu, Lys; experiment 2: Ser, Cys, Met, Leu, Lys). The complexity in interpreting the amino acid data is evidenced further by examining the first-order interactions. For example, the variables of treatment, time, and fraction were not significant for Phe and Asp in experiments 1 and 2, respectively. However, during the test period, the observed changes (Figure 5A,B) of these total amino acids were very complex which was further supported in each case by a significant first-order interaction of treatment times fraction.

In general, quantities of total amino acids of whole peanuts and soluble and insoluble fractions from uninoculated seeds showed only minor changes during the 18-day test period (Figure 5A,B). Quantities of Ser, Gly, Pro, Cys, and His clearly distinguish amino acid profiles of whole peanuts and soluble and insoluble fractions of uninoculated seeds during the test period. In experiment 2, quantitative changes of Ser, Glu, Gly, Pro, Ala, Cys, Leu, Tyr, Phe, His, and Lys in whole seeds and soluble and insoluble fractions were different in peanuts infected with *A. oryzae* as compared to preparations from uninoculated seeds (Figure 5A,B). In experiment 1 differences in Thr, Glu, Gly, Cys, Val, Tyr, and Lys were significant among these preparations of infected and uninoculated seeds. These changes in total amino acids within soluble and insoluble fractions would be expected to occur since alterations, both qualitative (Figure 3) and quantitative (Figures 1 and 2), in protein content between these preparations were noted.

Quantities of Cys and Met fluctuated similarly in inoculated whole seed and in soluble fractions of experiment 2. In the insoluble fractions, these total amino acids showed similar trends between days 2 and 9, after which Met increased while Cys declined at day 11. However, the changes in Cys were statistically significant while those of Met were not. In experiment 1, levels of Met increased in the soluble fraction and decreased in the insoluble portion of infected seeds between days 2 and 7; this trend was reversed at days 9 and 11. As similarly shown for Met in experiment 2, these changes were not statistically significant. In the whole seed, levels of Met remained approximately intermediate between those of soluble and insoluble fractions and Cys did not change throughout the test period. Arginine content showed a clear, but not significant, decline in the soluble fraction of infected peanuts between days 0 and 7 and then increased to levels intermediate between the whole seed and insoluble fractions during days 7 and 11. These data give further evidence of the complexity of this peanut–*A. oryzae* interrelationship.

Ser, Tyr, Asp, and Glu increased in soluble fractions of fungal mycelia/conidia while remaining the same or decreasing somewhat in insoluble preparations between days 7 and 11 (Figure 5). Similar increases were noted for Thr, Leu, His, and Arg in the insoluble preparations when compared to soluble fractions. Quantities of other total amino acids (Ala, Val, Ile, Leu, His, and Arg) in the fungal mycelia/conidia changed inversely to one another throughout the test period. Total amino acid variability between and within soluble and insoluble fractions reflects differences in ratios of amino acids which are known to exist in mycelia and conidia of aspergilli. Shifts in relative levels of amino acids would be expected to occur as conidia are formed late in the test period.

In summary, we have demonstrated that the rate and extent of hydrolysis of proteins in viable peanut seeds by *A. oryzae* differs from that previously reported for closely related *A. parasiticus* (Cherry et al., 1975). Changes in levels of specific amino acids in peanut proteins and in the free amino acid fractions of infected seeds indicate preferential hydrolysis and utilization by *A. oryzae*. This observation, in conjunction with similar observations reported earlier on proteolysis of defatted peanut meal by *A. oryzae* (Beuchat et al., 1975), implies that changes in nutritional value of protein in peanut substrates may occur as a result of infection or fermentation.

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Supplementary Material Available: Summaries of mean square values and level of significance for amino acid analyses of various samples (3 pages). Ordering information is given on any current masthead page.

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